CROSS-TALK OF SIGNALLING CASCADES IN THE MODULATION OF PRESYNAPTIC EXTRACELLULAR SIGNAL-REGULATED PROTEIN KINASE 1 AND 2 FUNCTION

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Abstract

Activation of extracellular signal-regulated protein kinases (ERK1&2) has emerged as a key signalling event in the modulation of presynaptic plasticity by neurotrophic factors. In this study, we investigated the cross-talk between presynaptic signalling pathways activating PKA or PKC and ERK signalling, underlying the modulation of neurotransmitter release. Purified, isolated cerebrocortical nerve terminals and phosphorylation state-specific antibodies were used to carry out this research. Stimulation of PKA by cAMP, produced in response to β-adrenoreceptor activation by isoproterenol, or direct stimulation of adenylyl cyclase by forskolin, resulted in a substantial increase in ERK phosphorylation/activity. Similarly, stimulation of PKC, by PDBu, also led to stimulation of ERK phosphorylation/activity. Metabotropic glutamate receptor activation by the Group I agonist DHPG, however, led to ERK 1,2 phosphorylation which showed a Ca^{2+}-dependency, though the role of PKC was less clearly defined. A key effector molecule for the regulation of neurotransmitter release by ERK activation is the small synaptic vesicle (SSV) protein synapsin I, which, through alteration in its phosphorylation-state, modulates SSV-actin cytoskeleton interactions and thereby controls the distribution of SSVs between release pools. Accordingly, with both PKA and PKC activation of ERKs, the downstream stimulation of synapsin phosphorylation at specific ERK-dependent sites was also detected. These results suggest that, together with the established roles of PKA and PKC acting on ion-channel targets to modulate of presynaptic excitability and/or voltage-dependent Ca-entry, downstream of these loci, the two kinase cascades may converge at the level of SSV mobilization to regulate the efficacy of glutamate release from cerebrocortical nerve terminals.

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Abbreviations

4-AP	4-aminopyridine
5'AMP	5' adenosine monophosphate
α/β-SNAPs	α/β-soluble-NSF-attachment proteins
A_1	adenosine receptor 1
AA	arachidonic acid
AC	adenylate cyclase
ADP	adenosine diphosphate
ANOVA	analysis of variance
AP2 or 180	adaptor protein 2 or 180
ATP	adenosine triphosphate
BDNF	brain-derived neurotrophic factor
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CAM	calmodulin
CaMKI	Ca^{2+}/calmodulin-dependent protein kinase I
CaMKII	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	cyclic adenosine monophosphate
DAG	diacylglycerol
DHPG	(R/S)-dihydroxyphenyl-glycine
DMSO	dimethylsulfoxide
EGTA	ethylene glycol-bis (β-aminoethyl ether) N,N,N',N' tetra acetic acid
EPSP	excitatory postsynaptic potential
ERK 1,2	extracellular signal-regulated protein kinases 1,2
Fura-2-AM	fura-2 acetoxymethylester
GABA	γ-aminobutyric acid
GAP	GTPase-activating protein
GDI	GDP-dissociation inhibitor
GDF	GDI-displacement factor
GDH	glutamate dehydrogenase
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GPCR	G-protein-coupled receptor
GRB2	growth-factor-receptor-bound protein 2
GTP	guanosine triphosphate
HBM	hepes-buffered medium
JNK	Jun N-terminal kinase

LTD	long term depression
LTP	long term potentiation
MAPK	mitogen-activated protein kinase
MEK/MKK	mitogen-activated protein kinase kinase
mGluR	metabotropic glutamate receptor
MKKK	MAPK kinase kinase or Raf
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NSF	N-ethylmaleimide sensitive factor
PDBu	phorbol 12,13 dibutyrate
PDE	phosphodiesterase
PIP_2	phosphatidylinositol 4,5 bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
Ras	rat sarcoma virus
RIM1a	Rab3-interacting molecule 1a
RTK	receptor tyrosine kinase
SAPK	stress-activated protein kinase
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
s.e.m.	standard error of the mean
SHC	SH2-domain-containing α2 collagen-related
SNAP-25	soluble-NSF-attachment protein-25
SNARE	soluble NSF-attachment protein receptor
SOS	son of sevenless
SSV	small synaptic vesicles
TBS	Tris-buffered saline solution
TBST	TBS with tween 20
TrkB	tropomyosin receptor kinase B
t-SNARE	target SNARE
VAMP	vesicle-associated membrane protein
VDCC	voltage-dependent Ca^{2+} channels
v-SNARE	vesicular SNARE

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Chapter 1

Introduction

1. Introduction

It was Ramon Y Cajal, in the early part of the twentieth century, who discovered that brain cells, or neurones, form junctions with each other. He observed that the junctions seemed to consist of three parts: the presynaptic terminal, the synaptic cleft (the space in-between), and the postsynaptic terminal. These junctions, or synapses, are now known to be crucial for regulating communication between neurones and for modulation of responses. Depolarising action potentials travel from the main body of the cell to the presynaptic nerve terminal where they activate voltage-dependent Ca^{2+} channels (VDCCs). The VDCCs are localised to membrane regions known as active zones where they can result in an increase in local Ca^{2+} concentration by up to as much as 100μM through the N-, P/Q- and R-type channels (Dunlap et al., 1995; Wu et al., 1998; Wu et al., 1999). This dramatic increase in the Ca^{2+} levels can stimulate the release of neurotransmitter from the active zone by way of exocytosis (or emptying) of synaptic vesicles into the synaptic cleft (Heidelberger et al., 1994). The neurotransmitter can then cross the cleft to act on receptors of the postsynaptic terminal in order to elicit all or nothing ionotropic responses, or modulatory metabotropic responses, from the neighbouring dendrite. The transmitter can also act on presynaptic receptors where it may have a modulatory role over further neurotransmitter release (Sanchez-Prieto et al., 1996).

A diverse array of signalling molecules can be released from cells to convey messages from one area to another, ranging from amino acids, to catecholamines, to fatty acids, to hormones. In the brain, the main excitatory neurotransmitter is the amino acid glutamate (Orrego and Villanueva, 1993). This thesis is concerned with the elucidation of the regulation/activation of extracellular-signal regulated protein kinases 1/2 (ERK1/2) phosphorylation, and their involvement in glutamatergic synaptic transmission. Accordingly, the subsequent focus of this introduction will be confined to glutamatergic neurotransmission, its regulation, and the cross-talk of kinase-mediated signalling pathways. Notwithstanding, glutamate release from nerve terminals can be regulated, through presynaptic receptors, by other neurotransmitters, such as noradrenaline and γ-aminobutyric acid (GABA), these aspects being discussed further in section 1.4.

1.1 Glutamate Release

There are currently believed to be four stages involved in the exocytosis/endocytosis pathway that constitute neurotransmitter release. These stages follow the filling of the small synaptic vesicle with neurotransmitter and translocation to the active zone, the specialised area of the nerve terminal where transmitter release takes place (Landis, 1988). The four stages are: docking of the synaptic vesicle to the release plasma membrane; priming of the vesicle; fusion of the vesicle with the release plasma membrane (including exocytosis); and, finally, recycling of the vesicle (endocytosis) (Brunger, 2000). Each stage in this cycle requires a great many proteins, which have specific, but sometimes overlapping, roles. Massive progress has recently been made in this field so that it is now possible to identify most of the proteins associated with vesicles, and to put forward hypotheses as to their potential involvements in release (Augustine, 2001). Central to the process are proteins known as SNAREs (soluble N-ethylmaleimide-sensitive factor [NSF]-attachment protein receptors), which are resident on the synaptic vesicle membrane (vesicular v-SNAREs) and plasma membrane (target t-SNAREs), and mediate the basic interaction between the two compartments (Söllner, 1995). The docking process is thought to initiate when nSec1 (also known as Munc18) dissociates from the plasma membrane bound t-SNARE, syntaxin (Pevsner et al., 1994). This dissociation has been shown to involve another protein, known as Munc-13 (mouse homologue of the *c. elegans* protein Uncoordinated-13), which is thought to either directly displace nSec1, or to induce a conformational change in the syntaxin/nSec1 complex leading to the dissociation of nSec1 (Sassa et al., 1999; Brose et al., 2000; Misura et al., 2000). This effects a switch in the primary conformation of syntaxin, from a 'closed' state bound to nSec1 (Dulubova et al., 1999), to an 'open' state enabling it to then bind to a partner t-SNARE, SNAP-25 (synaptosome-associated protein of 25kDa). This interaction of syntaxin and SNAP-25 is known as the binary conformation of SNAREs (Fasshauer et al., 1997a). The interaction between syntaxin and SNAP-25 has recently been reported to be the slow, rate-limiting step in SNARE formation (Fasshauer, 2003). The ternary conformation of SNAREs occurs when the v-SNARE, synaptobrevin (also known as vesicle-associated membrane protein, or VAMP) binds to the binary conformation of syntaxin and SNAP-25 (Otto et al., 1997; Fasshauer, 2003). This is thought to result in the formation of four, tight, α-helices in a

parallel arrangement (Fasshauer et al., 1997b; Poirier et al., 1998) which bring the synaptic vesicle within close proximity of the plasma membrane in a so called 'zipper model' of exocytosis (Hanson et al., 1997). The association of the three SNARE proteins is the docking phase of neurotransmitter release, which may be reversible to some extent. The next priming phase is where the vesicle is brought within close proximity of the plasma membrane in preparation for fusion and is a process that is putatively irreversible (Brunger, 2000). The protein Munc-13, is also believed to have a role central to vesicle priming, possibly through its interactions with RIM (Rab3-interacting molecule) (Betz et al., 2001).

The role of the SNARE proteins in the next stage of neurotransmitter release, the fusion of the vesicle with the plasma membrane, is the subject of considerable debate. As, although SNARE complexes have been shown to form readily *in vitro* (Fasshauer et al., 1997b; Sutton et al., 1998) and *in vivo* (Chen et al., 1999), their time course for enabling vesicle fusion *in vitro* is much slower than that in real life, probably as a result of the high energy barriers that need to be overcome. Other proteins are therefore believed to be involved at this stage of the cycle to facilitate the process, but Ca^{2+}-dependent roles for SNAREs are also beginning to be elucidated (Murthy and De Camilli, 2003, review). One protein that may well be involved in the Ca^{2+}-dependent fusion stage of exocytosis is synaptotagmin. It is a vesicle-bound protein which has two cytosolic domains known to interact with SNAREs (Schiavo et al., 1997), Ca^{2+} channels (Brose et al., 1992), and proteins involved with endocytosis (Chapman et al., 1998). Synaptotagmin has been shown to interact with SNARE proteins such as syntaxin in the presence (Chapman et al., 1995) or absence of Ca^{2+} (Sutton et al., 1999), but binding to lipid bilayers is greatly enhanced in the presence of Ca^{2+} (Davis et al., 1999). Consequently, synaptotagmin has been proposed as a 'clamp' of 'fusion-ready' vesicles, which is released on Ca^{2+}-influx, resulting in rapid exocytosis. Other proteins involved with vesicle fusion include the small G-protein, Rab3A, its effectors, rabphilin-3A and RIM, and its modulators, the GDP-dissociation inhibitor (GDI), and the GDI-displacement factor, GDF (Fischer von Mollard et al., 1990). Rab3A is bound to GDI when in the cytosol, thus preventing the exchange of GDP for GTP on the molecule, but when Rab3A associates with a synaptic vesicle, the GDI is replaced by GDF and GTP is allowed to bind and exchange for GDP (Geppert and Sudhof, 1998). The GTP-bound form of Rab3A can then interact with its effector proteins such as rabphilin-3A, which

binds to the synaptic vesicle, and RIM, which associates with the active zone of the plasma membrane (Stahl et al., 1996;Wang et al., 1997). RIM has also recently been shown to bind the novel cytomatrix at the active zone-associated protein, CAST, in association with the priming of vesicles (Takao-Rikitsu et al., 2004). The exact mechanisms by which these proteins elicit fusion and exocytosis are still not known. However, the rapid effect of Ca^{2+} on exocytosis suggests a conformational change in a component of the prefusion complex, rather than any indirect and likely slower, metabotropic effect (Murthy and De Camilli, 2003).

1.2 Endocytosis.

The final stage in the cycle on neurotransmitter release is endocytosis. The main proteins thought to be involved in the initial phase of this process are NSF (N-ethylmaleimide-sensitive factor) and α/β-SNAPs (soluble-NSF-attachment protein), all involved in the primary delineation and ultimately the nomenclatural definition of the SNAREs described above. Again, the exact mechanisms of action are not known but NSF, which as an ATPase binds and hydrolyses ATP, is key to providing the impetus necessary for SNARE dissociation (Whiteheart et al., 1994; Sumida et al., 1994). α/β-SNAPs, in their association with NSF, may act to form rigid levers to help with SNARE dissociation (Rice and Brunger, 1999). Following this there are two main hypotheses relating to endocytosis, neither of which totally counter the other. One hypothesis revolves around slow vesicle cycling whereby the synaptic vesicle becomes totally incorporated with the plasma membrane before being endocytosed. This means that all the transmitter contained within the vesicle is released and the whole cycle can take about 20 seconds (Koenig and Ikeda, 1996). Endocytosis in this situation is thought to occur following the binding of AP2 (adaptor protein 2) to synaptotagmin (Cremona and De Camilli, 1997), which has recently been identified as having a crucial role in vesicle endocytosis, aside from its role in exocytosis (Poskanzer et al., 2003). This then allows clathrin triskelia to assemble into a lattice of pentagons and hexagons (Abe et al., 1999) with the help of interactions with membrane lipids (Martin, 1998; Corvera et al., 1999). The lysophosphatidic acid acyl transferase, endophilin, is believed to be crucial in this stage of invagination of the plasma membrane (Ringstad et al., 1999), as it recruits and stabilises the phosphoinositide phosphatase, synaptojanin (Song and Zinsmaier, 2003). Endophilin has also been found to interact with the voltage-gated calcium channels

required for endocytosis (Chen et al., 2003). The size of the resulting clathrin coat, and so the synaptic vesicle, appears, at least in part, to be determined by the neuronal-specific protein AP180 (Zhang et al., 1998). Actin polymerisation, stimulated by GTPase or phosphoinositide pathways, seems to be involved in the narrowing of the neck region of the invagination (Merrifield et al., 1999; Gustafsson et al., 1998; Cremona et al., 1999), and cission seems to require the GTPase dynamin, amphiphysin, and endophilin (Abe et al., 1999; Ringstad et al., 1999; Graham et al., 2002; Holroyd et al., 2002). The second hypothesis relating to endocytosis is sometimes termed the 'kiss and run' theory, or 'fast endocytosis' (Koenig and Ikeda, 1996). This is where the synaptic vesicle does not become totally incorporated with the plasma membrane, so that not all neurotransmitter is released, but the whole cycle only takes about 6 seconds (Klingauf et al., 1998). Clathrin coating is also believed to be involved here but the exact mechanism remains to be elucidated (Takei et al., 1996). Also requiring elucidation are the signalling pathways involved in endocytosis but these have proved difficult to investigate due to the close coupling of endocytosis with exocytosis, however, calcium and the calcium-dependent phosphatase, calcineurin, are thought to be involved (Marks and McMahon, 1998), as well as cdk5 (Nguyen and Bibb, 2003).

1.3 Modulation of Glutamate Release

As discussed above, the processes involved in small synaptic vesicle exocytosis and endocytosis are highly regulated, but what about the trafficking of the synaptic vesicles preceding and following these events? It has been demonstrated that the distribution of vesicles in the presynaptic terminal is highly organised and consists of three distinct pools. In hippocampal neurones, the 'ready-releasable pool' is typically made up of 5-8 vesicles which are already docked to the plasma membrane, and have been correlated with the release-ready neurotransmitter quanta (Schikorski and Stevens, 2001). Juxtaposed to this is the 'reserve pool' of 17-20 synaptic vesicles which are tethered, close to the active zone, by a matrix of fine filaments. The resting pool of vesicles is normally kept about 200nm away from the active zone by tethering to microfilaments by members of the synapsin family. The 'resting pool' typically contains 180 vesicles (Garner et al., 2000; Sudhof, 2000, reviews).

From this description of glutamate release it is possible to postulate three distinct ways

of regulating transmitter release. One would be to regulate the excitability of the plasma membrane, thereby affecting the VDCCs and so either decreasing or enhancing the fusion of vesicles to the active zone. For example protein kinase C (PKC)-dependent phosphorylation is able to destabilise the membrane potential by inhibiting K^+ channels (Barrie et al., 1991). Protein kinase A (PKA) and PKC are also able to directly phosphorylate $Ca^{2\tau}$ channels and so facilitate their opening (Dolphin, 1995; Shearman et al., 1989; Farago and Nishizuka, 1990). Other potential targets for regulation are the proteins involved in mediating exocytosis and endocytosis, such as the SNAREs. In the control of SNARE function, NSF has been shown to undergo a depolarisation-induced and Ca^{2+}-dependent phosphorylation. This phosphorylation was believed to be mediated by PKC and to result in the inhibition of neurotransmitter release (Matveeva et al., 2001). The final potential target for modulation of transmitter release is the distribution of vesicles within the presynaptic terminal. The distribution could be altered either temporarily, or perhaps more permanently, as seen with some forms of long term potentiation (LTP) (Lynch et al., 1994). A key player in such a pathway could be synapsin I, one of a family of proteins involved in tethering small synaptic vesicles to the actin cytoskeleton in a phophorylation-dependent manner (Li et al., 1995; Hilfiker et al., 1998), which is known to have phosphorylation sites for several second messenger kinase cascades including the PKA (Huttner et al., 1981), Ca^{2+}/calmodulin-dependent protein kinases I and II (Choe and Wang, 2001b; Kennedy and Greengard, 1981) and extracellular signal-regulating kinases (ERK1/ERK2); also called p44 and p42 mitogen-activated protein (MAP) kinases (Jovanovic et al., 1996). In fact a pathway has in part been identified involving synapsin I in the regulation of neurotransmitter release. Brain-derived neurotrophic factor (BDNF) has been shown to increase glutamate release from cerebrocortical synaptosomes (Jovanovic et al., 2000) and to enhance hippocampal LTP (Figurov et al., 1996). The increase in glutamate release results from BDNF acting on the TrkB receptor to induce a second messenger cascade including the phosphorylation/activation of ERK1/ERK2 which in turn phosphorylate synapsin I on sites 4 and 5 (Jovanovic et al., 2000). The phosphorylation

of synapsin I regulates its interaction with the small synaptic vesicles and actin cytoskeleton (Jovanovic et al., 1996), and so perhaps can regulate glutamate release by making more vesicles available for release, through changing the balance of the aforementioned pools of synaptic vesicles.

One of the objectives of this thesis is to investigate whether this type of regulation may also be under the control of G-protein coupled presynaptic receptors (Parmentier et al., 2002) and whether these signalling pathways are able to cross-talk with ERK1 and 2 in nerve terminals, and if so, examine what the implications are for the regulation of glutamate release.

1.4 Regulation of Glutamatergic Nerve Terminals by Presynaptic Receptors

There are three main types of receptor shown to be involved in the regulation of glutamate release from nerve terminals: ionotropic, metabotropic G-protein coupled, and receptor tyrosine kinases (RTKs) (Jovanovic et al., 2000; Perkinton and Sihra, 1999; Sanchez-Prieto et al., 1996). Ionotropic receptors traverse the cell membrane and have a pore forming region, through which ions can flow into and out of the cells. The flow of ions can be regulated through phosphorylation of the receptor, which can induce conformational changes to mediate opening and closing of the pore region. In contrast to this, metabotropic G-protein coupled receptors and tyrosine receptor kinases transduce signals through coupling to intracellular molecules. Ligands will bind to external domains of the receptors, which can induce changes in the conformation or binding properties of the intracellular domains, resulting in stimulation of downstream effectors. Ionotropic receptors tend to be involved in rapid signalling processes, whereas metabotropic receptors tend to mediate slower, modulatory processes (Sugiyama et al., 1987; Schoepp et al., 1990; Nicoll et al., 1990; Kandel et al., 1991).

1.4.1 Ionotropic Receptors

There are three main ionotropic glutamate receptor subtypes; N-methyl-D-aspartate (NMDA) receptors, and two non-NMDA-type receptors known as kainate, and D-2-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA). These receptors were originally distinguished by their differing pharmacology and sensitivities to kainate and

AMPA (McLennan, 1983; Foster and Fagg, 1984; Monaghan et al., 1985; Sommer et al., 1992), however, more recent cloning experiments have also identified differences in the subunit composition of the receptors (Keinanen et al., 1990; Herb et al., 1992; Hollmann and Heinemann, 1994). The presence of AMPA and NMDA receptors in presynaptic locations is yet to be confirmed, despite a suggested role for the NMDA receptor in regulating the release of noradrenaline and dopamine, the majority of studies confine these two receptors to postsynaptic domains (Pittaluga and Raiteri, 1992; Wang, 1991; Nicoll, 2003, review). However, presynaptic roles for the kainate receptor have been elucidated in several systems, including cerebrocortical synaptosomes (Perkinton and Sihra, 1999).

1.4.1.1 Kainate Receptors

Kainate receptors are ligand-gated tetrameric ion channels, whose unitary conductance and affinity for kainate can be determined by their subunit composition (Rosenmund et al., 1998; Bowie and Lange, 2002). The different channel subunits have been divided into two groups, depending on their affinity for kainate. The low affinity subunits include GluR5, GluR6, and GluR7, which are also able to form homomeric channels, whereas the high affinity subunits include KA1 and KA2 and are only able to exist as part of heteromeric channels (Lerma et al., 2001, review). A characteristic of kainate receptors is to undergo rapid desensitisation, the time course of which is also dependent on subunit composition, as well as the cell type that the receptor is expressed in. In contrast to this, the subsequent recovery of the receptor is dependent on the agonist used, with more rapid recovery occurring with glutamate, and slower recovery with kainate (Paternain et al., 1998).

Kainate receptors are non-specific cation channels and so are able to carry ions such as K^+, Na^+ and Ca^{2+} across the cell membrane, leading to neuronal membrane depolarisation (Seeburg, 1993, review). Many of their presynaptic effects reflect these properties of the receptor, such as increasing glutamate release from cerebrocortical and CA3 synaptosomes (Malva et al., 1995; Malva et al., 1996; Perkinton and Sihra, 1999), and an initial enhancement in synaptic transmission at the Schaffer collateral and mossy fibre synapses in brain slices (Vignes et al., 1998; Schmitz et al., 2000). However, kainate has also been shown to decrease the release of glutamate from hippocampal synaptosomes (Chittajallu et al., 1996), and the synaptic transmission between various

hippocampal regions in rat brain slices (Kamiya and Ozawa, 1998; Vignes et al., 1998). The mechanism for this decrease has been proposed to occur at the level of Ca^{2+} influx, where Ca^{2+}-channels involved in the release process become inactivated following longer-term membrane depolarisation (Chittajallu et al., 1996; Kamiya and Ozawa, 1998). As well as regulating the excitatory synaptic transmission of a number of neurones, kainate receptors are also believed to be present on a subset of inhibitory nerve terminals (Lerma et al., 2001), where they have been shown to decrease the amplitude of the evoked inhibitory post synaptic potential (IPSP) (Rodriguez-Moreno et al., 1997; Min et al., 1999).

1.4.2 G-Protein Coupled Receptors

G-protein-coupled receptors (Parmentier et al., 2002) transduce signals from the cell surface to intracellular proteins through the activation of heterotrimeric G-proteins. They consist of seven α-helical transmembrane-spanning regions with an extracellular N-terminal domain and an intracellular C-terminal domain (Dohlman et al., 1987). There are over 1000 members of the GPCR family, making them the largest group of cell surface receptors encoded by the mammalian genome (Gutkind, 2000). Considering the size of the family, it is not surprising then, that they are responsible for mediating a diverse array of cellular signals stimulated by a wide range of agonists, including: hormones, phospholipids, neurotransmitters, vasoactive polypeptides, growth factors, odorants, photons, and taste ligands (Gutkind, 2000). Activation of GPCRs occurs following the binding of an extracellular ligand, which induces conformational changes in the α-helices and intracellular loops of the receptors. This results in the exposure of previously masked intracellular G protein binding sites. The interaction promotes the exchange of GDP on the G-protein for GTP, thereby effecting its activation (Wess, 1997;Bourne, 1997). The heterotrimeric G proteins consist of α, β and γ subunits. Binding of GTP to the α subunit of the G protein (Gα) causes it to dissociate from the βγ subunits by inducing conformational changes in three flexible 'switch regions' of Gα. This exposes the effector interaction sites on the βγ subunits and allows the targeting of the α subunits towards effector molecules such as adenylate cyclase (Lambright et al., 1994; Clapham and Neer, 1997).

There are currently believed to be about 20 different Gα subunits in existence, which have been divided into four families based on their sequence homology: Gs, Gi/o, Gq, and G_{12} (Wilkie et al., 1992). The four families have also been found to regulate different downstream effector molecules. Gαs, for example, stimulates adenylate cyclase resulting in the increase in intracellular levels of the second messenger, cAMP. Members of Gαi/o family, however, inhibit adenylate cyclase and so decrease the intracellular levels of cAMP (Simonds, 1999). Members of the Gαi/o family of α subunits have also been implicated in activating a variety of phospholipases and phosphodiesterases, as well as having direct interactions with some ion channels. They can directly increase K^+ channel activity as well as decrease Ca^{2+} channel activity (Hamm, 1998). Gαq family members have been linked with phospholipase C (PLC) activation, resulting in the increase in the production of the intracellular messengers: Ca^{2+} and diacylglycerol (DAG) (Rhee and Bae, 1997). The final family of Gα protein subunits, G_{12}, are believed to provide a link between GPCRs and the Rho family of small GTP-binding protein which are also involved in stimulating the activation of MAPK (Fromm et al., 1997; Fukuhara et al., 1999).

In contrast to Gα subunits, molecular cloning has so far only revealed the existence of 12 Gγ subunits and 6 Gβ subunits. Nonetheless, this still results in a substantial number of βγ dimers. It was originally thought that Gβγ subunits were passive in signalling terms, and that this was all carried out by the Gα subunit. Subsequent research is consistently finding more and more roles for the Gβγ subunit in signalling within cells; including activation of K^+-channels, inhibition of Ca^{2+} channels, and regulation of adenlyate cyclases, phosphatidyl-inositol-3-kinases (PI3Ks), phospholipases and receptor kinases (Clapham and Neer, 1997). It is also possible that there are distinct pools of Gβγ subunits, which mediate different processes in signal transduction depending on cell-type and/or subcellular localisation (Hamm, 1998).

The heterotrimeric G proteins involved in signalling downstream of GPCRs can, in part, be distinguished by the treatment of cells with specific bacterial toxins. Pertussis toxin catalyses the ADP-ribosylation of Gi and Go α-subunits resulting in their inactivation, however, it has no effect on Gq or Gs family members (Katada and Ui, 1982; Codina et al., 1983; Moss et al., 1984). Opposed to this treatment with pertussis toxin, Gs-type α-

subunits can be permanently activated by the toxin from Cholera vibrio (Kahn and Gilman, 1984b; Kahn and Gilman, 1984a).

1.4.2.1 Metabotropic Glutamate Receptors

Metabotropic glutamate receptors (mGluRs) were originally identified as a non-ionotropic component of glutamatergic signalling that could evoke phosphoinositide (PI) hydrolysis in rat brain slices (Nicoletti et al., 1986). The first mGluR to be identified, through expression cloning using a rat cerebellar library, was the receptor now known as rat mGluR1a (Houamed et al., 1991; Masu et al., 1991). Subsequent cloning experiments, using homology screening, led to the identification of 8 different mGluRs, which can be divided into 3 subgroups based on sequence homology and signal transduction systems (Nakanishi, 1992).

Group I contains the receptors; mGluR1, and its splice variants a, b, c, and e, and mGluR5 and its splice variants a and b. These receptors are primarily coupled to increased PIP_3 hydrolysis through Gq proteins (Pin and Duvoisin, 1995). Immunohistochemical evidence places these receptors at a post-synaptic location, however, there is more recent pharmacological evidence which suggests that these receptors may also be found presynaptically (Shigemoto et al., 1993; Herrero et al., 1998). The localisation of these receptors appears to be determined by 'homer' proteins, which selectively bind to Group I mGluRs and regulate their positioning near to intracellular messengers (Ciruela et al., 1999; Ango et al., 2000). Stimulation of presynaptic Group I mGluRs has been shown to increase glutamate release from nerve terminals, though coapplication of arachidonic acid is required in some systems (Herrero et al., 1998).

The Group II subgroup of mGluRs consists of mGluR2 and mGluR3 and are generally negatively coupled to adenylate cyclase (AC) through Gi proteins (Pin and Duvoisin, 1995). They have been located to perisynaptic areas, away from the site of transmitter release, in both pre- and post-synaptic neuronal locations (Petralia et al., 1997; Shigemoto et al., 1997). As well as this, mGluR3s have been found to be highly expressed in glial cells (Ohishi et al., 1993; Ohishi et al., 1994; Mineff and Valtschanoff, 1999). The perisynaptic localisation of mGluR2 on presynaptic terminals has been proposed to be important in the regulation of exocytotic cell death in

hippocampal neurones. The activation of these receptors appears to only occur following substantial glutamate release and results in the inhibition of further glutamate release, thus preventing excitotoxicity (Scanziani et al., 1997; Cartmell and Schoepp, 2000).

Group III contains the receptors; mGluR4, mGluR6, mGluR7 and mGluR8 and their splice variants. mGluR6 was originally believed to be exclusively expressed in ON bipolar cells in the retina, where it was found to have an important role in the amplification of visual inputs (Laurie et al., 1997; Ueda et al., 1997). However, more recent studies have also found mGluR6 to be expressed in microglia, where it may be involved in protecting microglia from over-stimulation-induced apoptosis (Taylor et al., 2003). mGluR4a, 7a, 7b and 8 have been identified in areas relating to presynaptic active zones in neurones (Shigemoto et al., 1997), with hippocampal mGluR7 being solely localised to glutamatergic neurones (Bradley et al., 1996). These receptors have been shown to be negatively coupled to AC, through Gi, and can reduce glutamate release from hippocampal neurones, as well as GABA release from striatal neurones (Lafon-Cazal et al., 1999) and cultured cortical neurones (Schaffhauser et al., 1998). Thus they are able to act both as autoreceptors and heteroceptors.

Not all the subtypes of mGluRs are found at every synapse, and their distribution can vary considerably even between terminals sharing the same axon. The presynaptic distribution of mGluRs appears to be heavily reliant on the postsynaptic identity (Scanziani et al., 1998; Shigemoto et al., 1996; Shigemoto et al., 1997). Despite these receptors being coupled to G-proteins and consisting of 7 transmembrane domains (7TMD), they have no sequence homology to the GPCRs mediated by 'classical' metabotropic neurotransmitters, such as muscarinic acetylcholine receptors. (Pin and Duvoisin, 1995). Rather mGluRs are members of the "Family 3" GPCRs, along with Ca^{2+}-sensing and $GABA_B$ receptors, and some putative olfactory, pheromone and taste receptors (De Blasi et al., 2001). These receptors have a large N-terminal extracellular domain (ECD) which consists of two lobes that are able to exist in an open or closed conformation. Agonist binding to the hinge region, located between the two lobes, is believed to stabilise the ECD in the closed conformation and bring about activation of the receptor. This mechanism for activation has become known as the 'venus fly-trap' model, as the opening and closing of the ECD in the absence and presence of agonists,

is believed to resemble the opening and closing of a venus fly-trap (Kunishima et al., 2000). The signal transduction downstream of glutamate binding to the ECD of mGluRs has been suggested to be conferred by the second intracellular loop of the 7TMD. This loop is believed to be important in coupling to G-proteins and in determining the transduction mechanism for the receptor subtype (Pin and Duvoisin, 1995; Gomeza et al., 1996). The third intracellular loop plays a crucial role in G-protein activation and controls the coupling efficacy in combination with the first intracellular loop and the C-terminal tail (De Blasi et al., 2001).

1.4.2.2 $GABA_B$ Receptors

GABA is believed to be the main inhibitory neurotransmitter found in the brain and is found to act at two different receptors, the ionotropic $GABA_A$ receptor and the metabotropic $GABA_B$ receptor (Stephenson, 1988; Bowery, 1989). It is the $GABA_B$ receptor which has been shown to be located presynaptically (as well as postsynaptically) and to regulate the release of neurotransmitters (Bowery et al., 1980; Hill and Bowery, 1981).

$GABA_B$ receptors have been found to be structurally related to the mGluR family and display the venus flytrap model of agonist binding (Kaupmann et al., 1997). Two distinct $GABA_B$ subunits have been identified; $GABA_{B1}$ and $GABA_{B2}$, which, combined with pharmacological studies, has led to the suggestion that the differing properties of pre- and postsynaptic $GABA_B$ receptors may be mediated by different subunits (Enna, 2001; Yamada et al., 1999). However, an absolute requirement for both these subunits in the formation of functional $GABA_B$ receptors has been demonstrated in knock out mice (Schuler et al., 2001), which, unusually for GPCRs, form heterodimers (Enna, 2001). Notwithstanding, it remains possible that as yet unidentified proteins are responsible for the observed differences (Enna, 2001).

The first presynaptic effect, now attributed to $GABA_B$ receptors, was demonstrated in spinal cord, where a decrease in neurotransmitter release from excitatory nerve terminals was observed (Pierau and Zimmermann, 1973). Subsequent electrophysiological studies conducted in the CA1 region of the hippocampus have supported this role for $GABA_B$ receptors (Lanthorn and Cotman, 1981; Potier and Dutar, 1993). As well as studies investigating the K^+-evoked release from purified

synaptosomes, which have found $GABA_B$ receptors to be involved in inhibiting GABA, glutamate and somatostatin release (Bonanno et al., 1991; Bonanno and Raiteri, 1992;Pende et al., 1993). More recent studies have reported on the mechanisms involved in $GABA_B$-mediated decreases in neurotransmitter release. Inhibition of glutamate release from cerebrocortical synaptosomes by the $GABA_B$ agonist, baclofen, was found to be dependent on the decreased conductance of the VDCCs linked to release (Perkinton and Sihra, 1998). These results have been supported by those obtained examining the calyx of held synapse, which also found that $GABA_B$ receptor activation led to inhibition of Ca^{2+}-channels via Gi/o proteins (Takahashi et al., 1998; Isaacson, 1998). Interestingly, and in contrast to postsynaptic $GABA_B$ receptors, none of these studies identified a GABA-mediated enhancement of K^+ conductance, which could, in theory, inhibit neurotransmitter release by reducing the nerve terminal excitability (Saint et al., 1990). As well as signalling through Gi/o to decrease adenylate cyclase activity, $GABA_B$ receptor signalling has also been shown to be regulated by PKC and to be able to activate ERK1/2 in the CA1 region of mouse hippocampus (Perkinton and Sihra, 1998; Vanhoose et al., 2002).

1.4.2.3 β-Adrenoceptors

There are currently believed to be three subtypes of β-adrenergic receptor, classified as β_1, β_2, and β_3. The most prevalent subtype found in forebrain regions, including the cortex, hippocampus and striatum, has been identified as the β_1 subtype, whereas β_2-adrenoceptors are the more prevalent in the cerebellum (Minneman et al., 1979; Garnier et al., 1997). These receptors have been shown to exist in both high and low affinity states, depending on their G-protein coupling. Activated β-adrenoceptors are generally believed to be in their high affinity conformation when bound to Gs, which results in the activation of adenylate cyclase leading to increased levels of cAMP and stimulation of PKA (De Lean et al., 1980; Rasenick et al., 1994). The third intracellular loop of β-adrenoceptors is believed to confer their G-protein binding specificity (Strader et al., 1994), with both the transmembrane segments 3 and 6 being involved in the activation of the G-protein (Ballesteros et al., 2001). Following activation, β-adrenoceptors have been demonstrated to undergo clathrin-coated endocytosis, possibly resulting in the stimulation of signalling cascades such as the ERK1/2 pathway (Daaka et al., 1998; Luttrell et al., 1999).

Activation of β-adrenoceptors has, for a while, been shown to be required for long term potentiation (LTP) in the dentate gyrus (Stanton and Sarvey, 1985b; Stanton and Sarvey, 1985a), a form of synaptic plasticity believed to be involved in mnemonic processes. More recent evidence in slices has suggested that β-adrenoceptors may be acting presynaptically to increase neurotransmitter release (Chavez-Noriega and Stevens, 1994; Huang and Kandel, 1996). This phenomenon has been confirmed by work using purified cerebrocortical nerve terminals, which found activation of β-adrenoceptors could enhance the depolarisation-induced release of glutamate (Herrero and Sanchez-Prieto, 1996; Wang et al., 2002). These downstream signalling effects of β-adrenoceptors have been demonstrated to be mediated through Gs activation of adenylate cyclase leading to increased levels of intracellular cAMP and subsequent activation of PKA (Huang et al., 1996; Huang and Kandel, 1996; Herrero and Sanchez-Prieto, 1996; Wang et al., 2002).

Recent evidence has suggested that β-adrenoceptors are able to cross-talk with other receptors in the control of signalling pathways. For example, activation of these receptors has been shown to result in the 'transactivation' of the epidermal growth factor receptor (EGFR) (Kim et al., 2002). The β_2-adrenoceptor has also been shown to be able to form heteromeric complexes with kappa opioid receptors, resulting in inhibition of β_2-adrenoceptor endocytosis and activation of ERK1/2 (Jordan et al., 2001).

1.4.2.4 Adenosine Receptors

Adenosine is a ubiquitous signalling molecule which appears to be released from all cells, including glia and neurones (Ribeiro et al., 2003). Intracellular adenosine is released through a nucleoside transporter which can also regulate reuptake, thus controlling the extracellular concentration of the nucleoside (Gu et al., 1995). As well as the intracellular synthesis of adenosine from 5'AMP, adenosine can also be synthesised in the extracellular space, from the breakdown of adenine nucleotides (Zimmermann and Braun, 1999). The location of adenosine production in cells may be of functional importance, as A_1 receptors have been shown to be preferentially activated

by released adenosine, whereas A_{2A} receptors were preferentially activated by the adenosine formed from adenine nucleotides (Cunha et al., 1996).

There are currently believed to be three different subtypes of adenosine receptor in cells: A_1, A_2, and A_3, with the A_2 subgroup further divisible into A_{2A} and A_{2B} (Klinger et al., 2002). Distribution studies have found A_1 to be highly expressed in the cerebral cortex, cerebellum, hippocampus and the dorsal horn of the spinal cord, whereas the A_{2A} receptors appear to be localised to striato-pallidal GABAergic neurones and the olfactory bulb, with lower levels of expression in the hippocampus, neocortex and thalamus (Reppert et al., 1991; Sebastiao and Ribeiro, 1996). This is in contrast to the A_{2B} and A_3 adenosine receptors which show low levels of expression throughout the brain (Dixon et al., 1996; Fredholm et al., 2001).

All the subtypes of adenosine receptor are asparagine-linked glycoproteins, which can couple to different G-proteins, with A_1 and A_3 found normally coupled to Gi/o, and A_{2A} and A_{2B} normally coupled to Gs (Feoktistov and Biaggioni, 1997; Linden, 2001; Ribeiro et al., 2003). This differential coupling of the adenosine receptor subtypes provides mechanisms for the expression of the diverse cellular effects mediated by these receptors. It can explain why the activation of adenosine receptors can lead to presynaptic inhibition and postsynaptic hyperpolarisation, mediated by A_1 and A_3 receptors, and to the potentiation of neurotransmitter release and postsynaptic depolarisation, following activation of A_{2A} or A_{2B} receptors (Klinger et al., 2002; Ribeiro et al., 2003). A_1 receptors can inhibit N-type voltage-gated Ca^{2+} channels, through a voltage-dependent and PTX-sensitive pathway (Park et al., 2001). A_2 receptors have been shown to increase levels of intracellular Ca^{2+} in some cells, and inhibit L-type Ca^{2+} channels in others (Gubitz et al., 1996; Goncalves et al., 1997; Stella et al., 2002). A_2 receptors can also inhibit Na^+ channels, through a mechanism possibly dependent on cAMP (Ribeiro and Sebastiao, 1987).

As well as a role in regulating general neuronal excitability, adenosine receptors have also been shown to directly modulate the activity of other cell surface receptors. For example, the coupling of adenosine A_3 receptors to Gq proteins, can lead to the stimulation of PKC, which in turn can lead to the inhibition of Group II and III mGluRs (Macek et al., 1998). Recent studies have found that neurotrophin TrkB receptors can

be activated in the absence of neurotrophin, but only as long as A_{2A} receptors are activated (Lee and Chao, 2001). A_{2A} receptors can also enhance the desensitisation of the nicotinic ACh receptor, possibly through a cAMP/PKA-dependent mechanism (Correia-de-Sa and Ribeiro, 1994). The G-protein coupling of adenosine receptors can leave them open to regulation by kinase signalling cascades, as mGluRs have been shown to inhibit A_1 activation through a mechanism dependent on PKC (de Mendonca and Ribeiro, 1997). The regulation of cell function by adenosine receptors is not limited to interactions between other types of receptor and the adenosine receptor, as A_{2A} receptors can also attenuate the actions of A_1 receptors through a PKC-dependent mechanism (Lopes et al., 1999).

Studies have suggested that adenosine is a molecule which has very important neuromodulatory roles in the CNS, and can both regulate the increase and decrease of neurotransmitter release from nerve terminals (Ribeiro et al., 2003).

1.4.3 Neurotrophin Receptors

There are five different neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and neurotrophin-6 (NT-6), which act at two different types of neurotrophin receptor: p75 receptors, that belong to the tumour necrosis factor (TNF) receptor superfamily; and tropomyosin-related kinase (Trk) receptors from the receptor tyrosine kinase (RTK) family (Poo, 2001). There are three main subtypes of Trk receptor found in cells; TrkA, B and C, which have also been found to exist as several different functional splice variants. p75 and Trk receptors are not thought to directly bind to each other, but signalling complexes have been found to form between them. p75 appears to confer increased ligand selectivity upon the Trks, for example, TrkB becomes specific for BDNF and TrkA specific for NGF, when complexed with p75 (Benedetti et al., 1993; Bibel et al., 1999; Lee et al., 2001).

Ligand binding to Trk receptors induces receptor dimerisation and autophosphorylation on tyrosine residues. Of the 10 tyrosine phosphorylation sites localised on the intracellular domain of Trk receptors, the three found on the autoregulatory loop are responsible for further activation of the kinase, whereas the remaining seven sites mediate the docking of adaptor proteins (Patapoutian and Reichardt, 2001).

Phosphorylation of tyrosine 490 has been shown to regulate the binding of the adaptor protein Shc, and tyrosine 785 can regulate interactions with PLC-γ1 (Obermeier et al., 1993; Cunningham et al., 1997). Although Trk receptors can be remarkably similar in structure, they are able to signal through different downstream cascades by interacting with different adaptor proteins. The recruitment of adaptor proteins such as Shc, or growth factor receptor-bound protein 2 (Grb2), can lead to the binding of guanine nucleotide exchange factors (GEFs) with proline-rich SH3 domain-binding sites, such as Sos1/2 (son of sevenless) and Ras-GRF (Li et al., 1993). The GEFs then catalyse the exchange of GDP for GTP in small guanine nucleotide binding proteins such as Ras, which are acting as molecular 'switches' by cycling between GDP and GTP bound forms. Ras then goes on the activate Raf, a broad specificity serine/threonine kinase which, in turn, activates mitogen- and extracellular-signal regulated kinase kinase (MEK) and ERK1/2 (Moodie et al., 1993). In conjunction with this well characterised activation of the ERK1/2 signalling cascade, Trk receptors can also activate PI3K, through Ras-dependent and –independent mechanisms, and stimulate PKCδ through activation of PLC-γ1, which can also lead to MEK activation (Burgering et al., 1993; Rodriguez-Viciana et al., 1994; Holgado-Madruga et al., 1997; Kaplan and Miller, 2000).

Following receptor activation, Trks can undergo endocytosis, a process which is believed to be crucial for some aspects of neurotrophin signalling (Poo, 2001). Neurotrophin-mediated signalling is implicated in a wide range of processes in cells; including regulation of cell survival, mediation of synaptic contacts during development, activation of gene transcription, maintenance of synaptic structure, and the regulation of synaptic plasticity (Poo, 2001; Kaplan and Miller, 2000). Of particular note with respect to this thesis, is the finding that BDNF-mediated activation of TrkB receptors on adult rat presynaptic cerebrocortical nerve terminals leads to increases in glutamate neurotransmitter release. This signalling pathway was found to be dependent on ERK1/2 activation and the downstream phosphorylation of sites 4/5 of synapsin I, which is believed to result in greater availability of synaptic vesicles for release (Jovanovic et al., 2000). The diversity of upstream signals which are able to stimulate ERK1/2 (Gutkind, 2000) has led to the possibility of a general role for this kinase in mediating cross-talk between signalling cascades in the regulation of glutamate release.

1.5 Signalling cascades downstream of Cell-Surface Receptors

Signalling cascades downstream of cell-surface receptors often involve the activation of protein kinases or phosphatases. Phosphorylation is an important means of posttranslational modification involving the transfer of a negatively charged phosphate group from ATP (or occasionally, GTP) to tyrosine or serine/threonine residues on substrate proteins (Engh and Bossemeyer, 2001). Modification by phosphorylation can induce substantial changes in proteins, resulting in alterations in their binding properties and/or conformation. In kinase signalling cascades, this can lead to activation, as in the case of extracellular signal-regulated kinase (ERK), or in some cases, can bring about deactivation, as with the phosphorylation by PKA on serine 259 of Raf-1 for instance (Dhillon et al., 2002), providing a very important means for regulating signalling in the cell. Enzymes that are able to mediate the addition of phosphate groups to substrates (phosphorylate) are termed protein kinases, whereas protein phosphatases catalyse the removal of phosphate groups (Sun et al., 1993).

1.6 Protein Kinases

There are believed to be over 1000 protein kinases in humans, with up to a third of all cytoplasmic proteins containing a covalently bound phosphate group at any one time. The catalytic domain structure of protein kinases is a highly conserved region (Hanks and Quinn, 1991) and all protein kinases require divalent metal ions, such as Mg^{2+}, for activity (Engh and Bossemeyer, 2001). The disregulation of protein kinases, often through spontaneous genetic mutation, has wide ranging implications in the process of serious diseases, such as cancer (Sherr, 1996). This current study investigates the relationships between three families of protein kinases: protein kinases A, protein kinases C and mitogen-activated protein kinases with respect to presynaptic function.

1.6.1 Protein Kinase A

Protein kinase A (PKA) is a broad specificity kinase which phosphorylates proteins on serine or threonine residues contained in the consensus sequence RRxS/Tx (Kemp and Pearson, 1990). PKA, in the inactive state, consists of two regulatory subunits (R) and two catalytic subunits (C) (Francis and Corbin, 1994). So far, four different isoforms of R have been identified, along with 3 different isoforms of C. The regulatory subunits have been shown to be able to form heterodimers, making it possible to have 24

different isoforms of PKA. However, not all the isoforms of the subunits exist in the same tissue, meaning the number of different PKAs found in reality is a lot less than that predicted on theoretical grounds (Beebe, 1994; Tasken et al., 1997).

1.6.1.1 Activation of Protein Kinase A

The understanding of how protein kinases are activated has been helped dramatically by obtaining crystal structures of the conformation and construction of a fully active protein kinase (Bossemeyer et al., 1993). The structure of a PKA catalytic subunit was one of the first to be determined (Knighton et al., 1991). In the inactive state, PKA exists as a tetramer, where the catalytic subunits are stabilised by the binding of pseudosubstrate regions found on the regulatory subunits. The pseudosubstrate motif consists of RRxG/Ax, where non-phosphorylatable glycine or alanine residues have replaced the serine or threonine residues found on the genuine substrates (Soderling, 1993). Each regulatory subunit also possesses two similar binding sites for 3',5'-cyclic adenosine monophosphate (cAMP), meaning each PKA enzyme requires four cAMP molecules for activation. Upon binding of one cAMP molecule, the affinity of the other binding sites for cAMP is increased, showing that cAMP binding is cooperative. Upon complete occupancy of the cAMP binding sites, the stability of the R_2C_2 complex declines and the R subunits dissociate from the C subunits, allowing the C subunits to phosphorylate their target substrates. Following dissociation of the subunits the affinity of the R subunits for cAMP decreases, resulting in the release of cAMP back into the cytoplasm (Scott, 1991; Taylor et al., 1993; Tasken et al., 1997). As well as requiring cAMP-binding for activation, PKA also requires the phosphorylation of a threonine residue found in the activation loop (Thr197) (Adams et al., 1995). Phosphorylation of this residue can either occur via phosphoinositide-dependent protein kinase (PDK1) (Cheng et al., 1998), or through autophosphorylation (Girod et al., 1996).

1.6.1.2 Roles of Protein Kinase A

PKA has been shown to be able to regulate gene transcription through phosphorylation of the cAMP response element binding protein (CREB). The increase in gene transcription mediated by CREB is thought to underlie the process of LTP and to regulate many of the longer term stimulus-induced post-synaptic changes (Bito et al., 1996). PKA can also phosphorylate and inhibit Group II mGluRs in the dentate gyrus

and CA3 area of the hippocampus, and Group III mGluRs in the dentate gyrus and CA1 area of the hippocampus. This effectively decreases the inhibitory effects of these receptors on glutamate release (Kamiya and Yamamoto, 1997; Schaffhauser et al., 2000).

1.6.1.3 Signalling Upstream of PKA: Cyclic Adenosine Monophosphate (cAMP) Second Messenger Production.

cAMP is synthesised from ATP (adenosine triphosphate), a common constituent of cells, by a reaction which is catalysed by the membrane bound adenylate cyclase (AC) (Tang and Hurley, 1998). The subsequent removal of cAMP is highly regulated by the action of phosphodiesterases which catalyse the conversion of cAMP into 5'-AMP (Drummond, 1973). cAMP was the first cellular second messenger to be identified, by Sutherland, following experiments looking at glycogenolysis in dogs (Berthet et al., 1957). Work carried out since then has identified signalling roles for the second messenger throughout evolution, ranging from the prokaryotes: Eubacteria and Archaea, through to single cellular eukaryotes like yeast, and then on to multicellular plants, rats and humans (Botsford and Harman, 1992; Thorner, 1982; Bolwell, 1995; Castagna, 1983; Scarpace et al., 1991). The diversity of systems in which cAMP is used as a messenger could perhaps be accounted for by its roles in mediating responses to starvation. cAMP is also able to regulate ion channels in some systems, such as in olfactory cells and in plants (Bradley et al., 1994; Li et al., 1994). More recent evidence has also implicated cAMP as having a direct signalling role to protein kinase cascades, other than PKA (de Rooij et al., 1998).

1.6.1.4 Signalling Upstream of Protein Kinase A: Adenylate Cyclase

Adenylate cyclase (AC) can be structurally divided into four main domains starting from the N-terminus; M_1, C_1, M_2 and C_2. M_1 and M_2 consist of six transmembrane α-helices each, whereas C_1 and C_2 are extensive cytoplasmic domains (Krupinski et al., 1989). The catalytic site for synthesis of cAMP from ATP is located within the C_1 and C_2 domains, along with several important regulatory sites (Tang and Gilman, 1995; Yan et al., 1997). To date, nine different isoforms of adenylate cyclase (AC) have been cloned and identified in mammals. They are labelled I-IX and have been divided into classes depending on their activation properties. Class 1 includes AC II, IV and VII;

Class 2 contains AC V and VI; and Class 3 consists of AC I, III, and VIII. All mammalian ACs identified so far can be activated by Gαs subunits and inhibited by so-called P-site inhibitors such as deoxy-3'-AMP (Tang and Hurley, 1998; Hurley, 1999).

Table 1.6.1 Properties of the Different Adenylate Cyclases.

Adenylate Cyclase	Localisation	Activators	Inhibitors
I	neural tissue	Gαs, forskolin, Ca^{2+}.Calmodulin	βγ subunits, Gαi
II	brain, lung	Gαs, forskolin, PKC, βγ (synergistically with Gαs)	
III	mainly olfactory	Gαs, forskolin, Ca^{2+}.Calmodulin	
IV	broad distribution	Gαs, forskolin, βγ subunits (synergistically with Gαs)	
V	mainly heart, some brain	Gαs, forskolin, PKC	PKA, Gαi, Ca^{2+}
VI	mainly heart, brain, some other tissue	Gαs, forskolin,	PKA, Gαi, Ca^{2+}, PKC
VII	broad distribution	Gαs, forskolin, PKC, βγ (synergistically with Gαs)	
VIII	neural tissue	Gαs, forskolin, Ca^{2+}.Calmodulin	
IX		Gαs	

It is possible that some types of AC may have the potential to act as coincidence detectors in some systems. For example, βγ subunits do not appear to stimulate AC II, IV, and VII on their own, but their presence results in a synergistic activation by Gαs. However, the concentration of βγ subunits needed to produce this effect is greater than the number produced by Gs alone, suggesting a need for simultaneous Gi/o activation.

This means, to elicit activation of AC with βγ subunits, the simultaneous stimulation of two types of GPCR (Gi/o and Gs) with different effectors, would be required (Sunahara et al., 1996). This is an example of short term coincidence detection by AC, but longer lasting mechanisms can be induced by phosphorylation of the enzyme. PKC activation through Gq-mediated pathways can result in the phosphorylation of type II, V, and VII AC, which may prepare the enzyme for the transient stimulation by Gαs (Summers and Cronin, 1986). Not all phosphorylations of AC are excitatory however, PKA has been shown to inhibit AC V and VI through a phosphorylation mediated by a negative feedback loop (Chen et al., 1997). PKC has also been shown to phosphorylate and inhibit AC VI (Lai et al., 1997).

1.6.2 Protein Kinase C

Protein kinase C (PKC) was initially identified as a cyclic nucleotide-independent protein kinase present in bovine cerebellum (Takai et al., 1977). Since then, molecular cloning has revealed the presence of a total of 12 different mammalian isoforms of PKC (Parker et al., 1986; Coussens et al., 1986). These isoforms have been split into three subgroups depending on their activation properties and sequence homology (Way et al., 2000). The first subgroup of PKCs has become known as the conventional PKCs (cPKC), these require Ca^{2+}, DAG and a phospholipid, such as phosphatidylserine, for activation. Included in this subgroup are the α, β1, β2 and γ isoforms of PKC. The second subgroup of PKCs are known as the novel PKCs (nPKC) and, like cPKCs, they also require DAG and phospholipid for activation, but are Ca^{2+}-independent. Four isoforms are included in this subgroup; δ, ε, η, and θ. The remaining subgroup of PKCs contains PKCλ, ι, ξ, and μ, these require phospholipid but are not activated by Ca^{2+} or DAG, which has led their being labelled as atypical PKCs (aPKC) (Majewski and Iannazzo, 1998).

1.6.2.1 Activation of Protein Kinase C

PKC proteins consist of 4 domains (with the exception of aPKC), identified as C1, C2, C3 and C4 when starting from the N-terminus, with C1 and C2 acting as regulatory domains and C3 and C4 as catalytic domains (Newton, 1995). Activation of cPKCs and nPKC occurs in four stages. The first stage involves the phosphorylation of the C4 domain at Thr-500 by a PKC kinase, such as PDK1. This subsequently allows

autophosphorylation to occur at sites Thr-641 and Ser-660, which are also found on the C4 domain (Keranen et al., 1995). These phosphorylations render the kinase catalytically competent but still inactive due to the proximity of a pseudosubstrate region found on the N-terminus of the protein. The third stage of activation occurs with DAG binding to the C1 domain, combined with Ca^{2+} binding to the C2 domain (when present). This renders the regulatory domain less hydrophilic, and in doing so, encourages interactions between the C2 domain and the phospholipid membrane of cells. The final stage of activation occurs when the pseudosubstrate region is distanced from the catalytic domain by the conformational change in the protein resulting from binding of the C2 domain to phospholipids. This exposes the catalytic domain of the PKC to intracellular substrates, so rendering the kinase active (Newton, 1997).

1.6.2.2 Signalling upstream of Protein Kinase C: Lipids

All PKCs require the binding of a phospholipid for activation (Bell and Burns, 1991). Phospholipids are common constituents of the membrane bilayer in cells. They all consist of a glycerol backbone which is esterified at its first and second hydroxyl groups to fatty acids. The third hydroxyl group contains a diester of phosphoric acid and an alcohol (Folch and Lebaron, 1956; Lebaron and Folch, 1957). The name given to the phospholipid is determined by the alcohol attached to the third hydroxyl group:

Table 1.6.2 Phopholipids

ALCOHOL	PHOSPHOLIPID
Choline	Phosphatidylcholine (PC)
Serine	Phosphatidylserine (PS)
Ethanolamine	Phosphatidylethanolamine (PE)
Inositol	Phosphatidylinositol (PI)

The most common phospholipid involved in the activation of PKCs is phosphatidylserine (PS), occurring when PKC interacts with the cell membrane, however, products of phospholipid metabolism can also be involved in regulating the activation of PKCs (Bell and Burns, 1991). Phosphoinositide (PI) can undergo two further phosphorylations by phosphatidylinositol 4-kinase (PI4K) and then phosphatidylinositol-4-phosphate 5-kinase (PI5K) to become phosphatidylinositol-4,5-

bisphosphate (PIP_2) (Fruman et al., 1998). PIP_2 is a major substrate for phospholipase C (PLC), which cleaves the phospholipid into diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP3) (Rhee and Bae, 1997). DAG is hydrophobic and, because of its relatively small size, it is able to travel laterally along the lipid membrane and go on to activate novel and conventional PKCs (Newton, 1997). However, DAG is rapidly metabolised, either into glycerol and fatty acids by DAG lipase, or into phosphatidate by DAG kinase, thus resulting in a transient activation of n and cPKCs (Topham and Prescott, 1999). IP3, in contrast to DAG, is a water-soluble second messenger which combines with specific receptors on membrane organelles (endoplasmic reticulum and mitochondria) to release calcium (Ca^{2+}) from endogenous stores (Berridge, 1993). This rise in intracellular Ca^{2+}-levels can also increase the activation of cPKCs, as well as modulating many other signalling pathways (Hug and Sarre, 1993; Verkhratsky and Petersen, 1998). As well as PIP_2 being an important source of DAG and IP3, it can itself be further phosphorylated by phosphoinositide-3-kinase (PI3K) to form the product phosphoinositol-3,4,5-triphosphate ($PI3,4,5P_3$). PI(4)P can also be phosphorylated by PI3K to form phosphoinositol-3,4-bisphosphate ($PI(3,4)P_2$) (Anderson et al., 1999). Both $PI3,4,5P_3$ and $PI3,4,P_2$ are able to increase the activation of the nPKCs, δ,ε, and θ, and of the aPKC, ζ (Toker et al., 1994).

Another phospholipid that can be metabolised into products which regulate PKC activation is phosphatidylcholine. This can be cleaved by phospholipase A_2 (PLA_2) to release the unsaturated fatty acid, arachidonic acid (AA). The effect of DAG upon cPKCs and nPKCs is believed to be potentiated by AA, negating the need for increases in intracellular Ca^{2+} concentrations. Lysophosphatidic acid and lyso-phosphatidylcholine can also enhance the activities of nPKCs and cPKCs (Sando and Chertihin, 1996). Phospholipase D (Pastorino et al., 2000) is also able to hydrolyse phosphatidylcholine to produce phosphatidate and choline. Subsequent removal of the phosphate group of phosphatidate by phosphatidate phosphohydrolase results in the production of DAG. The synthesis of DAG in this manner provides a less transient source of this second messenger (Martin, 1988).

1.6.2.3 Phospholipase C

There are currently believed to be three main classes of PLC: PLCβ, γ and δ. All these

PLCs possess a PH domain, EF-hand motifs, a C2 domain, and two catalytic subdomains known as X and Y. The PH domain is possibly involved in tethering the enzyme to the phospholipids membrane and the EF-hands may bind Ca^{2+} (Rebecchi and Pentyala, 2000). PLCδ is believed to be an ancestral form of PLC as it is simpler than the other enzymes and does not require receptor activation, though it does require Ca^{2+} (Essen et al., 1996; Singer et al., 1997). PLCβ isoforms have been linked with signalling downstream of Gq and Gi/o-coupled GPCRs (Zhu and Birnbaumer, 1996). The α-subunit from Gq-coupled receptors has been shown to interact with the extended C-terminal region of PLCβ, resulting in the activation of the enzyme (Smrcka et al., 1991). In contrast to this, it is the βγ subunit that activates PLCβ from Gi/o-coupled receptors, by binding to a site inbetween the X and Y subdomains (Kuang et al., 1996). Activation of the γ isoforms of PLC can be mediated by receptor tyrosine kinases (RTKs), due to extra SH3 and SH2 domains located in between the X and Y catalytic regions. The binding of a stimulatory ligand to RTKs results in the phosphorylation of a tyrosine residue found within the intracellular domain of the receptor. The SH2 domains of PLCγ enable it to bind to these phosphorylated intracellular domains and in doing so, become phosphorylated and activated (Meisenhelder et al., 1989; Anderson et al., 1990; Nishibe et al., 1990). PLCγ can also be phosphorylated and activated by accessory, non-receptor tyrosine kinases (Park et al., 1991b; Park et al., 1991a).

1.6.3 Mitogen-Activated Protein Kinase Family

The mitogen-activated protein kinases (Baldassa et al., 2003) are a specialised family of serine/threonine phosphorylating kinases which show a high degree of evolutionary conservation (Camps et al., 2000). Their signalling cascades are believed to have a major role in cells in providing a link between cell-surface receptors and important regulatory processes within the cell, such as gene transcription (Pages et al., 1993). The first members of the MAPK family to be identified were the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2). These were found to be phosphoproteins that were stimulated by mitogen and, as a consequence of this, are also known as p42 and p44 MAPK (Cooper and Hunter, 1983). Subsequent studies in the late 1980's further identified ERK1/2 as enzymes which phosphorylated myelin basic protein (MBP) and microtubule-associated protein 2 (MAP2) (Ray and Sturgill, 1987; Hoshi et al., 1988). Advances in drug development and gene cloning have led to the

identification of at least a further 3 major subgroups of MAPKs in mammals (Chang and Karin, 2001). These are the c-Jun N-terminal kinases/stress-activated protein kinases (JNK/SAPK 1, 2, and 3), p38 proteins (p38α, β, γ (also known as ERK6 or SAPK3) and δ), and ERK5 (or big mitogen-activated protein kinase 1, BMK-1, or SAPK5). These kinases are activated by a wide range of stimuli and, as a result, are involved in a wide range of cellular processes such as regulating cell morphology, survival and neurotransmitter release (Seger and Krebs, 1995;Jovanovic et al., 2000). A group consisting of ERK3 and ERK 7 has also recently been identified, but their roles in cells remain to be determined.

MAPKs are activated by a dual phosphorylation of a threonine and a tyrosine residue found within the activation loop of the kinase subdomain VIII (with the exception of ERK3) (Cobb and Goldsmith, 1995). The phosphorylations are not processive, but rather occur through a two-collision distributive mechanism, despite this, the tyrosine is most commonly phosphorylated first (Ferrell and Bhatt, 1997a; Burack and Sturgill, 1997). Removal of either phosphate group will inactivate the kinase, showing that the phosphorylation of both the threonine and the tyrosine residue is required for the activation of the kinases (Payne et al., 1991). The tyrosine and threonine residues are found in a TxY motif that is characteristic of MAPKs. The residue labelled x refers to glutamate in ERKs, proline in JNKs, and glycine in p38 MAPKs. When activated, these kinases in turn phosphorylate serine or threonine residues preceding a proline residue on their target proteins, with specificity being conferred by additional docking sites (Kallunki et al., 1996; Tanoue et al., 2000).

The dual specificity kinases responsible for the phosphorylation of MAPKs are termed mitogen activated protein kinase kinases (MKKs or MEKs). MKKs are also activated by phosphorylation, which is brought about by the serine/threonine MAPK kinase kinases (MAPKKK or MKKK), thus forming a three kinase cascade, characteristic of MAPK signalling. The reasons behind having a three tier kinase cascade are still under debate but one possibility is, in this case at least, that it results in an amplification of the original signal. The finding that cells contain higher levels of MKK compared to MKKK supports the idea of signal amplification. The levels of MKKs and MAPKs, however, have been found to be similar in cells, a property thought to provide a

mechanism for creating a threshold to filter out noise from subthreshold levels (Ferrell, 1997b). Cells have also been found to contain many more different types of MKKKs than MKKs, which is perhaps why such a diversity of signals is able to feed into the MAPK signalling cascades.

1.6.3.1 c-Jun N-Terminal Kinases

The JNKs were initially discovered in 1990 as a cycloheximide-activated proline-directed kinase and in 1993, as a protein which phosphorylated the N-terminal of c-Jun in response to UV light (Kyriakis and Avruch, 1990; Hibi et al., 1993). The activation of JNK1, 2 and 3 is regulated by just two MKKs; JNK kinase 1 and 2. JNKK1 is also known as SEK1, MKK4, or SKK1 whereas JNKK2 has also been named MKK7 and SKK4. These proteins kinases have been found to be activated following trophic factor deprivation, treatment with inflammatory cytokines and factors stimulating cell stress, such as heat and osmotic shock, UV light, oxidants and DNA damaging agents. Downstream of activation, the JNKs are involved in pathways controlling T-cell differentiation, apoptotic cell death, and in the production of inflammatory cytokines and eicosanoids (English et al., 1999; Gutkind, 2000). This can include the phosphorylation of Jun proteins to enhance the activation of transcription, without affecting DNA binding (Kallunki et al., 1996) and, mediating the stabilisation of IL-2 messenger RNA in T cells (Chen et al., 2000).

1.6.3.2 p38 Proteins

These proteins were initially discovered as a lipopolysaccharide-induced tyrosine phosphoprotein and as a target of a drug which had been developed to inhibit LPS-induced tumour necrosis factor-α biosynthesis (Han et al., 1994; Lee et al., 1994; Stawowy et al., 2003). The phosphorylation and activation of the four isotopes of p38, (α,β,γ and δ) is regulated by two upstream kinases: MKK3, also known as SKK2, and MKK6, which is also termed MEK6 and SKK3. Like JNKs these enzymes have been found to be activated by trophic factor deprivation, inflammatory cytokines and a number of cell stresses, such as heat and osmotic shock, UV light, oxidants and DNA damaging agents. As a result, they are also involved in pathways controlling T-cell differentiation, apoptotic cell death, and in the production of inflammatory cytokines and eicosanoids. As well as these roles, studies have also implicated the p38 proteins in

B cell proliferation and muscle cell differentiation, following the phosphorylation of MEF2 transcription factors (Craxton et al., 1998; Zhao et al., 1999).

1.6.3.3 Extracellular Signal-Regulated Kinases 3, 5 and 7

ERK3 is the enzyme in the MAPK family whose catalytic region shares the most sequence homology with ERK2. Unlike ERK2 and the other MAPKs, ERK3 contains a phosphorylation motif consisting of a serine and a glycine residue, rather than the standard threonine-tyrosine dual motif (Boulton et al., 1991). ERK3 is also constitutively localised to the nucleus, whereas ERK2 only translocates to the nucleus upon activation (Cheng et al., 1996). The spatial localisation of ERK3 is believed to be conferred by its C-terminal tail (Robinson et al., 2002). ERK3 mRNA is upregulated in a p38-dependent manner following treatment of cells with proteasome inhibitors and may also be involved in cell cycle arrest (Zimmermann et al., 2001), though downstream substrates are yet to be identified.

ERK 5 was first identified, through cloning, as a homologue of ERK1/2 and as a protein that interacted with MEK5 (Lee et al., 1995; Zhou et al., 1995). It has been reported to respond to both growth factors and stressful stimuli, but less is known about this kinase compared to the rest of the MAPK family. Studies have shown a requirement for ERK5 in cell proliferation induced by epidermal growth factor and an ability of the kinase to phosphorylate the transcriptional regulators, MEF2 and Myc (Kato et al., 1998; English et al., 1998). Like ERK3, ERK5 also possesses a long C-terminal tail, though its function remains to be determined.

ERK7 was first cloned in 1999, where it was identified as a novel, 61kDa, member of the MAPK family of proteins. It was not found to be activated by stimulators of the other members of the MAPK family, such as growth factors or stress, but was found to be constitutively active in serum-starved cells. This kinase was also found to contain a C-terminal tail domain, which is in contrast to the other MAPK family members (Abe et al., 1999). The purpose of the C-terminal domain is still under investigation but it is, so far, believed to convey localisation of ERK7 to the nucleus and to regulate its kinase activity through multiple interactions. ERK7 has also been shown to undergo autophosphorylation as a form of activation, in the absence of an upstream MEK (Abe et al., 2001)

1.6.3.4 Extracellular Signal-Regulated Protein Kinase 1 and 2

ERK1 and 2 are the main focus of this study, and hence shall be discussed in more detail than the other members of the MAPK family. The three dimensional structure of ERK1 and 2 consists of a larger C-terminal domain and a smaller N-terminal domain, linked by a crossover region (Zhang et al., 1994). ATP is found to bind at a site deep within the region formed between the interface of the two domains, termed the catalytic cleft. Protein substrates bind to the surface of the kinases (Zhang et al., 1994). The tyrosine and threonine residues phorphorylated by MEK1/2 are 185 and 183 respectively, on human ERK1 and 204 and 202 respectively, on mouse ERK2 (Ray and Sturgill, 1988; Payne et al., 1991). Phosphorylation is believed to provoke both local and global conformational changes in the enzyme, to bring about activation. In the low activity state, the position of the Thr-183 and Tyr-185 residue-containing phosphorylation lip of ERK2, reduces the accessibility of the active site to substrates. Phosphorylation of these residues changes the conformation of ERK2 and allows the exposure of the active site to downstream substrates. MEK1 and 2 are the protein kinases which mediate the phosphorylation of these residues in ERK1 and 2 (Zhang et al., 1994; Cobb and Goldsmith, 1995).

ERK1 and 2 were originally identified by their roles in developmental and differentiation processes, downstream of neurotrophin receptor activation. They were found to be involved in cell cycle progression and mitogenesis, and neuronal differentiation and survival, with constitutively active Ras/Raf/ERK pathways taking on oncogenic properties (Gutkind, 2000). However, high levels of ERK1 and 2 persist in the post-mitotic, adult nervous system, implicating a post-developmental role for the kinases (Thomas and Hunt, 1993). A substantial amount of work has been carried out investigating the role of ERK1/2 signalling in long term potentiation (LTP) and long term depression (LTD) in the CA1 region of the hippocampus (English and Sweatt, 1997; Norman et al., 2000). LTP and LTD are used as a cellular model of learning and memory as they refer to the ability of various signals to modulate synaptic strength over long periods of time (Bear and Malenka, 1994). The involvement of ERK1 and 2 in the postsynaptic changes associated with LTP and LTD have been well documented, where they have been found to mediate the transcription of immediate early genes (Adams et al., 2000). ERK1/2 have been shown to be stimulated downsteam of receptor activation

and of increases in intracellular Ca^{2+} concentration, as well as being involved in cross-talk with the PKA and PKC signalling cascades, amongst others (Roberson et al., 1999, Sweatt, 2001). It is only recently that ERK1 and 2 have become associated with signalling pathways that are independent of nuclear translocation and regulation of gene transcription. The abundant presynaptic protein, synapsin I, has been identified as a substrate of ERK1/2 in nerve terminals, where it is believed to regulate the interactions of synapsin I with actin (Jovanovic et al., 1996). The synapsins are proteins which tether neurotransmitter-containing vesicles to the actin cytoskeleton, away from the plasma membrane (Hilfiker et al., 1999). Phosphorylation of synapsin by ERK1,2 has been suggested to be a mechanism for increasing glutamate release from presynaptic nerve terminals (Jovanovic et al., 2000). Figure 1.1 illustrates the established trkB/ERK1,2/synapsin pathway which has been identified in nerve terminals, along with possibilities for further interactions of this pathway with other signalling cascades, as discussed later in this introduction.

1.7 Signalling Upstream of ERK 1,2

The kinases involved in phosphorylating, and hence activating, ERK 1,2 are MEK1 and MEK2 (Seger et al., 1992). These are, in turn, activated by the phosphorylation of serine or threonine residues, generally brought about by Raf-1 or B-Raf (MKKKs) (Kyriakis et al., 1992; Dent et al., 1992). The MKKKs are quite a diverse family of protein kinases. Structurally, they can contain Pleckstrin homology (PH) domains, binding sites for GTP-binding proteins, leucine-zipper dimerisation sequences, proline-rich sequences for binding Src homology-3 domains (Ringstad et al., 1999), and phosphorylation sites for tyrosine and serine/threonine kinases (Garrington and Johnson, 1999). The three main types of Raf kinases that have been identified are c-Raf-1 (Raf-1), B-Raf, and A-Raf. Raf-1 is ubiquitously expressed in mouse, whereas B-Raf and A-Raf have much more restricted distributions, with B-Raf being localised to the brain and testis and A-raf to urogenital tissue (Hagemann and Rapp, 1999). As well as different distributions, these Raf kinases are also subject to differential regulation by upstream Ras family proteins. For example, the activity of Raf-1, downstream of Ras activation has been shown to be antagonised by the competitive binding of Rap1 to the Raf-1 site normally activated by Ras. This is in contrast to B-Raf, which was found to be activated by Rap-1 (Hagemann and Rapp, 1999). More recent studies have

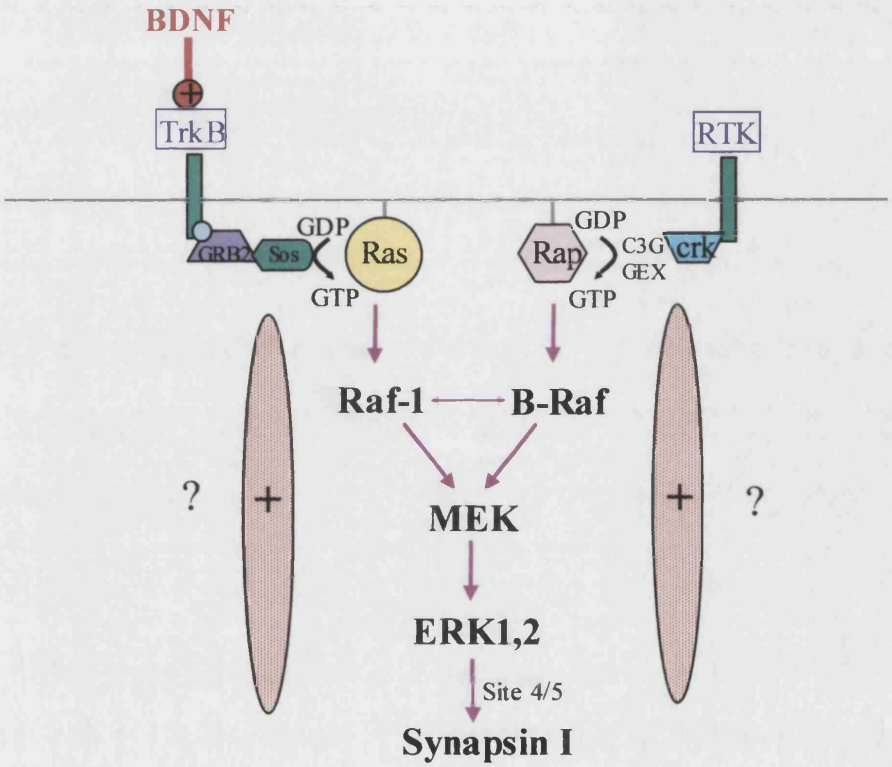


Figure 1.1 Schematic Diagram Illustrating the Mechanisms for Receptor-Tyrosine Kinase Activation of ERK 1,2 in Nerve Terminals.
BDNF has been shown to stimulate trkB receptors leading to the downstream activation of ERK 1,2 and synapsin I, and the enhancement of depolarisation-induced glutamate release. The specific roles for Raf-1 and B-Raf remain to be clarified. The large ovals indicate the potential for signalling cascades to cross-talk with this pathway at multiple loci. Abbreviations: BDNF = brain-derived neurotrophic factor, GDP = guanosine diphosphate, GTP = guanosine triphosphate, RTK = receptor tyrosine kinase, MEK = mitogen activated protein kinase kinase, ERK = extracellular signal-regulated protein kinase.

uncovered functional roles for these different signalling pathways within neuronal cells, where it has been suggested that activation of Raf-1 mediates transient signalling pathways, and that activation of B-Raf leads to a more sustained enhancement in ERK 1,2 phosphorylation (York et al., 1998). Both these pathways have also been shown to be regulated by cAMP/PKA signalling, whereby phosphorylation of Raf-1 by PKA leads to inhibition of ERK 1,2 activation, and stimulation by cAMP or PKA of a B-Raf-mediated pathway leads to enhanced activation of ERK 1,2 (Gutkind, 2000; Dhillon et al., 2002).

In contrast to Raf kinases, the Ras family of small GTP-binding proteins are highly conserved and are able to bind the same effectors and upstream GEFs. However, they do contain a 24 residue C-terminal hypervariable region which is believed to regulate the membrane microlocalisation of the protein, leading to biological differences in signalling (Prior and Hancock, 2001). As well as targeting Raf activation, Ras proteins also have the capacity to bind to and activate at least two other families of downstream effectors, including PI3K and Ral-specific GEFs (Ral-GEFs) (Shields et al., 2000). The Ras/Ral-GEF pathway has been shown to be involved in promoting cell cycle arrest following treatment with nerve growth factor, a complete opposite to the Ras/Raf pathway, which was found to promote cell proliferation. The Ras/Raf pathway has also been known to be stimulated for hours at a time, whereas Ras/Ral-GEF stimulation occurs as a short lived activation which is terminated within minutes by a PKC-dependent mechanism (Rusanescu et al., 2001).

1.8 Factors Determining Signalling Specificity of MAPKs

Considering the diversity of signals which are able to feed into the MAPK signalling cascades there must be some mechanisms in place which are able to regulate the specificity of the signalling, to ensure the correct pathways are followed for each stimulation. There are currently believed to be three mechanisms through which signalling specificity can be determined: through scaffolding proteins, sequential physical interactions, and through spatial localisation.

Scaffolding proteins have been found to be involved in both positive and negative regulatory mechanisms. They can bring proteins closer together for interaction, or they keep proteins apart, preventing interaction. For example, the scaffolding protein, MEK

partner 1 (Schaeffer et al., 1998), has been shown to bind specifically to MEK1 and ERK 1, leading to the facilitation of their activation (Schaeffer et al., 1998), and the kinase suppressor of Ras (Morrison, 2001) scaffold seems to localise MEK to the plasma membrane in a manner dependent on Ras activation, where it promotes the assembly of a multiprotein signalling complex to bring MEK into close proximity with its activators and substrates (Morrison, 2001). The differential expression of scaffolding proteins can also contribute to the control of subcellular localisation of some proteins and, like protein kinases, scaffolding proteins can also be controlled by post translational modification (Garrington and Johnson, 1999).

As well as scaffolding proteins, highly specific sequential physical interactions can also regulate the specificity of MAPK signalling cascades, as for example, MEKs have been found to be extremely specific for their MAPK substrates. This results in minimum variability in MAPK/MEK interactions, conferring specificity of the pathway (Garrington and Johnson, 1999). The sequential nature of the signalling cascades also help to confer specificity, where each interaction is disrupted on the activation of the downstream kinase (Xia et al., 1998).

The final mechanism for conferring the specificity of MAPK signalling cascades is through spatial localisation. Different MKKKs can be localised to different areas of cells. For example, cytoskeletal rearrangements may stimulate MEKK1 activity when MEKK1 is colocalised with elements of the cytoskeleton, leading to modulation of cell motility (Yujiri et al., 1998; English et al., 1999; Xia et al., 2000). Ras and Rap have also been shown to have different subcellular distributions, with Ras being mainly localised in the plasma membrane whereas Rap has also been found in the mid-Golgi, endocytic vesicles and lysosomes distribution, depending on cell type (Zwartkruis and Bos, 1999).

1.9 Phosphatases

Phosphatases are proteins that mediate the removal of a phosphate group from their substrates. This can result in changes in the binding properties or conformation of the substrate, leading to activation or deactivation. In the past, protein kinases have been maintained as the focus for regulating cell signalling, however, the finding of increasingly complicated mechanisms for the regulation of phosphatases, implies that

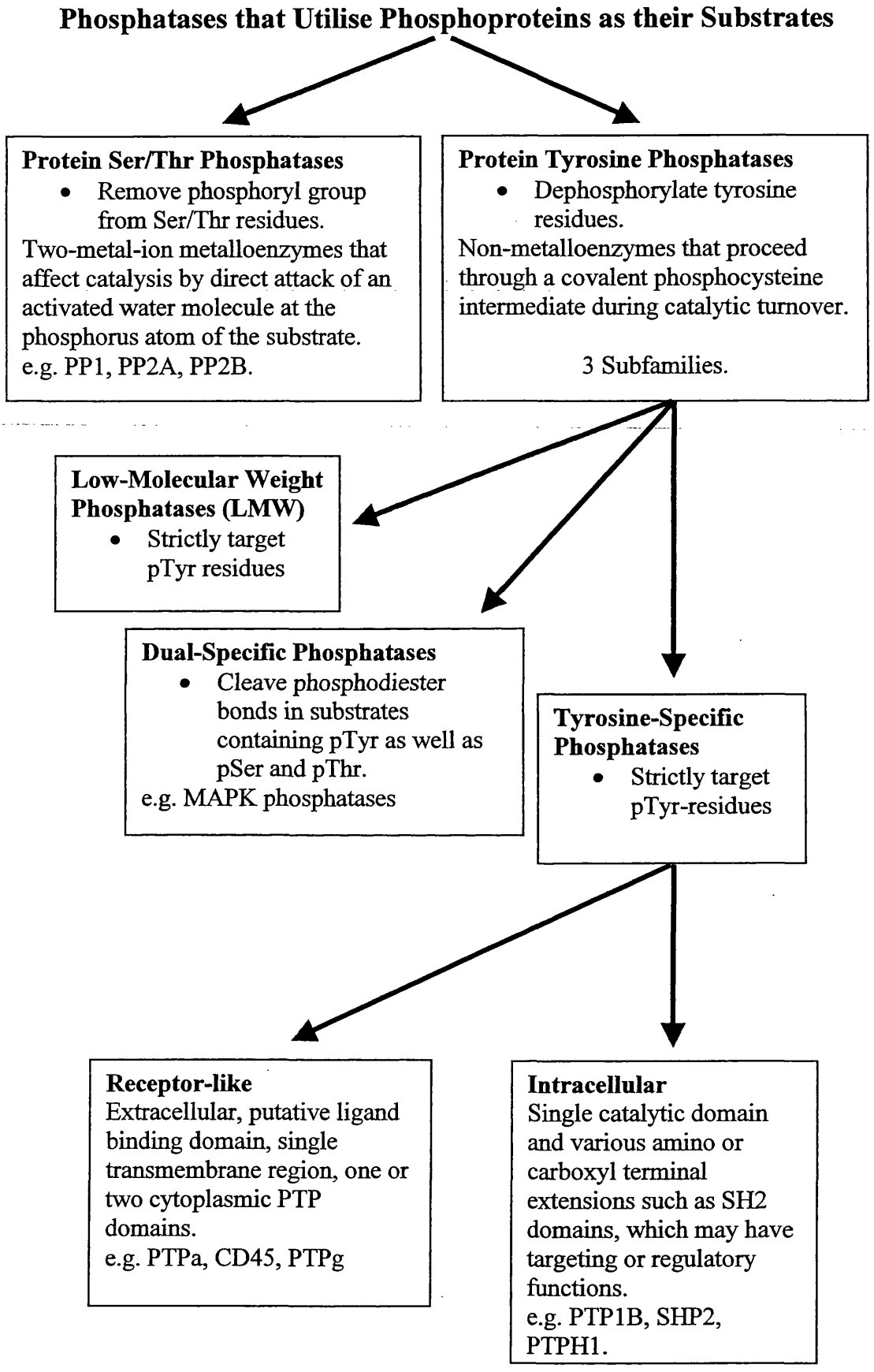


Figure 1.2 Different Families of Protein Phosphatases. Information collated from (Zhang et al., 2002).

they may be more important than originally thought. Figure 1.2 describes the different families of phosphatases that have so far been identified, as well as their target substrates (Zhang et al., 2002). The phosphatases involved in regulating the above signalling pathways are discussed in more detail in the following section.

1.9.1 Protein Phosphatase 1

Protein phosphatase 1 (PP1) is a widely expressed enzyme which is able to remove the phosphate from phosphorylated serine and threonine residues (Price and Mumby, 1999). The catalytic subunit of PP1 exists in multiple isoforms which are differentially distributed, with the α, β, γ1 and γ2 being expressed in brain and γ1 being located to presynaptic terminals in cortical pyramidal cells (Strack et al., 1999). Regulation of PP1 activity can occur through several mechanisms, including: direct inhibition of PP1, subcellular compartmentalisation, and direct protein-protein interactions (Winder and Sweatt, 2001). Several proteins have been identified in neurones, which are able to bind to and inhibit PP1 activity, such as Inhibitor 1 (I-1) and DARPP-32 (Walaas et al., 1983; Gustafson et al., 1991). Phosphorylation of DARPP-32, by PKA, promotes binding to PP1 resulting in PP1 inhibition, a process which can be reversed by the dephosphorylation of DARPP-32 by PP2B (Winder and Sweatt, 2001). As well as indirect inhibition of PP1, PKA can also indirectly activate PP1, by decreasing the binding of the inhibitory regulatory protein neurabin through mediating its phosphorylation (McAvoy T et al., 1999). This not only suggests that the activity of PP1 is highly regulated in cells, but also that regulation of its activity can be the point of cross-talk of some signalling pathways.

The regulatory proteins which bind to PP1 are also able to determine the subcellular localisation of the enzymes, for example, spinophilin can localise PP1 to actin, post-synaptic densities and D2 dopamine receptors (Allen et al., 1997; Smith et al., 1999). PP1 has been shown to be involved in dephosphorylating many proteins, including CREB, GluR1 subunits and membrane bound CaMKIIa (Bito et al., 1996; Strack et al., 1997; Lee et al., 2000). ERK1/2 phosphorylation has also been shown to be sensitive to PP1/2A inhibitors in the hippocampus (Runden et al., 1998). The ability of PP1 to dephosphorylate protein kinases has led to roles for the phosphatase in enhancing the induction hippocampal LTP and in increasing synaptic transmission. These

experiments have led to the suggestion that PP1 is transiently involved in synaptic plasticity (Winder and Sweatt, 2001).

1.9.2 Protein Phosphatase 2A

Protein phosphatase 2A (PP2A) is an amazingly ubiquitous and evolutionarily conserved family of enzymes. They can account for as much as 1% of total cellular proteins and form the majority of serine/threonine phosphatase activity in most cells and tissues (Cohen, 1997). PP2As can be differentially regulated, show distinct distribution patterns and have restricted substrate specificity, depending on their subunit composition. All PP2As consist of a scaffolding subunit (A), a regulatory subunit (B), and a catalytic subunit (C), however different forms of these subunits exist, which is what determines the different regulatory properties of the enzymes. As well as this, the different subunits can be regulated by phosphorylation. The C subunit contains two phosphorylation sites (Thr304, Tyr307), the first of which can be phosphorylated by "autophosphorylation-activated protein kinase", and the second by downstream of epidermal growth factor or insulin receptors (Guo and Damuni, 1993; Brautigan, 1995). Phosphorylation of these sites results in inactivation of the enzyme, a process that can be reversed by its unique ability to catalyse intramolecular autodephosphorylation (Guo and Damuni, 1993; Brautigan, 1995). In contrast to this, phosphorylation of the B subunit by PKA can result in the activation of PP2A (Usui et al., 1998). These studies have provided evidence for the dynamic regulation of phosphatase activation in cells, a property which was previously confined to protein kinases (Sontag, 2001). The dephosphorylation of downstream substrates, induced by activation of PP2A, can also have bidirectional effects on signalling pathways. For example, inhibitory phosphorylation of Raf-1 by PKB/Akt can be reversed by PP2A, resulting in Raf-1 activation. However, dephosphorylation by PP2A can also inactivate a diversity of kinases in vitro, including PKB (Andjelkovic et al., 1996), PKCs (Keranen et al., 1995), MEK and ERK (Sontag et al., 1993). Roles have been identified for PP2A in cell cycle regulation, cytoskeletal regulation and gene expression. This has led to an involvement of PP2A signalling regulation in diseases such as cancer, Alzheimer's disease and AIDs (Sontag, 2001).

1.9.3 Protein Phosphatase 2B/Calcineurin

PP2B is a eukaryotic Ca^{2+}- and calmodulin-dependent serine/threonine protein phosphatase. Wang and Desai were the first to detect PP2B, as a column fraction that inhibited the calmodulin-dependent cyclic nucleotide phosphodiesterase, through a mechanism which is now believed to be due to Ca^{2+}-dependent competition for calmodulin (Wang and Desai, 1977). PP2B is heterodimeric in structure and, in mammals, consists of a 57-59kDa catalytic subunit (A) and a 19-20kDa regulatory subunit (B). The calmodulin binding site is located within the catalytic subunit, whereas the regulatory subunit contains four Ca^{2+}-binding EF-hand motifs. As well as requiring Ca^{2+} and calmodulin for activation, PP2B also requires the cofactors Fe^{3+} and Zn^{2+} to catalyse the removal of phosphate groups from substrates (Rusnak and Mertz, 2000).

PP2B is widely distributed in mammals, with the highest levels identified in brain (Anthony et al., 1988). Elucidation of the subcellular localisation of PP2B has found it to be enriched in the cytoplasm and in nerve terminals, although it can also be localised to the nucleus (Jiang et al., 1997; Pujol et al., 1993). PP2B is tonically inhibited in cells through an α-helical autoinhibitory domain which binds to the active site cleft. Activation is mediated by pathways which lead to increases in intracellular Ca^{2+} concentrations, resulting in Ca^{2+} binding–induced conformational changes in calmodulin, thereby allowing the binding of calmodulin to PP2B. The binding of calmodulin to PP2B is likely to involve a conformational change in the enzyme which exposes the active site. The binding of Ca^{2+} to the regulatory subunit of PP2B is also believed to have a role in mediating the activation of the phosphatase activity of the enzyme (Rusnak and Mertz, 2000; Winder and Sweatt, 2001).

The localisation of PP2B to nerve terminals has led to the elucidation of several roles of this enzyme in the regulation of intercellular signalling and neurotransmitter release. This phosphatase has been shown to dephosphorylate sites 4, 5 and 6 on the synaptic vesicle tethering protein, synapsin, leading to the suggestions of an involvement in regulating vesicle availability for release (Jovanovic et al., 2001). PP2B has also been implicated in the regulation of endocytosis, through relieving the ERK2 phosphorylation-mediated inhibition of the dynamin-microtubule association (Earnest et al., 1996; Cousin et al., 2001). Numerous studies have also found a requirement for

PP2B activity in the regulation of memory processes and synaptic plasticity, and in the regulation of ion channel function (Winder and Sweatt, 2001).

1.9.4 Mitogen Activated Protein Kinase Phosphatases

Mitogen activated protein kinase phosphatases (MKPs) are members of the dual-specificity phosphatases, which means they are able to dephosphorylate both the phospho-tyrosine and the phospho-serine/threonine residues. Despite the broad function of the MKPs, they demonstrate greater substrate specificity than the other groups of phosphatases and individual MKPs have been shown to differentially recognise MAPK family members *in vitro*. To date, nine members of the dual-specificity phosphatases have been cloned and found to be effective in mediating the inactivation of MAPKs. They are: MKP-1, PAC1, MKP-2 or hVH-2, B23, M3/6 or hVH-5, MKP-3, B59, MKP-4 and MKP-5 (Camps et al., 2000).

Out of all the dual-specificity phosphatases to be identified, only hVH-5, MKP-1, B59, MKP-3, and B23 have been found in the brain, with MKP-1 and B23 expression being confined to the nucleus, and only MKP-3 being found in the cytosol (Muda et al., 1996a). It is believed that specific sequences within the amino-terminal region of the phosphatases could be responsible for their subcellular localisation, as well as their substrate specificity (Camps et al., 2000). MKP-3 is able to completely inactivate ERK1/2 at low concentrations, but not JNK or p38, whereas M3/6 is able to completely inactivate JNK and p38, but not ERK1/2 (Muda et al., 1996b). Other MKPs also show preference for one MAPK or another, with MKP-4 being more proficient at inactivating ERKs and MKP-1, despite being originally identified as an ERK1/2 phosphatase, has been shown to prefer JNK and p38s (Muda et al., 1997; Sun et al., 1993; Franklin and Kraft, 1997).

The first MKP to be identified was MKP-1, as an immediate early gene in mice (Charles et al., 1992). Further investigation has since found that other members of the MKPs, such as MKP-2 and PAC-1, are also immediate early genes, whose expression can be induced by activation of MAPKs (Brondello et al., 1997; Grumont et al., 1996; Bokemeyer et al., 1996). This suggests a mechanism for negative feedback of MAPK signalling cascades, and a way of inhibiting pools of MAPKs which have not yet been

activated, adding evidence to the hypothesis that the activation of these kinase cascades is highly regulated (Camps et al., 2000).

1.10 Phosphoprotein Substrates and Synaptic Plasticity.

As research into neurotransmitter release progresses, it is becoming evident that many of the proteins involved in regulating synaptic vesicle exocytosis and endocytosis are themselves subject to regulation by phosphorylation. For example, the t-SNARE, SNAP-25, has been shown to be phosphorylated by PKA (Risinger and Bennett, 1999; Hepp et al., 2002) which, in chromaffin cells at least, results in an increased number of primed synaptic vesicles ready for release (Nagy et al., 2004). The active zone protein Rim1a has also been shown to be phosphorylated by PKA, resulting in the induction of LTP in cerebellar parallel fibre synapses (Lonart et al., 2003). As well as this, the dephosphins, a family of proteins involved the endocytosis of synaptic vesicles, are known to be regulated by phosphorylation. However, they tend to be highly phosphorylated in resting states and undergo rapid dephosphorylation following nerve terminal depolarisation (Cousin and Robinson, 2001b). An example of a dephosphin is dynamin, which is phosphorylated by PKC (Cousin et al., 2001a; Powell et al., 2000) and following rapid dephosphorylation, mediates vesicle fission (Cousin and Robinson, 2001b). Many more components of the synaptic vesicle cycle are beginning to be seen to undergo phosphorylation, such as cysteine string protein, rabphilin, and snapin (Chheda et al., 2001; Evans et al., 2001b; Lonart and Sudhof, 2001), though the exact roles for these phosphorylations are awaiting further elucidation. The synaptic vesicle tethering protein, synapsin, is also a phosphoprotein whose phosphorylation state is known to be regulated by several protein kinases and phosphatases, including ERK 1,2 (Jovanovic et al., 2001). As a result of the regulation by ERK 1,2, this family of phosphoproteins, and their role in regulating neurotransmitter release, is described in more detail in the following section.

1.10.1 Synapsins

Synapsins were initially identified as proteins found in the brain which could act as substrates for multiple protein kinases (Johnson et al., 1971; Krueger et al., 1977). Synapsins were subsequently found to be abundant presynaptic proteins, accounting for up to 6% of the total vesicular protein present. There are three separate genes which can encode for synapsin in vertebrates (I, II and II), which can be alternatively spliced

to produce a, b and b-like isoforms (Sudhof et al., 1989; Hosaka and Sudhof, 1998; Kao et al., 1998). Invertebrates seem to only have one gene for the encoding of synapsin, but are able to produce multiple isoforms through alternative splicing (Klagges et al., 1996). The synapsin isoforms found in mammals have been shown to be differentially distributed, but the physiological relevance of this has yet to be determined.

Further investigations into the roles of synapsin have identified binding interactions with synaptic vesicles, actin, microtubules, neurofilaments and brain spectrin. Binding to vesicles occurs with high affinity in a two stage process, involving both protein and phospholipid interactions. The polar head region of synapsin first contacts with the surface of the vesicle membrane through electrostatic interactions. This is followed by a conformational change in the protein which leads to the penetration of specific regions within the C-terminal domain into the hydrophobic core of the vesicle lipid membrane bilayer. It is estimated that each vesicle can bind between 20 and 30 synapsin molecules at any one time (Hilfiker et al., 1999). These binding properties of synapsin have led to suggestions of the involvement of these proteins in the tethering of synaptic vesicles to the actin cytoskeleton, in the formation of the reserve pool of vesicles (Hilfiker et al., 1999). Further studies, involving genetic modification and localisation, have provided evidence for a causal link between synapsin-mediated regulation of the reserve pool of vesicles and regulation of neurotransmitter release (Jovanovic et al., 2000). The interaction between synapsin and actin has been shown to be dependent on the phosphorylation state of synapsin. Thus it has been suggested that, upon phosphorylation of synapsin, vesicles can be untethered from the reserve pool and migrate to the readily releasable pool where they act to increase neurotransmitter release (Jovanovic et al., 1996). Fairly recent experiments have finally provided some direct physiological evidence for the role of synapsin I as a phosphorylation-state-dependent regulator of synaptic vesicle mobilisation. Results obtained using green fluorescent protein-labelled synapsin Ia in hippocampal cell cultures, confirmed that synapsin I can indeed dissociate from synaptic vesicles during action potential firing. Synapsin I was found to disperse into axons, which correlated with vesicle pool mobilisation, and then recluster to synapses following cessation of the stimulation (Chi et al., 2001).

The interactions of synapsin I with actin are regulated by the phosphorylation of multiple sites on synapsin. Phosphorylation of synapsin can be induced following

depolarisation or by extracellular stimuli, and can decrease the affinity of the phospho-protein for actin. There are currently believed to be seven different phosphorylation sites found on synapsin I, which can be regulated by different protein kinases and phosphatases, as well as by Ca^{2+}, and have been summarised in the following table (Jovanovic et al., 2001; Sakurada et al., 2002).

Table 1.10: Regulation of Synapsin I Phosphorylation Sites.

Phosphorylation Site	Site Terminology	Kinases	Phosphatase	Response to Ca^{2+} influx
Serine-9	1	PKA CaMKI/IV	PP2A	↑
Serine-566	2	CaMKII PAK1	PP2A	↑
Serine-603	3			
Serine-62	4	ERK1,2	PP2B	↓
Serine-67	5			
Serine-549	6	ERK1,2 Cdk1,5	PP2B	↓
Serine-511	7	Cdk5		

Data obtained from (Jovanovic et al., 2001) and (Sakurada et al., 2002).

PI3-K has also been shown to associate with synapsin in intact nerve terminals, though its exact site is yet to be identified. Disruption of either PI3-K activity or its interaction with synapsin was found to inhibit replenishment of the readily releasable pool of vesicles from the reserve pool (Cousin et al., 2003).

The phosphorylation of synapsin I by ERK1/2 is particularly relevant to this thesis. BDNF has been shown to increase depolarisation-induced glutamate release from cerebrocortical nerve terminals. It is believed to do this through increasing vesicle availability following synapsin site 4/5 phosphorylation mediated by ERK1/2, which is activated downstream of trkB stimulation (Jovanovic et al., 2000). Phosphorylation of synapsin I by ERK1/2 has been shown to regulate interactions with the actin cytoskeleton (Jovanovic et al., 1996) and more recently, to regulate interactions with

synaptic vesicles following a range of electrical stimuli (5-20Hz) *in vitro* (Chi et al., 2003). ERK1/2 has also been shown to mediate synapsin site 4/5 phosphorylation *in vivo* (Yamagata et al., 2002), adding increasing evidence to the suggestion that ERK1/2 activation is able to increase glutamate release from nerve terminals through increasing vesicle availability following the phosphorylation of synasin I. This thesis is investigating other pathways, which can also increase glutamate release, to see if they could be stimulating ERK1/2 and synapsin site 4/5 phosphorylation as a mechanism for this modulation.

The identification of the role of synapsins in the regulation of neurotransmitter release has led to a plethora of studies investigating the participation of synapsin in human diseases of the central nervous system. Specific decreases in the levels of synapsin expression have been associated with Alzheimer's disease, schizophrenia, bipolar disorders and amyotrophic lateral sclerosis (Qin et al., 2004; Vawter et al., 2002; Ikemoto et al., 2002). Other diseases have been associated with abnormalities in synapsin phosphorylation, including Huntington's disease and schizophrenia (Lievens et al., 2002; Porton et al., 2004).

As well as a fairly well documented role in regulating vesicle availability in the modulation of neurotransmitter release, synapsin has also been linked with the regulation of endocytosis. In a study using the giant synapse in lamprey to examine synapsin distribution during synaptic activity, synapsin was found to migrate from the vesicle cluster to the endocytic zones, where it appeared to be involved in vesicle recycling (Bloom et al., 2003).

There is also a growing body of evidence to suggest that synapsins have a broad role in neuronal development. They have been found to participate in the formation and maintenance of synaptic contacts between central neurones, as well as having specific roles in the elongation of undifferentiated processes and their consequent differentiation into axons and dendrites (Ferreira and Paganoni, 2002). In addition, the role in neuronal development may not be limited solely to young, developing nervous systems. The location of synapsin III to the cell bodies and processes of 'plastic' neurones in mouse adult hippocampi and olfactory bulbs, suggests an involvement of synapsin III in adult neurogenesis (Pieribone et al., 2002). Synapsin I may also be involved in the

translocation of vesicles, by fast axonal transport, to the elongated membranes in regenerating sprouts following ligation crush injury of sciatic nerves in rats (Kwon et al., 2000).

1.11 Multiple Mechanisms of ERK 1,2 Activation Downstream of GPCR Signalling

Ligands that act at GPCRs can regulate signalling to ERK1/2 in many different ways. The mechanisms involved seem to be determined by the composition of the G-protein subunits coupled to the receptor and by the subcellular localisation of the receptors (English et al., 1999).

1.11.1 Gs-Coupled Receptors

These receptors are linked to adenylate cyclase activation upon agonist binding. The resulting increase in cAMP levels has been shown to decrease, increase, or have no effect on ERK1/2 phosphorylation levels. The differences in effects on ERK1/2 activation appear to be dependent on the cell type being investigated, though other factors may also be involved.

In rat 1hER fibroblasts, forskolin-stimulated increases in cAMP levels were found to block EGF-induced stimulation of MAPK phosphorylation. Further investigation found that PKA was phosphorylating Raf-1 on serine 43 in the regulatory domain, which reduced the apparent affinity of Raf-1 for Ras (Wu et al., 1993). Subsequent studies have identified further inhibitory PKA phosphorylation sites on Raf-1, including serine 621, which appears to be essential for catalytic activity and can interfere with Ras binding (Mischak et al., 1996), and serine 259. It has recently been confirmed that PKA is able to phosphorylate all these sites on Raf-1 *in vivo*, with Raf-1 inhibition mainly being mediated through serine 259 (Dhillon et al., 2002).

In neuroendocrine cells, the increase in cAMP levels produced by Gαs stimulation of adenylate cyclase has been found to enhance ERK1/2 activation. This signalling pathway is believed to involve the recruitment of Rap-1 and B-Raf, rather than Ras-1 and Raf-1, in the stimulation of ERK1/2. Guanine nucleotide-exchange factors have been identified for Rap-1 which can be stimulated directly by cAMP, such as Epac (de Rooij et al., 1998). Rap-1-GTP then goes on to activate B-Raf, which can feed into the

ERK1/2 cascade at the level of MEK1/2. PKA has also been found to directly phosphorylate Rap-1, leading to sequential B-Raf/MEK/ERK1,2 activation (Lerosey et al., 1991; English et al., 1999). However, in some systems Rap-1 has been shown to inhibit Ras signalling to Raf-1 by sequestering Raf-1, thus inhibiting the activation of ERK1/2 (English et al., 1999). Some studies have suggested that the nature of the signalling through Rap-1, whether through B-Raf leading to stimulation of ERK1/2, or through Raf-1 leading to inhibition of ERK1/2, is determined by the expression levels of B-Raf (Erhardt et al., 1995). Another mechanism for mediating Gαs stimulation of ERK1/2 is just recently beginning to be elucidated. Signalling through Gαs has been shown to enhance ERK1/2 phosphorylation through activation of Raf-1 via Src. This has been shown to be through a direct interaction between Gαs and Src *in vitro*, though its physiological relevance is yet to be determined (English et al., 1999).

1.11.2 Gi-Coupled Receptors

Evidence for the phosphorylation of ERK1/2 following Gi/o receptor activation suggests it is the βγ subunit of the heterotrimeric G-protein complex that is involved in mediating the ERK1/2 activation (Koch et al., 1994). This process is believed to involve Src, but it is not known whether this is through a direct interaction, or through a PI3K intermediary (Luttrell et al., 1996; Lopez-Ilasaca et al., 1997). G-protein βγ subunits have also been shown to activate the MAP kinase pathway at the level of GEFs (Crespo et al., 1994), calmodulin can also do this but only via an increase in intracellular calcium (Farnsworth et al., 1995).

1.11.3 Gq-Coupled Receptors

These receptors are known to stimulate the activity of phospholipase Cβ (PLCβ), thus producing the second messengers DAG and IP3, which can lead to the activation of some PKC isoforms (as described in section 1.6.2). PKC was originally shown to directly phosphorylate Raf-1 in a manner which enables it to go on to stimulate MEK1/2 activation, thus providing a Ras-independent mechanism for PKC-mediated activation of the ERK1/2 signalling cascade (Kolch et al., 1993). However, other studies found that phosphorylation of Raf-1 by PKC was not sufficient in itself to increase Raf-1 phosphorylation of MEK (Macdonald et al., 1993). More recent studies suggest that PKC can stimulate ERK1/2 activation through a mechanism distinct from

that of the receptor tyrosine kinases, by phosphorylating Raf-1 to facilitate its full activation upon binding to Ras (Marais et al., 1998). It should be noted that the Ras-dependency of PKC signalling to ERK1/2 could be dependent upon cell type and so further studies need to be conducted to clarify this.

Gαq-coupled receptors have also been found to stimulate the ERK1/2 cascade in a PKC-independent manner. Ras-GEFs have been identified which are directly stimulated by the binding of the second messenger, DAG, such as CalDAG. Ca^{2+}-dependent Ras-GEFs, such as Ras-GRF (Ras-guanine-nucleotide-releasing factor) are also found in neurones. These can be stimulated by the rises in intracellular Ca^{2+} levels brought about by the other product of PLC-mediated hydrolysis of PIP_2, IP_3. IP_3 can act on intracellular receptors to mediate the release of Ca^{2+} from intracellular stores (English et al., 1999).

1.12 Preparations Used to Study Presynaptic Function

This thesis focuses on the cross-talk of signalling cascades in the modulation of presynaptic function. There are several preparations that could be used to examine this phenomenon, namely: brain slices, primary neuronal cultures, and isolated nerve terminals (synaptosomes). Brain slices contain mixed poopulations of neurones with some *in situ* neuronal connections remaining intact. This makes it a particularly good preparation for examining postsynaptic changes brought on by presynaptic electrical stimulation. Although it is possible to examine transmitter release with this preparation, the presence of the postsynaptic cell makes it difficult to establish whether the activation of signalling cascades is brought about by pre- or postsynaptic receptor modulation. The same problem is encountered when using primary neuronal cell cultures which, although can provide a purer population of neurones, does not lend itself to the examination of the presynaptic nerve terminal in complete isolation from the postsynaptic nerve terminal. The isolated nerve terminals preparation, however, is an acute procedure which results in the production of isolated presynaptic nerve terminals, thus making it possible to examine the presynaptic signalling cascades without the ambiguity of signalling from receptors on the cell body. It is for this reason that the preparation chosen here, to examine the cross-talk of signalling pathways in the modulation of presynaptic function, is the isolated nerve terminal preparation.

1.12.1 The Isolated Nerve Terminal (Synaptosome)

Synaptosomes are small (0.5-1.0μm) plasma membrane delimited nerve terminals which have been pinched off from axons during homogenisation and isolated using differential centrifugation. Electron microscopy has shown the synaptosomes to contain 1-2 mitochondria and a high density of small synaptic vesicle (SSVs), clustered around the active zone (Surchev, 1987; Dunkley et al., 1988). These nerve terminals are metabolically active, being able to produce ATP, and can maintain a resting membrane potential of between –60 and –70mV (Nicholls, 1989). They are also able to control intracellular Ca^{2+} concentrations, by way of a Ca^{2+}-ATPase, with 0.1-0.3mM levels being maintained within polarised synaptosomes (Nicholls et al., 1987). The advantages for using synaptosomes are that signalling cascades and the Ca^{2+}-dependent release of multiple transmitters can be examined in isolation form the cell body. However, it should be noted that these preparations contain a heterogenous population of neurones, making it difficult to measure responses of a particul subset of nerve terminals in isolation. Although synaptosomes are metabolically active, the absence of a nucleus means they can only be used as an acute preparation, lasting 4-6 hours, and so cannot be used to examine long term changes in transmitter release, or to examine the effects of changes in gene or protein expression.

Electrical stimulation can be used to evoke transmitter release from synaptosomes but this results in a Ca^{2+}-independent release and so biochemical methods are usually favoured (Bradford, 1970b; Bradford, 1970a; Bradford et al., 1973; De Belleroche and Bradford, 1977). KCl is often used to cause depolarisation of isolated nerve terminals and neurotransmitter release, through clamping of the membrane potential, however this is not thought to be particularly physiological as it is not sensitive to the Na^{+} channel blocker tetrodotoxin (Tibbs et al., 1989). 4-aminopyridine (White et al., 2003) is a K^{+} channel blocker which elicits nerve terminals depolarisation through destabilising the membrane potential. This method for evoking release is sensitive to tetrodotoxin, but will result in random depolarising events, rather than the global depolarisation obtained with KCl (Tibbs et al., 1989). Ionophores, such as ionomycin and A23187, can also be used to examine neurotransmitter release. Application of ionomycin or A23187 results in random Ca^{2+} influx into the nerve terminals, allowing for the examination of release downstream of Ca^{2+} channel modulation. However, this is not quite the same as the

stimulation of release following Ca^{2+} influx through voltage-dependent Ca^{2+} channels, which leads to very localised increases in Ca^{2+} at the active zone, rather than the "delocalised" increases in Ca^{2+} concentration resulting from the treatment with ionophores (McMahon and Nicholls, 1990; Sihra et al., 1992; Verhage et al., 1991).

1.13 PhD Aims

The above discussion has highlighted the importance of ERK 1,2 as post-mitotic signalling molecules, that are not only activated through the traditional trk.Ras/Raf/MEK/ERK pathway but are also open to regulation via cross-talk with the cAMP/PKA and PKC signalling cascades. This evidence, combined with studies implicating all three of these signalling cascades in the regulation of glutamate neurotransmitter release, has led me to examine the hypothesis of whether cAMP/PKA and PKC signalling cascades could be cross-talking with ERK in the regulation of presynaptic function. More specifically, I aim:

- To investigate the cross-talk of presynaptic signalling cascades in the regulation of ERK 1,2 activation in cerebrocortical nerve terminals.

- To identify the involvement of upstream presynaptic receptors in regulating the cross-talk of signalling cascades with ERK 1,2 in cerebrocortical nerve terminals.

- To elucidate the possible roles of signalling cross-talk with the ERK 1,2 cascade in the modulation of presynaptic function, through the examination of the activation of the downstream effector, synapsin I, and the regulation of glutamate release.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1 Preparation of Rat Brain Synaptosomes

Isolated nerve terminals were prepared from the cerebrocortices of 150-200g male Sprague-Dawley rats using the previously described methods of differential centrifugation and Percoll gradient purification (Dunkley et al., 1986; Sihra, 1997). A Percoll purified preparation was used, rather than the P2 level of isolation, as this process removes the majority of the free mitochondria, myelin and broken postsynaptic membranes that contaminate the P2 preparation (Dunkley et al., 1986; Dunkley et al., 1988). All steps in this section were carried out at 4°C.

Percoll gradients were prepared in advance consisting of a lower layer of a solution containing 23% Percoll, a middle layer solution containing 10% Percoll, and an upper layer consisting of a 3% Percoll solution. As well as containing 3, 10, or 23% Percoll, these solutions also contained 320mM sucrose, 1mM EDTA, and 250μM DTT and had a pH of 7.4 (Dunkley et al., 1986; Sihra, 1997).

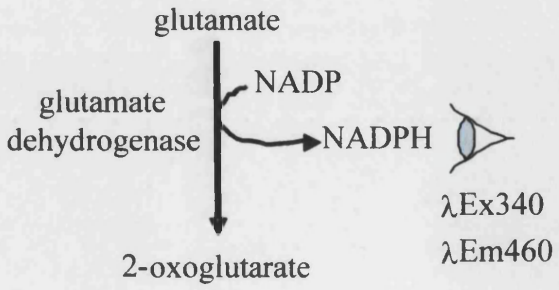
Animals were killed by stunning followed by decapitation according to Schedule 1 procedures under the Home Office Animals (Scientific Procedures) Act of 1986. The brains were removed rapidly onto ice and the cerebrocortices dissected out. Each hemisphere was homogenised in 30ml of 320mM sucrose solution (pH 7.4) using a Potter-Elvehjem tissue grinder and Teflon pestle with 0.1-.015mm clearance (spinning at 900rpm). The homogenate was then centrifuged at 3,000 x g for two minutes and the resulting supernatant (S1) transferred to a fresh tube and recentrifuged at 14,500 x g for 11 minutes. Following this centrifugation the supernatant (S2) was discarded and the remaining crude synaptosomal pellet (P2) was resuspended in the sucrose solution and homogenised using a loose, glass-to-glass, Dounce homogeniser to disperse the nerve terminals. The resuspension of crude synaptosomes from two P2 pellets (equivalent to one brain) was diluted to 8ml with sucrose solution and 2ml carefully layered onto the top, 3%, layer of each of the Percoll gradients. The gradients were resolved by centrifugation at 32,500 x g for 6 minutes. The synaptosomal fraction, found between the 23% and 10% layers, was harvested into 40ml HBM (Hepes-buffered medium containing: 20mM Hepes, pH7.4, 140mM NaCl, 5mM KCl, 5mM $NaHCO_3$, 1.2mM

Na_2HPO_4, 1mM $MgCl_2$, 10mM Glucose) and centrifuged at 27,000 x g for 10 minutes as a wash step. The resulting pellet was resuspended to 2-3ml using HBM before duplicate aliquots of 5 and 10µl were assayed for protein concentration using the Bradford method (Bradford, 1976). Aliquots of synaptosomal resuspension were placed into tubes containing 8ml of HBM, as a final wash before centrifugation at 3,000 x g for 10 minutes to obtain the final pellets. Pellets were stored on ice (4°C), until required and all synaptosome pellets were used within four hours of purification. All centrifugation steps were carried out using a Beckman J2-21M/E centrifuge with a JA-20 rotor (or JA-20.1 rotor for the final pelleting step) (Dunkley et al., 1986; Sihra, 1997).

2.2 Measurement of Glutamate Release

A direct cumulative measurement of glutamate release was conducted using an enzymatic assay system whereby glutamate was metabolised to 2-oxoglutarate by exogenous glutamate dehydrogenase with exogenous NADP as a cofactor. This process of oxidative deamination resulted in the reduction of NADP to the fluorescent NADPH. By measuring the level of fluorescence increase of the system, the amount of released and metabolised glutamate could be thereby be ascertained assuming a direct 1:1 correlation between the NADPH produced and the glutamate released (see Figure 2.1).

Figure 2.1 Glutamate Release Assay



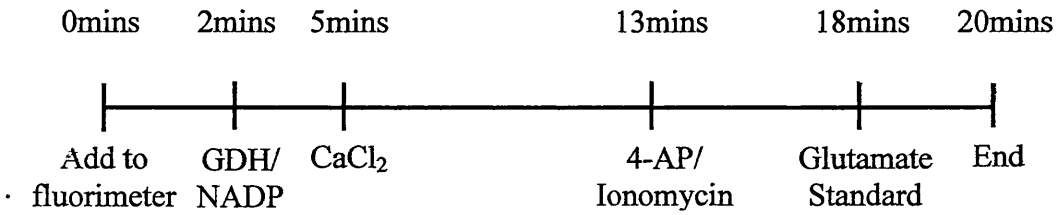
To calibrate this fluorescence response, 5nmol of exogenous glutamate standard was routinely added at the end of each incubation, and the steady-state fluorescence response induced by this addition used to carry out a point-by-point calculation of the

foregoing glutamate release as nmol/mg synaptosomal protein. This assay for measuring glutamate release was originally developed using synaptosomes purified from guinea-pig cerebral cortices (Nicholls and Sihra, 1986). Subsequent studies have also found this protocol useful for measuring glutamate release from synaptosomes purified from the cerebral cortices of rats and mice, as well as from other brain regions such as the hippocampus (Sanchez-Prieto et al., 1987; Jovanovic et al., 2000; Rodriguez-Moreno and Sihra, 2004).

2.2.1 Measurement of Glutamate Release from Synaptosomes

Synaptosomes were stored on ice as 0.3 mg pellets and resuspended in HBM containing1mg/ml bovine serum albumin (BSA), to give a final synaptosomal protein concentration of 0.2mg/ml. The resuspension was transferred to a magnetically stirred cuvette in a Perkin-Elmer LS3B Spectrofluorometer (Perkin-Elmer, Emeryville, CA) and maintained at 37°C. Excitation and emission wavelengths were 340nm and 460nm respectively. Glutamate dehydrogenase (GDH, 50U/ml) and nicotinamide adenine dinucleotide phosphate (NADP, 2mM) were added after 2 minutes, followed by the addition of 1mM $CaCl_2$ after 5 minutes, as a standard procedure. Either 1mM 4-aminopyridine (White et al., 2003) or 5μM ionomycin was used to stimulate glutamate release after 13 minutes of incubation, which was calibrated and measured using 5nmol exogenous glutamate standards applied 18 minutes after commencing the 37°C incubation (see Scheme 2.1). To allow direct comparisons of effects on depolarisation-induced release to be made, release traces were aligned horizontally and vertically at the point of secretogogue addition and initiation of glutamate release. Data was analysed using Lotus 1,2,3 spreadsheet and Origin (Microcal) graphical software. This protocol has been adapted from others which have been previously published (Nicholls and Sihra, 1986; Perkinton and Sihra, 1999).

Scheme 2.1 Standard Protocol for Measuring Glutamate Release.



The drugs whose effects were to be tested on glutamate release were applied prior to the addition of the secretogogue, with times and concentrations specified in the relevant chapter.

2.3 Measuring Intracellular Calcium Concentration (Fura-2-AM)

Fura-2 is a fluorescent Ca^{2+}-chelating agent which has been used to study intracellular Ca^{2+} concentrations in many tissues. It is a ratiometric dye and, upon Ca^{2+}-chelation, exhibits an excitation shift from 380nm to 340nm, making it possible to measure Ca^{2+} concentrations through examining the ratio between the two excitation values. Emission values are measured at a wavelength of 510nm. Ratiometric dyes are advantageous compared to single wavelength dyes, as the resultant evaluation of Ca^{2+} is rendered independent of differences in dye loading and thereby unaffected by factors such as cell/preparation thickness/depth. The addition of an acetoxymethyl ester group to Fura-2 enables it to cross cell membranes into the cell, where the acetoxymethyl ester group is removed from the ester by cytosolic esterases, thereby trapping the charged (Foster and Fagg, 1984) indicator within the synaptosome (Grynkiewicz et al., 1985).

2.3.1 Fura-2-AM Loading of Synaptosomes

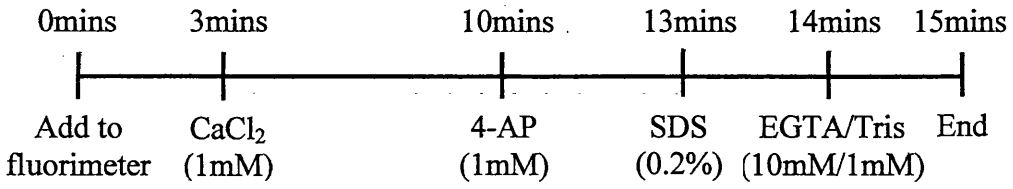
Pellets of synaptosomes (0.5mg) were resuspended at 4°C using 1ml of HBM containing 1mg of BSA. The synaptosomes were then put to preincubate at 37°C for 20 minutes, with magnetic stirring. Fura-2-AM (5μM) was added immediately after transferring the synaptosomes to the waterbath and $CaCl_2$ (0.1mM) was added following 3 minutes of incubation. A 20 minute preincubation protocol was used to ensure adequate incorporation of the Fura-2 into the nerve terminals, to achieve reproducible measurements of cytosolic Ca^{2+}. Following this preincubation, the synaptosomes were spun at 10,000 x g for 30 seconds using a microcentrifuge, to remove the unincorporated Fura-2 from the extracellular medium. Pellets were resuspended using 1.5ml of HBM and the washing procedure repeated once more to remove any residual indicator (Perkinton and Sihra, 1999).

2.3.2 Intracellular Ca^{2+} Measurements

Synaptosomal pellets loaded with Fura-2 were resuspended in 1.5ml HBM and incubated in a Perkin-Elmer LS3B Spectrofluorometer (Perkin-Elmer, Emeryville, CA)

at 37°C with magnetic stirring, for a total of 15mins, with the following additions being made: (see Scheme 2.2)

Scheme 2.2 Standard Protocol for Measuring Intracellular Calcium.



Excitation wavelengths were cycled between 340nm and 380nm with fluorescent values being taken at the emission wavelength of 505nm, every 3.5secs and recorded digitally on a computer. Internal calibrations were made for every synaptosome incubation, defining the maximum and minimum Fura-2 fluorescence levels. Maximum fluorescence levels were obtained by the addition of 0.2% SDS to lyse the nerve terminals and thereby allow the complete saturation of Fura-2 in the incubation with the excess 1mM Ca^{2+} present in the lysate. Minimum levels were measured following the subsequent chelation of this Ca^{2+} by 5mM Na-EGTA buffered with 1M tris. The Tris buffer was added in conjunction with EGTA to neutralise the increase in $[H^+]$ resulting from chelation of Ca^{2+} by EGTA, which would otherwise affect the fluorescent response of the Fura-2/Ca^{2+} adduct. This method for internal calibration in synaptosomes has been previously described (Sihra et al., 1993). Free cytosolic Ca^{2+} concentration was subsequently calculated using the following equation (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_c = K_d \times \frac{(R-R_{min})}{(R_{max}-R)} \times \frac{F_{min\lambda 2}}{F_{max\lambda 2}}$$

K_d = 224nM at 37°C

R = Experimental fluorescence ratio value (340/380)

R_{min} = Minimum fluorescence ratio value

R_{max} = Maximum fluorescence ratio value

$F_{min\lambda 2}$ = Minimum fluorescence value at 380nm

$F_{max\lambda 2}$ = Maximum fluorescence value at 380nm

2.4 Identification of Downstream Effectors

Synaptosomal pellets were resuspended at 4°C to a concentration of 1mg/ml using HBM containing 1mg/ml BSA. $CaCl_2$ (1mM) was added immediately before transfer of the resuspended synaptosomes to the 37°C waterbath. The exact incubation protocols used varied in detail depending on the aim of the individual experiment and are described in more detail below. Following the 37°C incubation, samples were stopped using 5x SDS-PAGE STOP buffer (1% (w/v) sodium dodecyl sulphate, 6.25mM Tris, pH 6.8, 5% 2-mercaptoethanol, 10% (v/v) glycerol, and 0.001% (w/v) bromophenol blue) before being boiled for 5 minutes, in preparation for protein separation using SDS-PAGE (PolyAcrylamide Gel Electrophoresis) (Laemmli, 1970).

2.4.1 Standard Incubation Protocol

Following synaptosomal resuspension and addition of 1mM $CaCl_2$, synaptosomes were split into individual incubation tubes for each condition being tested. The tubes were transferred to the 37°C waterbath for a total of 10 minutes with the maximum possible time in the presence of a drug also being 10 minutes. Specific drug incubations are described in the methods section of each chapter. Each incubation was terminated with the addition of 5x SDS-PAGE STOP buffer (Jovanovic et al., 2000).

2.4.2 Preincubation Protocol

Resuspended synaptosomes containing 1mM $CaCl_2$ were divided into two preincubation tubes (control and test) before both tubes were put to incubate at 37°C, with stirring, for 20 minutes. Drugs were added at different stages during this preincubation, depending on the specific drug used (as described in each chapter). Following this preincubation, both tubes were removed from the waterbath and centrifuged using a benchtop microcentrifuge (10,000 x g, 30 seconds) to pellet the synaptosomes. At this stage the supernatant was removed and either kept, or discarded as specified in each chapter. The synaptosome pellet was cooled to 4°C for 10 minutes before following the standard 10 minute incubation protocol. The aim of this preincubation protocol was to introduce drugs into the synaptosomes which would normally take longer than 10 minutes to cross the plasma membrane in sufficient quantity to be effective.

2.4.3 SDS-Polyacrylamide Gel Electrophoresis

A Hoeffer gel system was used with gels consisting of separate stacking and resolving dimensions. Resolving gel mixture routinely contained 7.5% acrylamide, 0.2% bis-acrylamide, 0.1% SDS and 325mM tris (pH 8.8), and were polymerised using 75µl of 10% ammonium persulphate (APS) and 50µl TEMED (N,N,N',N'-tetramethylethylenediamine) per 30ml of resolving gel mix. Stacking gel mixture routinely contained 3.75% acrylamide, 0.1% bis-acrylamide, 0.1% SDS and 125mM tris (pH 6.8) and were polymerised using 50µl of 10% APS and 10µl of TEMED per 10ml of stacking gel mix. Sample wells were formed using combs inserted during the polymerisation of the stacking gel, ensuring a minimum of 1cm migration for samples in the stacking phase. Gels were loaded with either 10µg or 20µg of synaptosomal protein (according to Bradford protein quantification), depending on the antibody used for immuoblotting and electrophoretic progress determined using visibly labelled molecular weight markers (Rainbow Markers, Amersham Pharmacia Biotech) ranging between 14.3KDa and 220KDa and including a marker at 46KDa. Gels were run in a buffer containing 25mM tris (pH 7.2), 192mM glycine and 0.1% SDS at a setting of 80V through the stacking gel and 150V through the resolving gel (Laemmli, 1970).

2.4.4 Immunoblotting

Following separation using gel electrophoresis the synaptosomal proteins were transferred onto nitrocellulose membrane (Schleicher and Schuell) by electroblotting at 200mA overnight. The transfer buffer contained 25mM tris (pH 7.2), 192mM glycine and 20% methanol to fix the proteins. The nitrocellulose was then dried before being labelled with Ponceau S (0.2% Ponceau Red, 3% TCA and 3% sulfosalicyclic acid) to verify even protein loading and transfer. Procedures for immunolabelling of the proteins varied for each antibody as detailed below (Burnette, 1981).

2.4.4.1 Phospho-p44/p42 MAP Kinase (Thr202/Tyr204) Antibody (NEB)

The nitrocellulose membranes used for labelling with this antibody had been obtained from gels loaded with 20µg of synaptosomal protein per lane. The nitrocellulose was first washed to remove any traces of Ponceau S, using three 10 minute incubations with tris-buffered saline (TBS: 20mM tris, pH 7.6, and 137mM NaCl) containing 0.1% Tween-20 (TBST). All incubations occurred at room temperature, unless otherwise

specified, with constant agitation provided by a rocking platform (Stuart Scientific). Following washing, the membranes were incubated for 1 hour in blocking buffer containing 1x TBST and 5% w/v nonfat dry milk (Marvel Foods) to minimise non-specific binding of the antibody. Blocked membranes were then labelled with the primary phospho-p42/p44 MAP kinase antibody (NEB) diluted 1:1000 in blocking buffer, by overnight incubation at 4°C. Following primary incubation, excess, unbound, antibody was washed off using one 5 minute incubation, proceeded by two 15 minute incubations, in blocking buffer. In order to detect and quantitate levels of primary antibody binding, membranes were secondarily labelled by incubation with ^{125}I-labelled protein A diluted in blocking buffer (18500dps/10ml of buffer) for one hour. Following secondary incubation, excess ^{125}I-protein A was removed using one 5 minute wash step, proceeded by one 15 minute incubation in blocking buffer. In a final wash, milk protein was removed from the nitrocellulose membrane using one 5 minute incubation and a subsequent 15 minute incubation in TBST. The labelled membranes were then allowed to dry for 30 minutes before being exposed to a phosphorimager screen for radiolabel detection (Dai et al., 2001; Jovanovic et al., 2000).

2.4.4.2 Phospho-Synapsin I Site 1, Site 2/3, and Site 4/5

From herein the various synapsin antibodies are assigned a terminology relating to the original immunoglobulin production batch number. Thus, the synapsin antibody specific for site 1 phosphorylation is termed G257, site 2/3 termed RU19, and site 4/5 termed G526 (Czernik et al., 1991; Jovanovic et al., 1996).

A similar protocol was followed for labelling nitrocellulose membranes with these phospho-synapsin antibodies, as for the phospho-p42/p44 MAP kinase antibody. The major differences included the composition of the tris-buffered saline (50mM tris (pH 7.5) and 200mM NaCl, with 0.05% Tween-20 added to make TBST), the blocking buffer (1x TBST with 2.5% w/v nonfat dried milk), and the length of the antibody incubation. Both RU19 and G526 were used at a dilution of 1:500 in an incubation lasting for 90 minutes at room temperature, whereas G257 was diluted to 1:200 for an incubation also lasting 90 minutes. Also, the nitrocellulose membranes used were obtained from gels resolving 10μg of synaptosomal protein per lane when labelling with RU19 and G257, and 20μg per lane when labelling with G526. These loadings have

been characterised as producing linear antibody responses to increasing protein concentrations of the antibody in synaptosomal lysates (Czernik et al., 1991; Jovanovic et al., 1996).

The phospho-state specific antibodies used in this study were created using synthetic peptides containing permanently phosphorylated residues at positions corresponding to the phosphorylatable residues in the native protein. These synthetic phosphor-peptides were used to immunise rabbits and the resulting antibodies were harvested and purified using Protein A-Sepharose affinity chromatography to obtain IgG fractions based on Fc binding (Czernik et al., 1991). All such antibodies used in this study were either kind donations or purchased from companies, the details of which can be found in section 2.7.3.

2.4.4.3 B-Raf Antibody

A similar protocol was used for labelling nitrocellulose membranes with this B-Raf antibody as for the phospho-synapsin I antibodies. TBST solution was standard, as described above, but the blocking buffer contained higher nonfat dried milk (5% w/v rather than 2.5%). The B-Raf primary antibody was used at a dilution of 1:250, with the incubation being conducted overnight at 4°C. Because this commercially acquired B-Raf antibody was raised in sheep, it binds poorly to Protein A (Harlow and Lane, 1988), and so 125I-labelled protein G (37000dps per 10ml of buffer) was used to probe for primary antibody binding instead (Moodie et al., 1994).

Following labelling all membranes were allowed to dry before being exposed to a phosphorimager screen either overnight or for 48 hours. Phosphorimage data was collected using Imagequant (Molecular Dynamics) and analysed using Origin (Microcal) graphical software.

2.5 Immunoblot Analysis

Radioactive Western blots were analysed by exposing a phosphorimager screen (Kodak) overnight and developed using a Typhoon 7100 phosphorimager/scanner (Molecular Dynamics or BioRad) to retrieve the images. Densitometric evaluation of the bands from the images was carried out using Imagequant software (Molecular Dynamics). Definitions of bands as "regions of interest" for evaluation was carried out using

uniform-sized boxes to reduce band-to-band error due to differential contribution of background levels of radioactivity. The same sized region/box was also used to sample the background level of radioactivity on each blot, this being subtracted from each sample reading prior to further analysis. Following subtraction of background, the densitometry reading for each band was normalised to the 37°C control reading for that blot using the equation:

$$\text{Normalised value (\%)} = \left[\frac{100}{(37^{\circ}\text{C control} - \text{background})}\right] \text{X } (\text{sample} - \text{background})$$

The data were normalised to obviate the inevitable blot-to-blot variability in absolute levels of blotting response. This normalisation also accounted for changes in reading levels due to changes in phosphorimager efficiency and, indeed, the use of different phosphorimager instruments, as was necessitated during the course of this work.

2.6 Statistical Analysis

All statistical analysis was carried out on data normalised as described in the previous section, with the exception of release data. Although analysis should ideally be carried out on raw data, it was not possible in this case due to large variability in the sensitivity of different phosphoimagers resulting in type II statistical errors. In order to try and prevent any reduction in validity, efforts were made to normalise the data in such a way so as to remove variation in the basal signalling levels obtained between blots, but to leave intact the differences between samples on any one blot.

The test used for assessing the confidence levels of any significant differences between two data sets was the Student's paired t-test. An analysis of variance (ANOVA), followed by post hoc analysis with Duncan's test, was used for assessing the confidence levels of differences between multiple data sets. Calculations were carried out using Excel spreadsheets (Microsoft) and WinStat (Microsoft).

2.7 Reagents List

All reagents, with the exception of those listed below, were obtained from BDH Chemicals Ltd, Poole, Dorset and were of AnalaR grade.

2.7.1 Glutamate Release/Fura-2-AM Reagents

Glutamate dehydrogenase (GDH) type II from bovine liver (catalogue number G-2626), nicotinamide adenine dinucleotide (NADP), ionomycin (from Streptomyces conglobatus), L-glutamic acid, and ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N',-tetraacetic acid (EGTA) were all obtained from Sigma-Aldrich Company, Gillingham, Dorset. 4-aminopyridine (White et al., 2003) was purchased from Tocris Cookson Ltd, Avonmouth, Bristol and Fura-2-AM was acquired from Molecular Probes c/o Cambridge BioScience, Cambridge.

2.7.2 Synaptosomal Preparation Reagents

Sucrose and purified water were obtained from BDH Chemicals Ltd, Poole, Dorset, but were of molecular biology grade and HPLC grade, respectively. Percoll, bovine serum albumin (BSA) and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma-Aldrich Company, Gillingham, Dorset.

2.7.3 Gel Electrophoresis/Immunoblotting Reagents

Phospho-p44/p42 MAP kinase (Thr202/Tyr204) antibody (phospho-ERK1,2 antibody), catalogue number 9101L, was purchased from New England Biolabs, Hitchin, Hertfordshire. This polyclonal antibody was produced by immunizing rabbits with a human phosphor-peptide containing the Thr202/Tyr204 region, and cross-reacts with rat forms of the protein. The B-Raf antibody, catalogue number 07-453, was purchased from Upstate Biotechnology, Milton Keynes. This antibody was raised in sheep using a peptide derived from the human form of B-Raf, and cross-reacts with the rat form of the kinase. The phospho-specific synapsin I antibodies were raised in rabbits at the Rockefeller University, New York, and were a kind gift from Professor Paul Greengard (Rockefeller University, New York), Dr. Andrew Czernik (Rockefeller University, New York) and Dr. Jasmina Jovanovic (London School of Pharmacy). ^{125}I-labelled protein A and protein G were obtained from Amersham Pharmacia Biotech, Buckinghamshire. Acrylamide and bis-acrylamide were purchased as ready-made solutions (30% and 2%, respectively) from (National Diagnostic, Hull, UK).

Chapter 3

Forskolin/IBMX-Mediated Modulation of Presynaptic Function: Cross-Talk of cAMP and ERK 1, 2 Signalling Cascades.

3.1 Introduction

The strength of synaptic connections within the brain has been shown to be a highly regulated phenomenon, in which both pre- and post-synaptic nerve terminals can play a part. Changes occur which can both enhance or diminish the transduction of signals across synapses, resulting in either short-term or long-term modifications. This process of synaptic plasticity has been shown to be regulated by numerous different modulators, including protein kinase A (PKA) (Roche et al., 1994; Greengard, 2001; Sheng and Kim, 2002; Waltereit and Weller, 2003).

Roles for cAMP/PKA have been described for short-term plastic phenomena, such as temporary decreases or increases in neurotransmitter release from presynaptic nerve terminals. For example, activation of the cAMP/PKA pathway in Lymnaea neurones has been shown to result in reduced synaptic efficacy during early synaptogenesis and plasticity (Munno et al., 2003), and increases in cAMP in the hippocampus have been shown to result in the depression of excitatory post synaptic potentials (EPSPs). This depression was believed to be due to adenosine receptor activation, following cAMP metabolism to adenosine (Gereau and Conn, 1994). However, increases in cAMP/PKA activation have also been linked with the enhancement of neurotransmission (in the form of glutamate release) in the CA3 region of the hippocampus (Lonart et al., 1998), at granule cell to Purkinje cells synapses in the cerebellum (Chen and Regehr, 1997), and in layer V of the entorhinal cortex (Evans et al., 2001a). In addition to glutamate, cAMP/PKA activation has been shown to increase the release of other neurotransmitters, for instance, Sciancalepore et al., found that the metabotropic glutamate receptor agonist, trans-ACPD, increased GABA release from hippocampal interneurones in a PKA-dependent manner (Sciancalepore et al., 1995).

Long-term changes in synaptic strength can also be dependent on cAMP/PKA activation and roles for PKA have been described in the regulation of long-term depression (LTD) and in long-term potentiation (LTP). In the nucleus accumbens, activation of the PKA signalling cascade was shown to result in LTD, mediated by a PKA-dependent long-term reduction in Ca^{2+} influx through P/Q-type channels (Robbe et al., 2002). Activation of PKA and PKC was also found to be required for the

induction of LTD in the medial perforant path of the dentate gyrus (Huang et al., 1999), whereas the CA3-CA1 synapse in the hippocampus was found to undergo cAMP/PKA-mediated LTP and LTD (Yu et al., 2001). An involvement of PKA in mossy fibre LTP was first identified in 1994, where Ca^{2+}-influx was believed to activate type I adenylate cyclase, resulting in the downstream activation of PKA and a persistent enhancement in evoked glutamate release (Weisskopf et al., 1994). Since then, PKA activation has been shown to be required for the induction of cerebellar LTP (Salin et al., 1996; Linden and Ahn, 1999) and for corticothalamic LTP (Castro-Alamancos and Calcagnotto, 1999). PKA activation has also been shown to be able to increase the number of functional release sites in cultured neurones from the hippocampal region (Ma et al., 1999; Kohara et al., 2001).

cAMP/PKA-dependent signalling has been shown to regulate the enhancement of release from presynaptic nerve terminals through four different mechanisms. The first mechanism is through PKA-mediated regulation of membrane excitability, through the modification of ion channels. This form of regulation results in an increased excitability of the membrane leading to an increase in the propensity towards vesicle release (Dunwiddie et al., 1992; Herrero and Sanchez-Prieto, 1996). Secondly, direct PKA-regulation of Ca^{2+} channels could lead to increases in Ca^{2+} influx, and thus enhancement of Ca^{2+}/exocytosis coupling (Catterall, 2000; Lonart et al., 1998). The third mechanism through which cAMP/PKA could increase glutamate release is via the regulation of the coupling of exocytotic machinery (Chen and Regehr, 1997; Lonart and Sudhof, 1998). PKA phosphorylation of components of the release machinery can lead to enhanced Ca^{2+}-sensitivity and so increased probability of vesicle release (Trudeau et al., 1996). Consistent with this, some of the key members of the secretory apparatus and associated proteins have been shown to be substrates for PKA. These include the t-SNARE, SNAP-25 (Nagy et al., 2004), as well as Rab3A and RIM1a, two proteins implicated in mossy fibre LTP (Lonart et al., 1998; Castillo et al., 2002). The fourth, and final, mechanism through which cAMP/PKA activation could result in the enhancement of glutamate release would be through increasing the availability of synaptic vesicles for exocytosis. PKA can phosphorylate the synaptic vesicle tethering protein, synapsin I, to decrease its interactions with actin thus allowing increased numbers of vesicles to migrate to the proximity of the active zone (Chi et al., 2001).

Indeed, cAMP/PKA has been shown to increase the number of vesicles available for release during mossy fibre LTP (Lonart et al., 1998).

As research better defines the plastic changes occurring at synapses it is becoming evident that the specific involvement of PKA may vary between different synapses. For example, increases in cAMP levels were found to increase release at granule cell to purkinje cell synapses in rat cerebellum. The increase in cAMP was not found to alter Ca^{2+} influx, or change the presynaptic action potential, but it was found to increase the probability of release from existing active zones (Chen and Regehr, 1997). In contrast to this, the increase in cAMP/PKA activation in the CA3 region of the hippocampus has been shown to increase glutamate release through three separate mechanisms: through increasing the size of the readily releasable pool, through increasing Ca^{2+} influx and through enhancing Ca^{2+} action on the secretory apparatus (Lonart et al., 1998). This chapter focuses on the downstream pathways of cAMP/PKA signalling from synaptosomes purified from the rat cerebrocortex. Research to date, using this preparation, has identified several possible roles for PKA in the enhancement of depolarisation-induced glutamate release. It is possible that PKA may be regulating membrane excitability in this system, though this is yet to be confirmed (Herrero and Sanchez-Prieto, 1996). A more recent study, however, has shown that activation of PKA enhanced glutamate release from this system through increased Ca^{2+} influx from Ca^{2+}-channels, as well as through mechanisms occurring downstream of Ca^{2+} entry, but not via regulation of membrane excitability (Wang and Sihra, 2003). This chapter aims to focus on the possible mechanisms of the cAMP/PKA mediated enhancement of glutamate release that is occurring downstream of Ca^{2+} entry, and whether the cross-talk of protein kinase signalling cascades could be involved in this regulation.

The neurotrophin, BDNF has been proposed to enhance glutamate release from purified cerebrocortical nerve terminals through ERK1,2-mediated phosphorylation of synapsin I (Jovanovic et al., 2000). This represents a mechanism for increasing glutamate release downstream of Ca^{2+} channel regulation. It is possible that direct and/or indirect phosphorylation of synapsin I by PKA represents a major modulatory contribution to enhanced release downstream of Ca^{2+} entry. However, given the examples of cAMP/PKA signalling cross-talk with the ERK1,2 cascade in many other systems

devoid of the synapsins as substrates, this is unlikely to be the sole mechanism, even in nerve terminals.

PKA has been shown to both stimulate and inhibit ERK1,2 phosphorylation in several systems. Inhibition of ERK1,2 activation can result from PKA-mediated inhibition of kinases shown to be upstream of ERK1,2 in signalling cascades. For example, PKA has been shown to inhibit Raf-1 through phosphorylation of several different serine residues, including Ser-259 and Ser-621 (Mischak et al., 1996; Dhillon et al., 2002), and also to inhibit B-Raf signalling (Sidovar et al., 2000; Konig et al., 2001). However, PKA has also been shown to stimulate ERK1,2 signalling through activation of the small GTPase proteins Ras-1 and Rap-1 (Schmitt and Stork, 2002; Norum et al., 2003), as well as through direct activation of B-Raf (Yamaguchi et al., 2003). The effect of PKA on ERK1,2 signalling has been found to be dependent on cell type and on serum conditions (Stork and Schmitt, 2002; Dhillon et al., 2002). As well as cAMP-dependent activation of PKA leading to the regulation of the ERK1,2 signalling cascade, cAMP has also been shown to stimulate ERK1,2 signalling in a manner independent of PKA. cAMP can directly activate a Rap-1 GEF known as Epac, leading to the formation of GTP-bound Rap-1 and activation of B-Raf/MEK/Erk1,2, a pathway which has been found to exist in several different systems (Laroche-Joubert et al., 2002; Fujita et al., 2002; Berruti, 2003). cAMP can also directly stimulate Ras in melanocytes, which also results in the activation of B-Raf/MEK and ERK1,2 (Busca et al., 2000). More recently, roles have been identified for the coupling of cAMP signalling to Rap-1, including an involvement in the control of early and late LTP in pyramidal cells in the hippocampus (Morozov et al., 2003).

This chapter focuses on the presynaptic roles of the cAMP/PKA signalling cascade in the regulation of glutamate release, and on its cross-talk with the ERK1,2 cascade. It aims to investigate whether cAMP/PKA could be stimulating ERK1,2 phosphorylation in cerebrocortical nerve terminals, and whether this could lead to downstream synapsin I phosphorylation. If so, this could provide a basis for cAMP/PKA modulation of glutamate release through increasing the availability of synaptic vesicles in cerebrocortical nerve terminals. This would not only suggest an alternative mechanism for cAMP/PKA modulation of glutamate release downstream of Ca^{2+} entry, but would also imply that distinct receptor-mediated signalling pathways coupled to protein

phosphorylation could crosstalk with each other, to produce a convergent mechanism for the modulation of glutamate release.

3.2 Method

Synaptosomes were prepared as described in section 2.1 (Sihra, 1997)

3.2.1. Glutamate Release

The standard glutamate release protocol was followed, as described in section 2.2 (Nicholls and Sihra, 1986; Perkinton and Sihra, 1999). When used, forskolin (100μM) was added 4 minutes prior, IBMX (50μM) 2 minutes prior, as described by Wang et al., 2003 (Wang and Sihra, 2003) and U0126 (10μM) 30 minutes prior (Brogley et al., 1999), to the addition of the secretogogue. The secretogogues used were 4-AP (1mM) or ionomycin (5μM). All incubations took place in the presence of 1mM $CaCl_2$.

3.2.2 Fura-2-AM

Synaptosomes were loaded with Fura-2-AM using established protocols, as described in chapter 2 in section 2.3, adapted from Perkinton et al., 1999 (Perkinton and Sihra, 1999). The protocol for measuring intracellular Ca^{2+} levels was followed, with U0126 (20μM) or DMSO (0.2%) being added to the synaptosomes after 3mins 30secs of incubation, 6mins and 3secs prior to the addition of 4-AP (1mM). Ratios were converted into cytosolic Ca^{2+} concentrations using calibration procedures and formulae given in more detail in section 2.3.2 and described previously (Grynkiewicz et al., 1985; Sihra et al., 1993).

3.2.3 Standard Incubation for ERK 1,2 Phosphorylation/Activation

The synaptosomes were kept at 4°C whilst being resuspended to a final synaptosomal concentration of 1mg/ml using HBM containing BSA. A final concentration of 1mM $CaCl_2$ was added prior to splitting the synaptosomes into individual incubation tubes. Forskolin (100μM), IBMX (50μM), H-89 (10μM), KT-5720 (1μM) or Rp-cAMPS (50μM or 100μM) were added immediately before transferring the incubation tubes to the 37°C waterbath. Incubations lasted for a standard time of 10 minutes before being terminated through addition of 5X SDS-PAGE sample stop buffer. This incubation protocol for measuring ERK 1,2 phosphorylation has previously been described by Jovanovic et al., 2000 (Jovanovic et al., 2000).

3.2.4 Preincubation

Synaptosomes were resuspended at 4°C to a concentration of 1mg/ml using HBM containing 1mg/ml of BSA. $CaCl_2$ (1mM) was added to the test tube containing the synaptosomes immediately prior to the transfer of the tube to the 37°C waterbath. The incubation lasted 20 minutes before the synaptosomes were recentrifuged using a microcentrifuge (10,000 x g for 30 seconds). The supernatant was discarded and the pellet cooled back to 4°C for 5 minutes. The standard incubation protocol was then utilised with the exception of forskolin (100μM) and IBMX (50μM) being added 6 minutes and 30 seconds prior to the termination of the reactions, instead of 10 minutes prior.

Samples were processed for ERK 1,2, B-Raf and synapsin phosphorylation levels as described in the main methods in chapter 2, section 2.4, following previously published methods (Dai et al., 2001; Moodie et al., 1994; Jovanovic et al., 1996).

3.2.5 Reagents

Forskolin: Adenylate cyclase activator ([3R-(3α,4aβ,5β,6β,6aα,10α,10aβ,10bα)]-5-(Acetyloxy)-3-ethenyldodecahydro-6,10,10b-trihydroxy-3,4a,7,7,10a-pentamethyl-1H-naphtho[2,1-b]pyran-1-one) (Seamon and Daly, 1981).
Dissolved in DMSO to a concentration of 100mM to form the stock solution. This was further diluted with water to a concentration of 10mM, immediately prior to use.

IBMX: Phosphodiesterase inhibitor with antagonistic properties at adenosine receptors (3-isobutyl-1-methylxanthine) (Smellie et al., 1979; Dunwiddie et al., 1981).
This was dissolved in DMSO to give a 100mM stock solution, which was diluted with water immediately prior to use to give a 5mM working solution.

U0126: MEK 1,2 inhibitor (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) (Favata et al., 1998).
20mM stock solutions were obtained by dissolving in DMSO, the 2mM working solution was obtained through addition of water to the stock solution.

Fura-2-AM: Cell permeable Ca^{2+} fluorophore (1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methyl-phenoxy)ethane-N,N,N',N'-tetraacetic acid, pentaacetoxymethyl ester) (Grynkiewicz et al., 1985).
Dissolved in DMSO to give a stock solution of 1mM, which was further diluted with water to a concentration of 500μM immediately prior to use.

H-89: PKA inhibitor acting at the catalytic site (N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride) (Chijiwa et al., 1990).
This was dissolved with DMSO to give a stock concentration of 10mM, which was further diluted with water prior to use to give a concentration of 1mM.

KT-5720: PKA inhibitor acting at the catalytic site ((9R,10S,12S)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-Kl]pyrrolo[3,4-i][1,6]benxodiazocine-10-carboxylic acid, hexyl ester) (Kase et al., 1987; Gadbois et al., 1992).
This was dissolved to a concentration of 1mM in DMSO and was further diluted before use to 100μM with water.

Rp-cAMPS: PKA inhibitor acting at the regulatory site (Rp-adenosine 3',5'-cyclic monophosphorothioate triethylammonium salt) (Botelho et al., 1988).
This was dissolved in water to a concentration of 10mM.

All the above drugs were purchased from the Sigma-Aldrich Company, Gillingham, Dorset, with the exception of U0126, which was obtained from Promega, Southampton, and Fura-2-AM, which was purchased from Molecular Probes, c/o Cambridge BioScience, Cambridge.

Unless stated, all drugs were added using 100x stock solutions to minimise volume changes and only final concentrations are quoted in the text forthwith.

3.3 Results

This chapter aimed to investigate whether the PKA and ERK1,2 signal transduction pathways could be cross-talking in the regulation of glutamate release from cerebrocortical nerve terminals. The first step towards this aim was to investigate whether stimulation of PKA could result in increased glutamate release and, perhaps more importantly, whether this could occur downstream of Ca^{2+} channel regulation.

Two drugs were used in combination, both of which increase PKA activation through increasing intracellular levels of cAMP. Forskolin is a membrane permeable diterpenoid, which activates adenylyl cyclase to increase levels of cAMP (de Souza et al., 1983). IBMX increases cAMP levels through inhibiting the phosphodiesterase which would otherwise catalyse the breakdown of cAMP into 5'-AMP (Corbin and Francis, 2002). Addition of forskolin and IBMX prior to the secretogogue, 4-AP, elicited significantly higher cumulative levels of glutamate release than observed in the presence of the secretogogue alone (Figure 3.1) (mean ± s.e.m. cumulative glutamate release calculated from 8 independent experiments: control = 14.10 ± 1.3 nmol/mg; forskolin/IBMX = 25.04 ± 3.0 nmol/mg). This is in complete agreement with previous studies examining the role of PKA in neurotransmitter regulation, which have also found increases in release following PKA stimulation (Herrero and Sanchez-Prieto, 1996; Wang and Sihra, 2003). One of these previous studies has also shown that PKA can increase glutamate release through the modulation of Ca^{2+} channel activation (Wang and Sihra, 2003). The following experiment was used to identify whether PKA could also be acting via an alternative mechanism, downstream of Ca^{2+}-channel regulation, to increase glutamate release from cerebrocortical nerve terminals.

Ionomycin is an ionophore, which allows the influx of Ca^{2+} ions into nerve terminals independently of Ca^{2+} channels. Effects of agents that would normally modulate synaptic excitability and/or Ca^{2+} channel function to regulate glutamate release would be obviated under these circumstances, making it possible to look for alternative mechanisms of release modulation downstream of Ca^{2+} influx. Under these conditions, with ionomycin used as the secretogogue, forskolin and IBMX were still able to significantly enhance glutamate release from cerebrocortical nerve terminals (Figure

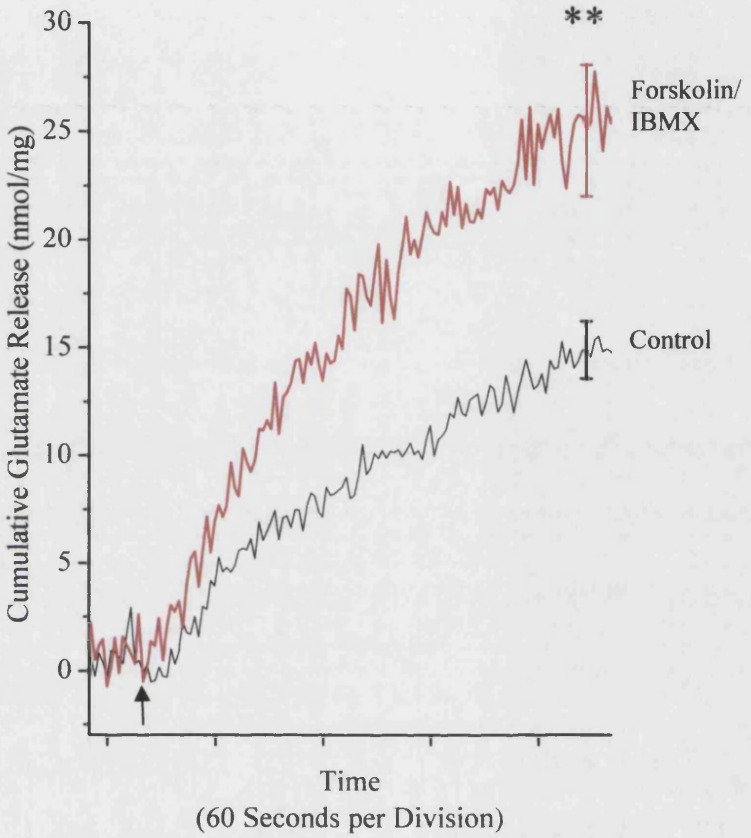


Figure 3.1 Forskolin/IBMX Enhances 4-AP-Evoked Glutamate Release. Synaptosomes were incubated in the presence of 1mM $CaCl_2$ as indicated in section 3.2.1. Glutamate release was evoked with the addition of 4-AP (1mM, arrow) in the absence (control), or presence (+Forskolin/IBMX) of 100μM and 50μM forskolin and IBMX, respectively, with forskolin added 2 min prior, and IBMX added 4 min prior to the addition of the secretogogue. The mean traces from eight independent experiments, measuring accumulative release at 2 sec intervals, are shown. Mean ± s.e.m. release was calculated at each time point, but the cumulative glutamate release values following 4 min of incubation with the secretogogue were used for statistical analysis **P<0.01; different from control (Student's paired t-test; n=8).

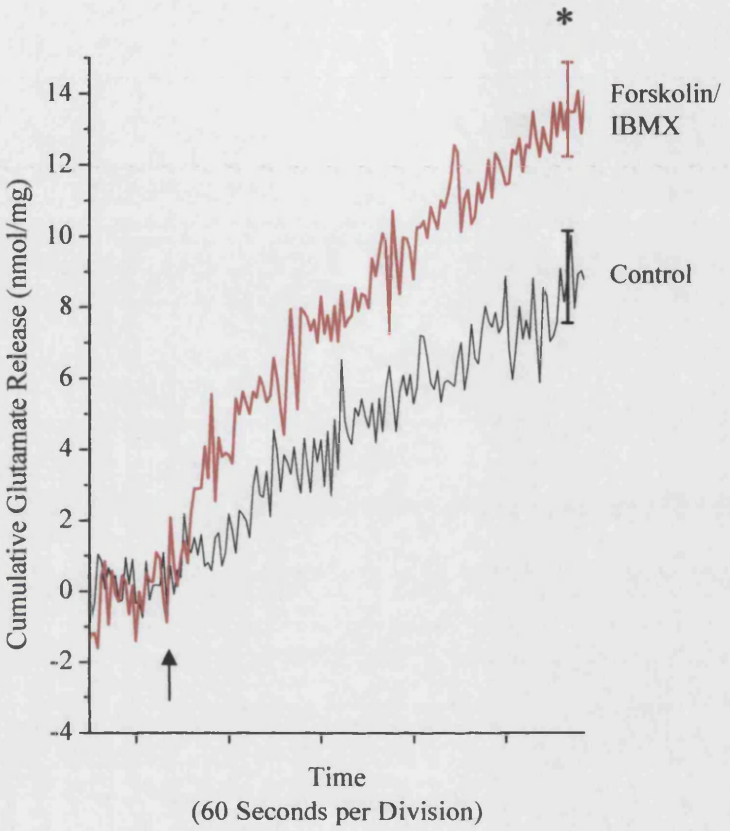


Figure 3.2 Forskolin/IBMX Enhances Ionomycin-Elicited Glutamate Release. Synaptosomes were incubated in the presence of 1mM $CaCl_2$ as described in section 3.2.1. 100μM forskolin and 50μM IBMX were added 4 min and 2 min, respectively, prior to the addition of the secretogogue. Glutamate release was elicited by addition of ionomycin (5μM, arrow) in the absence (control) or presence (+Forskolin/IBMX) of forskolin/IBMX. The mean traces from four independent experiments, measuring accumulative release at 2 sec intervals, are shown. Mean ± s.e.m. release was calculated at each time point, but the cumulative glutamate release values following 4 min of incubation with the secretogogue were used for statistical analysis *$P<0.05$; different from control (Student's paired t-test; n=4).

3.2) (mean ± s.e.m. cumulative glutamate release calculated from 4 independent experiments: control = 8.86 ± 1.3 nmol/mg; forskolin/IBMX = 13.56 ± 1.3 nmol/mg). This suggests that, as well as modulating Ca^{2+} channels, the cAMP/PKA signalling cascade is also able to act at an alternative locus, downstream of Ca^{2+} entry, to increase glutamate release from cerebrocortical nerve terminals.

The ERK1,2 signalling pathway was examined as potentially being the downstream site of action of cAMP and PKA signalling in nerve terminals. This pathway was specifically chosen as it has previously been shown to be involved in modulating glutamate release from nerve terminals (Jovanovic et al., 2000), as well as being open to regulation by PKA in some systems (Schmitt and Stork, 2002; Norum et al., 2003). Specific ERK1,2 inhibitors are not currently commercially available, but there are inhibitors of MEK1/2, the activating kinase directly upstream of ERK1,2. The next experiment in this chapter therefore looked at the effect of a MEK inhibitor, U0126 (Favata et al., 1998), on glutamate release from nerve terminals. The ultimate aim of this approach was to test whether the inhibition of the ERK 1,2 cascade could attenuate, or abrogate the forskolin/IBMX enhancement of glutamate release and thereby directly implicate ERK1,2 activation downstream of cAMP production.

Synaptosomes were incubated at 37°C for 30 min with 10μM U0126, prior to the addition of the secretogogue, 4-AP. As can be seen from the graph in Figure 3.3, treatment with U0126 alone was able to significantly enhance the glutamate release elicited by 4-AP. This unexpected finding could be explained through one of two mechanisms; either that U0126 is acting non-specifically to increase glutamate release, or that the ERK1,2 signalling pathway has an inhibitory influence on glutamate release under the incubation protocols used herein. Although studies conducted in other labs have found U0126 to be relatively specific with respect to other kinases (Davies et al., 2000), several irregularities have been noted with its use in nerve terminals. For example, U0126 was found to non-specifically potentiate depolarisation-induced Ca^{2+}-independent glutamate release from hippocampal synaptosomes (Pereira et al., 2002). Use of this MEK inhibitor has also resulted in shifts in the activation of dendritic K^+ channels to more hyperpolarized potentials (Watanabe et al., 2002), an effect that would also be expected to lead to enhanced excitability. In order to obviate

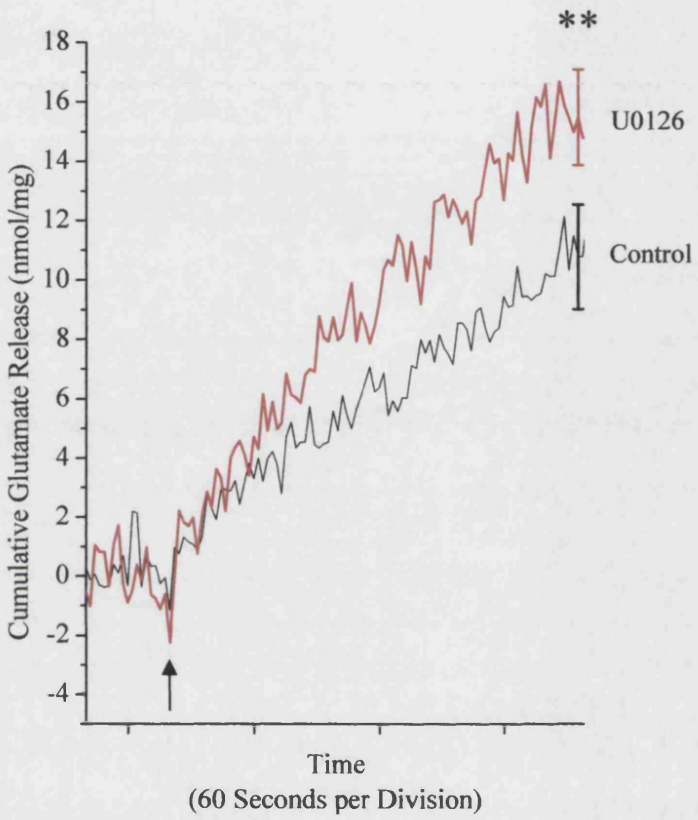


Figure 3.3 U0126 Enhances 4-AP-Evoked Glutamate Release.
Synaptosomes were incubated in the presence of 1mM $CaCl_2$ as indicated in section 3.2.1. Glutamate release was evoked using 4-AP (1mM, arrow) either in the absence (control) or presence (Pereira et al., 2002) of 10μM U0126, added 30 min prior to the addition of 4-AP. The mean traces from three independent experiments, measuring accumulative glutamate release at 2 sec intervals, are shown. Mean ± s.e.m. release was calculated at each time point, but the cumulative glutamate release values following 4 min of incubation with the secretogogue were used for statistical analysis **P<0.05; different from control (Student's paired t-test; n=3).

the possible non-specific effects of U0126 on glutamate release, PD98059, the first established MEK inhibitor, (Dudley et al., 1995), could be considered as an alternative. Unfortunately however, although PD98059 has had obvious utility and value as a relatively specific inhibitor of MAP kinase pathways in many systems, the high intrinsic fluorescence and quenching properties of this compound makes it impossible to use with this enzyme-linked fluorescence assay for measuring cumulative glutamate release (Jovanovic et al., 2000). Additionally, PD98059 has also been shown to non-specifically regulate N and P/Q-type voltage-dependent Ca^{2+} channels (Pereira et al., 2002), complicating its relevance for use in examining the involvement of the ERK1,2 signalling cascade in the regulation of glutamate release. Similarly, in control experiments looking at intracellular Ca^{2+} concentrations in synaptosomes, an effect of U0126 in increasing basal Ca^{2+} levels (Figure 3.4) was also indicated, which could potentially suggest a locus for the unexpected facilitation of glutamate release seen with the use of this compound. Given the individual and overlapping problems encountered with the available MEK inhibitors, alternative approaches for investigating the roles of the MAPK signalling pathway were warranted. To most directly invoke a role for the kinase cascade in presynaptic modulation, in the following experiments we correlated actual levels of ERK1,2 activation in response to the application of upstream regulators.

ERK1 and ERK2 are believed to be activated when they undergo dual phosphorylation of threonine and tyrosine residues (Payne et al., 1991). Antibodies have now been developed which are able to specifically detect the dually phosphorylated forms of these kinases, making it possible to detect levels of ERK1,2 activation in cells (Dai et al., 2001). Thus, as an alternative to the foregoing problematic inhibitor studies, this antibody could be used to determine whether ERK1,2 activation/phosphorylation levels are enhanced when synaptosomes are treated with forskolin/IBMX. This would identify whether elements of the cAMP/PKA signalling cascade could be cross talking with the MAPK signalling cascade.

Figure 3.5 shows the results obtained using the phospho-specific ERK1,2 antibody when synaptosomes were incubated with forskolin and IBMX. As forskolin has been shown to inhibit ERK in some systems, albeit through a PKC-dependent mechanism (Siddhanti et al., 1995), both the combined and individual effects of forskolin and

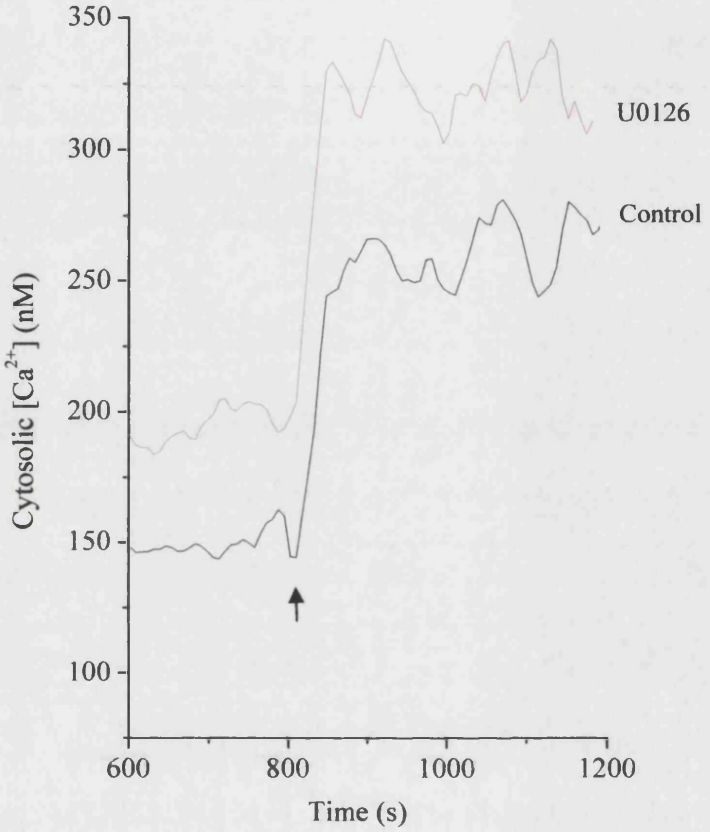


Figure 3.4 U0126 Can Increase Intracellular Ca^{2+} Concentration in Synaptosomes. Synaptosomes were loaded with Fura-2-AM as described in chapter 2.3 and then incubated with 1mM $CaCl_2$ either in the presence (Pereira et al., 2002) or absence (control) of 20μM U0126. The arrow indicates the addition of 4-AP (1mM) to the nerve terminals. Excitation wavelengths of 340nm and 380nm were used, with fluorescence readings collected every 3.5sec at 505nm using a fluorimeter (Perkin-Elmer). Maximum and minimum Ca^{2+} levels were obtained and cytosolic Ca^{2+} concentrations were calculated. The graph shows representative synaptosomal cytosolic Ca^{2+} concentrations obtained in the presence and absence of the MEK inhibitor, U0126.

IBMX on ERK1,2 phosphorylation levels were examined. No significant differences were found between the changes in phosphorylation levels of ERK1 compared to ERK2 (data not shown), therefore henceforth, data are presented in terms of combined total ERK phosphorylation/activation levels.

The results show that both the treatments with IBMX alone, and IBMX combined with forskolin, were able to significantly increase ERK phosphorylation levels (mean ± s.e.m. in % compared to 100% 37°C control levels of total ERK phosphorylation: IBMX = 122 ± 2; Forskolin/IBMX = 144 ± 13). The increase in ERK activation in the presence of forskolin was not found to be significantly different from control levels (mean ± s.e.m. in % compared to 100% 37°C control: Forskolin = 116 ± 4), however, it did not mediate the decrease in phosphorylation levels that has been observed in other systems. Of note from these experiments was the observation that when forskolin and IBMX were added in conjunction with each other, ERK1,2 phosphorylation levels were significantly higher than when either drug was added alone ($P<0.05$, ANOVA followed by Duncan's *post hoc* analysis). This implies an aspect of additivity to their signalling pathways, suggesting that the two drugs were working together to increase levels of ERK1,2 activation in nerve terminals. These results are in complete agreement with other studies looking at forskolin/IBMX-mediated enhancement of glutamate release from cerebrocortical nerve terminals. The studies examining the properties of glutamate release found that application of forskolin on its own was unable to enhance 4-AP-elicited release, and IBMX was only able to cause a minimal increase in 4-AP-evoked release. However, combined application of both drugs induced a substantial increase in 4-AP-elicited glutamate release from these nerve terminals (Wang and Sihra, 2003). As well as inhibiting phosphodiesterase activity, IBMX has also been shown to inhibit adenosine A_1 receptor activation in synaptosomes (Wang and Sihra, 2003). A_1 receptors, in turn, have been shown to have inhibitory influences in the central nervous system and are believed to be able to suppress signalling pathways (Lorenzen et al., 1998; Park et al., 2001; Linden, 2001). Accordingly, in the aforementioned glutamate release studies, specific inhibitors of both A_1 receptors and phosphodiesterase activity were able to enhance forskolin effects in the same manner as IBMX. This suggests that forskolin-mediated effects on glutamate release can be pharmacologically enhanced through two different means; via reduced catabolism of cAMP levels, and by

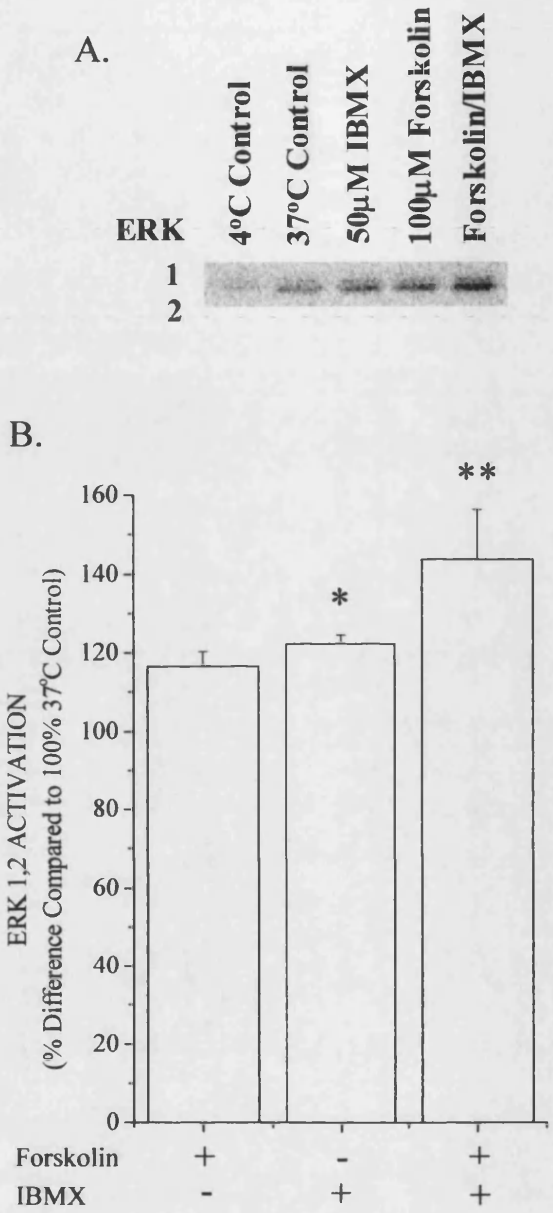


Figure 3.5 Forskolin and IBMX-Mediated Stimulation of ERK 1,2 Phosphorylation. Synaptosomes were incubated at 37°C with 1mM $CaCl_2$ for 10 min either in the absence (control) or presence of 100μM forskolin and/or 50μM IBMX (+forskolin and/or +IBMX). 7.5% polyacrylamide gels were run. Immunoblots were probed using phospho-MAPK antibody (1:1000, NEB) and reported using ^{125}I-labelled protein A. Levels of ^{125}I-labelling were detected and analysed using phosphorimager spectroscopy (Molecular Dynamics). Mean ± s.e.m. ERK1,2 phosphorylation levels were obtained from four separate experiments and normalised to individual 37°C controls. *P<0.05, **P<0.01 compared to 37°C control (ANOVA followed by Duncan's post hoc analysis, n=4). **A.** Phosphorimage of forskolin/IBMX effect. **B.** Quantification of ERK1,2 phosphorylation levels.

the relief of inhibitory adenosine tone (Wang and Sihra, 2003). These effects of forskolin and IBMX on glutamate release have made it possible to invoke three possible pathways leading to the increases seen in ERK1,2 phosphorylation in nerve terminals. The first pathway would involve increased levels of cAMP resulting in greater activation of PKA, and therefore greater PKA-mediated activation of ERK1,2 (Lerosey et al., 1991; English et al., 1999). The second pathway would involve a PKA-independent mechanism, where increased levels of cAMP lead to direct activation of a Rap-GEF, such as Epac, and subsequent stimulation of an ERK1,2 signalling cascade (de Rooij et al., 1998). The third mechanism by which forskolin and IBMX could be increasing nerve terminal ERK1,2 phosphorylation could be through the lifting of an A_1-mediated inhibitory influence, such as removing the inhibition of a Ca^{2+} channel (Park et al., 2001). These pathways are illustrated in Figure 3.6, wherein it is notable that, given the additivity of the forskolin and IBMX effects on ERK1,2 phosphorylation, it is highly likely that multiple influences acting in conjunction elicit the observed effect on ERK 1,2 phosphorylation/activation.

The next series of experiments conducted in this chapter investigated the involvement of pathway 1 (Figure 3.6) in the enhancement of ERK1,2 activation, focussing on whether the forskolin/IBMX mediated increases in ERK1,2 phosphorylation were occurring in a PKA-dependent or –independent manner. This was done through introducing PKA inhibitors to the incubation medium and examining the ERK1,2 phosphorylation levels in the absence and presence of forskolin/IBMX stimulation.

The first PKA-inhibitor used was H-89, a compound which binds competitively, with respect to ATP, to the catalytic subunit of PKA (Chijiwa et al., 1990). The ERK1,2 phosphorylation levels obtained in the presence of this inhibitor are shown in Figure 3.7. As can be seen from this figure, treatment of synaptosomes with H-89 alone induced a significant decrease in basal ERK1,2 phosphorylation levels (mean ± s.e.m. in % compared to 100% 37°C controls: H-89 = 60 ± 1). The effect of forskolin/IBMX treatment on ERK1,2 phosphorylation levels switched from a significant increase in the presence of forskolin/IBMX alone, to a significant decrease when H-89 was also added (mean ± s.e.m. in % compared to 100% 37°C control: forskolin/IBMX = 128 ± 6;

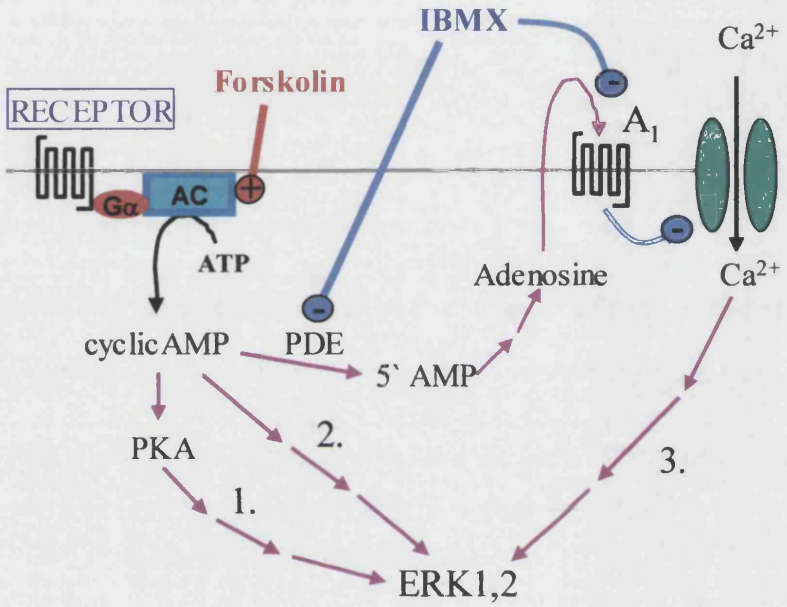


Figure 3.6 Possible Pathways Involved in the Forskolin/IBMX Mediated Increases in ERK1,2 Phosphorylation. **1)** Increased levels of cAMP, through activation of AC by forskolin and inhibition of PDE by IBMX, could be leading to increased activation of PKA and subsequent phosphorylation of ERK1,2. **2)** Increased levels of cAMP through activation of AC by forskolin and inhibition of PDE by IBMX could be leading to direct activation of the ERK1,2 signalling cascade through cAMP interactions with Rap-GEFs. **3)** IBMX-mediated inhibition of the A_1 receptor could be leading to the lifting of an inhibitory influence on the ERK1,2 signalling cascade. Blue arrows indicate an inhibitory pathway, whereas pink arrows indicate a stimulatory effect. Abbreviations: AC = adenylate cyclase; ATP = adenosine triphosphate; cyclicAMP = cyclic adenosine monophosphate; PKA = protein kinase A; PDE = phosphodiesterase; 5'AMP = 5'-adenosine monophosphate; A_1 = inhibitory adenosine receptor type I; ERK1,2 = extracellular regulated protein kinase 1,2.

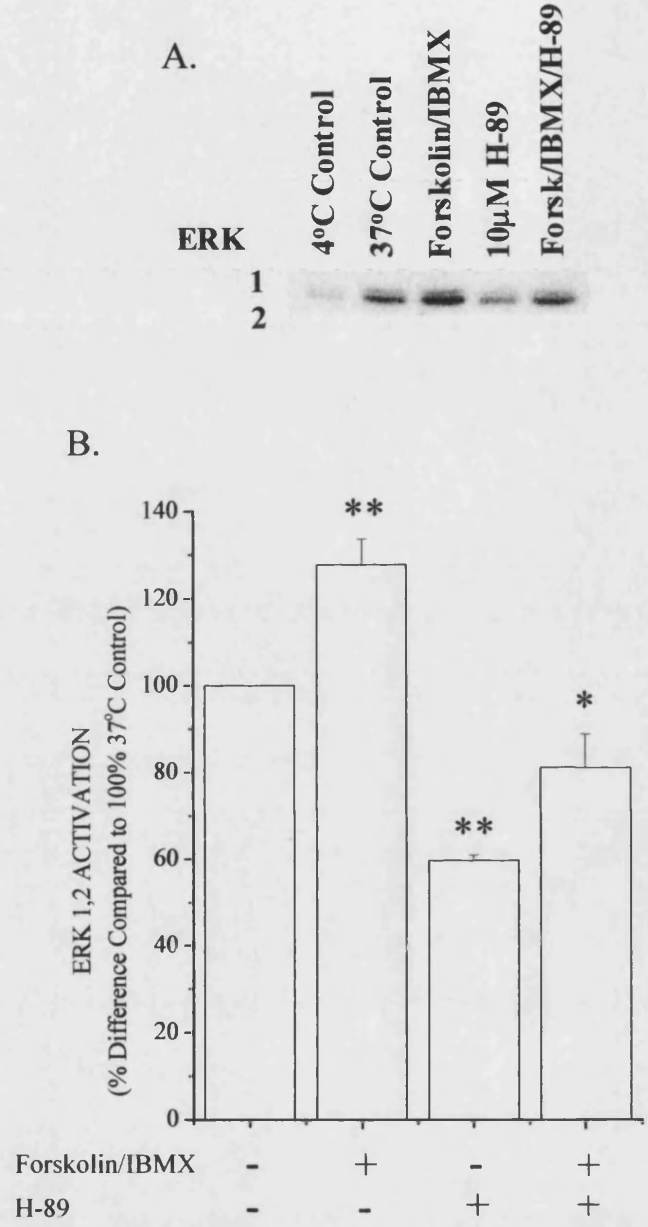


Figure 3.7 H-89 Decreased Basal Levels of ERK1,2 Phosphorylation But Not Forskolin/IBMX-Mediated Phosphorylation. Synaptosomes were incubated at 37°C with 1mM $CaCl_2$, for 10 min either in the absence (control) or presence of 10μM H-89 (+H-89), and/or 100μM forskolin and 50μM IBMX (+forsk/IBMX). 7.5% polyacrylamide gels were run. Immunoblots were labelled with phospho-MAPK primary antibody (1:1000, NEB) and probed using ^{125}I-labelled protein A. Levels of ^{125}I from several independent experiments were detected and analysed using phosphorimager spectroscopy (Molecular Dynamics) *P<0.05, **P<0.01 compared to 100% 37°C controls (ANOVA followed by Duncan's post hoc analysis, n=6). **A.** Phosphorimage of H-89 and/or forskolin/IBMX effect. **B.** Quantification of ERK1,2 phosphorylation levels.

forskolin/IBMX/H-89 = 81 ± 8). Notably however, forskolin/IBMX in the presence of H-89 was still able to significantly enhance ERK1,2 phosphorylation (compared to activation in the presence of H-89 alone, $P<0.01$, ANOVA with Duncan's *post hoc* analysis). Judging from this evidence therefore, the reduced activation of ERK 1,2 with forskolin/IBMX in the presence of H-89 may simply reflect the basal effects of H-89 on ERK 1,2 activation, rather than inhibition of a forskolin/IBMX-mediated pathway. If this is true, the implication would be that while there may be a PKA-dependent component to basal ERK1,2 phosphorylation levels (H-89 inhibited), the actions forskolin/IBMX may be occurring through a PKA-independent route to increase ERK 1,2 activation in nerve terminals. Alternatively, the apparent differential activity of H-89 under basal conditions compared to the effect in the presence of forskolin/IBMX may reflect on the specificity of action of the inhibitor. It is known that H-89 is not an entirely specific inhibitor of PKA, having been shown, *in vitro*, to inhibit at least three other kinases, sometimes with a potency similar to or greater than that for PKA, including mitogen and stress activated protein kinase 1 (MSK1), p70 ribosomal protein S6 kinase (S6K1) and Rho-dependent protein kinase (ROCKII) (Davies et al., 2000). While these alternative targets may not be present in nerve terminal, the possibility that the reduction in basal levels of ERK1,2 activation by H-89 could be mediated through the inhibition of a kinase other than PKA remains tenable. The relative lack of effect of the inhibitor on forkolin/IBMX-stimulated PKA activity may reflect the fact that, H-89, as a competitive inhibitor of the ATP-binding site on PKA, can be displaced under conditions of high cAMP production as might be postulated to occur in the presence of forskolin/IBMX. In order to try and clarify the roles of PKA in mediating ERK1,2 phosphorylation levels in cerebrocortical nerve terminals, the effects of a second PKA inhibitor were examined.

KT-5720 has been described as a cell permeable, potent and specific inhibitor of PKA and, like H-89, also appears to work at the catalytic site of PKA (Kase et al., 1987; Gadbois et al., 1992). The results obtained with this inhibitor, in the presence and absence of forskolin/IBMX, are shown in Figure 3.8. Like H-89, KT-5720 also significantly decreased basal levels of ERK1,2 phosphorylation (mean ± s.e.m. in % compared to 100% 37°C control values: KT-5720 = 74 ± 4). KT-5720 also appeared to inhibit forskolin/IBMX induced increases in ERK1,2 activation, when compared to the

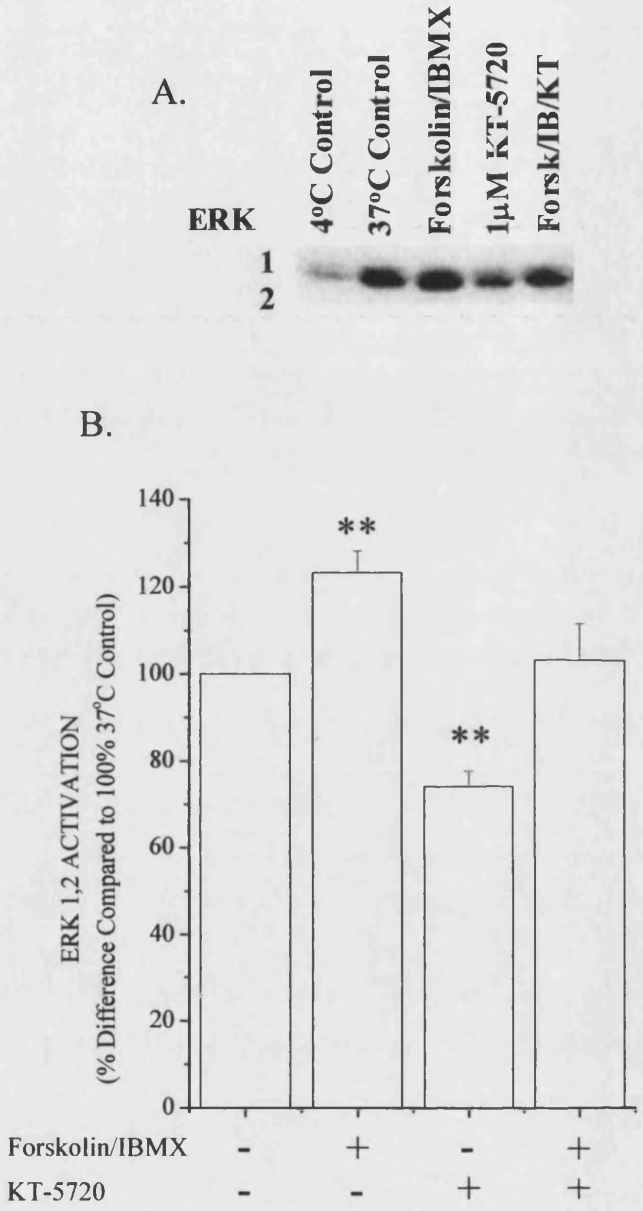


Figure 3.8 KT-5720 Decreased Basal Levels of ERK 1,2 Phosphorylation But Not Forskolin/IBMX-Mediated Phosphorylation. Synaptosomes were incubated for 10 mins with 1mM $CaCl_2$, either in the absence (control) or presence of 1μM KT-5720 (+KT-5720) and/or 100μM forskolin and 50μM IBMX (+Forskolin/IBMX). 7.5% polyacrylamide gels were run. Immunoblots were labelled with phospho-MAPK primary antibody (1:1000, NEB) and probed using ^{125}I-labelled protein A. Levels of ^{125}I from several separate blots were detected and analysed using phosphorimager spectroscopy (Molecular Dynamics). **P<0.01 compared to 100% 37°C controls (ANOVA followed by Duncan's *post hoc* analysis, n=5). **A.** Phosphorimage of KT-5720 and forskolin/IBMX effects on ERK 1,2 phosphorylation. **B.** Quantification of ERK 1,2 phosphorylation levels.

100% 37°C control values (mean ± s.e.m. in %: forskolin/IBMX = 123 ± 5; forskolin/IBMX/KT-5720 = 103 ± 8). However, again, because treatment of synaptosomes with forskolin/IBMX in the presence of KT-5720 was found to still significantly increase ERK1,2 phosphorylation levels when compared to the KT-5720 control ($P<0.01$, ANOVA with Duncan's *post hoc* analysis), the data would suggest that the lack of net change in ERK1,2 phosphorylation levels reflects a simple additivity of the negative effects of KT-5720 and the positive effects of forskolin/IBMX. These results are congruent with those obtained using H-89 and indeed, like H-89, KT-5720 has also been recently shown to inhibit other protein kinases, these notably being MEK1 and ERK2, members of the signalling cascade being addressed (Davies et al., 2000). Thus, notwithstanding the fact that the concentrations of inhibitor used in the latter studies were 10x higher than those used here, it is possible that the inhibitory effects of KT-5720 on basal ERK1,2 phosphorylation levels are due to a direct interaction with the ERK signalling cascade, rather than through PKA inhibition (Davies et al., 2000).

Both the PKA inhibitors used so far have utilised binding of the catalytic site of PKA to mediate their inhibition, and both were found to decrease basal levels of ERK1,2 phosphorylation. It is possible that these effects of the inhibitors on the ERK1,2 signalling cascade are as a result of non-specific binding, conducive to the design of the inhibitors as interactors with the ATP binding site of the catalytic domain of PKA. Other PKA inhibitors are available which have been designed to act at the regulatory subunit of PKA, and so should not have the same non-specific effects as H-89 and KT-5720 (Davies et al., 2000). The following experiments conducted in this chapter investigated the effects of one such inhibitor, Rp-cAMPS, on ERK1,2 phosphorylation levels in the presence and absence of forskolin/IBMX.

Rp-cAMPS binds to the regulatory subunit of PKA in the place of cAMP, and in doing so prevents cAMP-mediated dissociation of the regulatory subunits from the catalytic subunits, thus resulting in the inhibition of PKA catalytic activity (Botelho et al., 1988). Figure 3.9 shows the measure of ERK1,2 activation obtained when synaptosomes were incubated in the presence and absence of Rp-cAMPS (50μM) and/or forskolin/IBMX. ERK1,2 phosphorylation levels were found to be significantly increased both in the presence of forskolin/IBMX and when Rp-cAMPS was added in conjunction

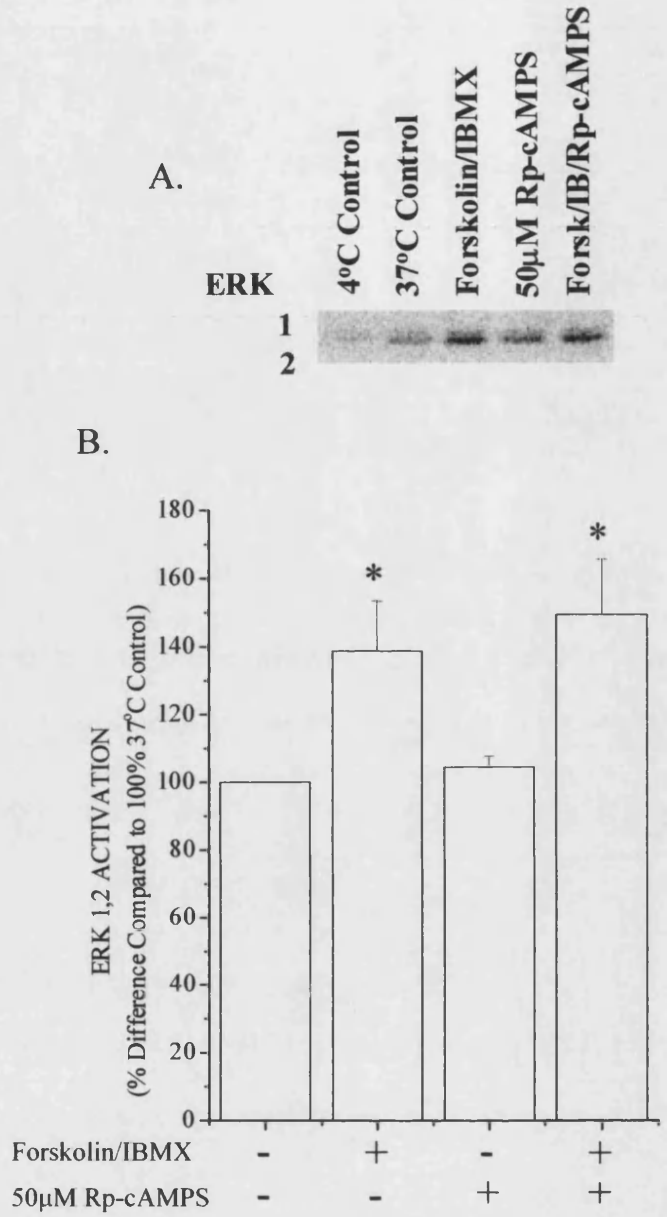


Figure 3.9 Rp-cAMPS (50μM) Does Not Inhibit Forskolin/IBMX Stimulation of ERK 1,2 Phosphorylation. Synaptosomes were incubated for 10 min with 1mM $CaCl_2$ either in the absence (control) or presence of 50μM Rp-cAMPS (+Rp-cAMPS) and/or 100μM forskolin and 50μM IBMX (+forskolin/IBMX). 7.5% polyacylamide gels were run. Immunoblots were labelled with phospho-MAPK primary antibody (1:1000, NEB) and probed using ^{125}I-labelled protein A. Levels of ^{125}I from several separate blots were detected and analysed using phosphorimager spectroscopy (Molecular Dynamics). *P<0.05 compared to 100% 37°C controls (ANOVA followed by Duncan's *post hoc* analysis, n=4). **A.** Phosphorimage of Rp-cAMPS and forskolin/IBMX effects on ERK 1,2 phosphorylation. **B.** Quantification of ERK 1,2 phosphorylation levels.

with forskolin/IBMX (mean ± s.e.m. in % compared to 100% 37°C controls levels: forskolin/IBMX = 138 ± 14; forskolin/IBMX/Rp-cAMPS = 149 ± 16). These results are in agreement with those obtained with the other PKA inhibitors, with the finding that forskolin/IBMX-mediated increases in ERK1,2 phosphorylation levels were not decreased by PKA inhibition. In contrast to the experiments conducted with H-89 and KT-5720, Rp-cAMPS by itself was not found to significantly decrease basal levels of ERK1,2 phosphorylation (mean ± s.e.m. in % compared to 100% 37°C control: Rp-cAMPS = 104 ± 3). As it is possible that the lack of inhibitory effects seen here with Rp-cAMPS are due to competitive displacement of Rp-cAMPS by high levels of cAMP, a higher concentration of this inhibitor was also tested.

Figure 3.10 illustrates the effects found on ERK1,2 activation following treatment with the higher concentration of Rp-cAMPS (100μM). Both forskolin/IBMX and forskolin/IBMX/Rp-cAMP treatments were found to significantly enhance ERK1,2 phosphorylation levels with respect to control levels (mean ± s.e.m. in % compared to 100% 37°C control: forskolin/IBMX = 132 ± 8; forskolin/IBMX/Rp-cAMPS = 136 ± 6). They were also found to be significantly different from the levels of ERK1,2 phosphorylation obtained in the presence of Rp-cAMP on its own (mean ± s.e.m. in % compared to 100% 37°C control: Rp-cAMPS (100μM) = 112 ± 3) (*$P<0.05$, ANOVA followed by Duncan's *post hoc* analysis). However, no significant difference was found between ERK1,2 activation in the presence of Rp-cAMP and the levels obtained in control conditions.

Rp-cAMPS is not a very membrane soluble compound so it is possible that a 10 minute incubation is not long enough for this PKA inhibitor to be incorporated into the synaptosomes. Although this compound has been shown to be active in isolated nerve terminals, and to inhibit forskolin/IBMX-mediated enhancement of glutamate release, long (30 minute) preincubation times were used (Wang and Sihra, 2003). Likewise, recently developed and very specific PKA inhibitor peptides with membrane-permeable acetoxymethyl ester groups have also been shown to require a 30 minute preincubation, to allow incorporation into synaptosomes. As a view to these paradigms, a preincubation protocol was devised which would allow substantial loading of nucleotide inhibitor into synaptosomes, with the aim of clarifying the involvement of PKA in

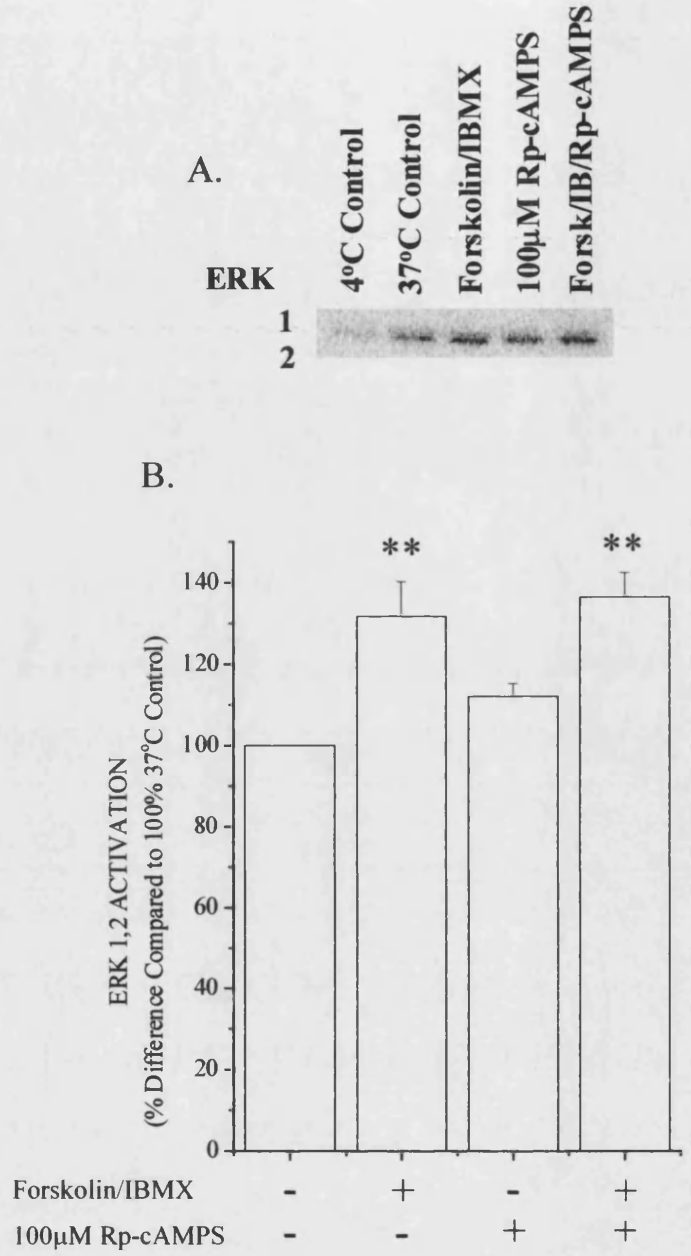


Figure 3.10 Raising the Concentration of Rp-cAMPS Does Not Change its Effect on Forskolin/IBMX-Mediated ERK 1,2 Phosphorylation. Synaptosomes were incubated with 1mM $CaCl_2$ for 10 min either in the absence (control) or presence of 100µM Rp-cAMPS (+Rp-cAMPS) and/or 100µM forskolin and 50µM IBMX (+forskolin/IBMX). 7.5% polyacrylamide gels were run. Immunoblots were labelled with phospho-MAPK antibody and probed using ^{125}I-labelled protein A. Phosphorimager spectroscopy (Molecular Dynamics) was used to measure and analyse levels of ^{125}I labelling. **P<0.01 compared to 100% 37°C controls (ANOVA followed by Duncan's *post hoc* analysis, n=5). **A.** Phosphorimage of Rp-cAMPS and forskolin/IBMX effects on ERK 1,2 phosphorylation. **B.** Quantification of ERK 1,2 phosphorylation levels.

forskolin/IBMX-mediated enhancement of ERK1,2 phosphorylation.

Synaptosomes were incubated at 37°C for 20 minutes with 1mM $CaCl_2$ before being pelleted and cooled back to 4°C. Upon resuspension, a standard 10 minute incubation protocol was used. The results are shown in Figure 3.11 and, as can be seen from the graphs, forskolin/IBMX was no longer found to enhance ERK1,2 phosphorylation levels following the preincubation protocol. Instead, treatment of the synaptosomes with forskolin/IBMX resulted in a significant decrease in ERK1,2 activation, the complete opposite to the increase observed with the short incubation protocol (mean ± s.e.m. in % compared to 100% 37°C controls: preincubation = 77 ± 8; short incubation = 143 ± 8). These results suggests a bidirectional effect of forskolin/IBMX treatment on ERK1,2 activation, which is dependent upon incubation conditions. This could possibly help explain why some studies have identified an inhibitory effect of forskolin application on ERK1,2 phosphorylation, whereas others have found stimulatory effects (Siddhanti et al., 1995; Dhillon et al., 2002; Kanterewicz et al., 2000).

The results so far gleaned from putative PKA inhibitor studies appear to suggest that activation of ERK1,2, following the enhancement of cAMP levels and A_1 receptor inhibition, could be occurring independently of PKA. To begin to examine this possibility, the next experiment investigated the possible presence of a key player putatively involved in pathways which, directly through cAMP action, potentially lead to the stimulation of ERK1,2 phosphorylation/activation. cAMP has been shown to directly activate the ERK1,2 signalling cascade in other systems, namely in spermatid-enriched cell cultures and in kidney cortical collecting duct cells, through the activation of a guanine nucleotide exchange factor (GEF) known as Epac (Laroche-Joubert et al., 2002; Berruti, 2003), with more recent studies suggesting that this pathway may also exist in neurones (Lin et al., 2003). This cAMP-dependent GEF catalyses the transformation of Rap-1 from its GDP-bound form to its GTP-bound form, which results in the subsequent activation of an isoform of Raf known as B-Raf, and the sequestering of MEK (de Rooij et al., 1998; York et al., 1998). Synaptosomes were immunoblotted for B-Raf in order to investigate whether or not this pathway could possibly be leading to cAMP-mediated ERK1,2 activation in cerebrocortical nerve terminals. Figure 3.12 illustrates that B-Raf isoforms are indeed present in cerebrocortical nerve terminals, leading to the possibility that this pathway could be

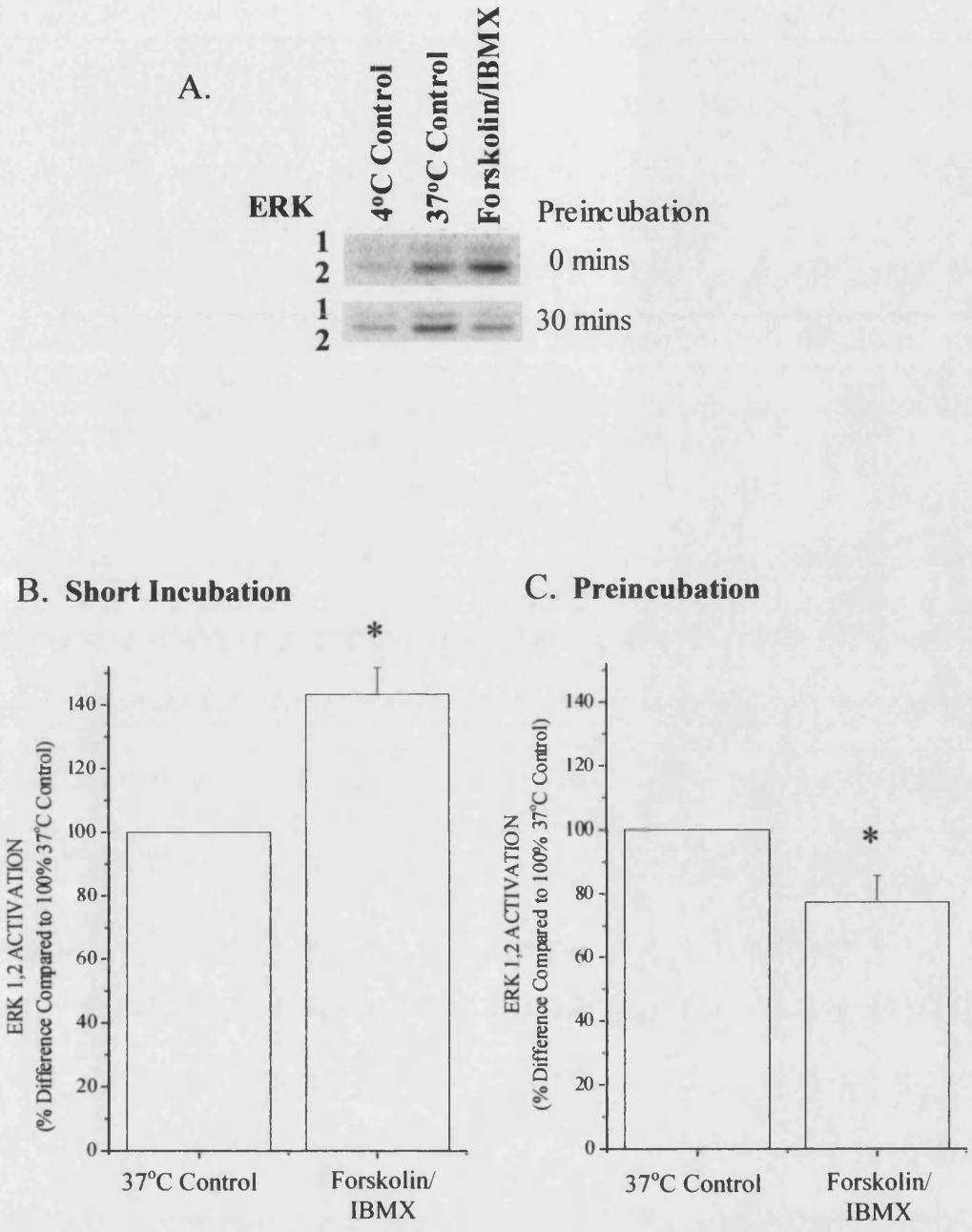


Figure 3.11 Forskolin/IBMX-Mediated Regulation of ERK 1,2 Activation is Bidirectional. Short Incubation: synaptosomes were incubated for a total of 10 min with 6 min in the absence (control) or presence of 100μM forskolin/50μM IBMX (+forskolin/IBMX). Preincubation: synaptosomes were preincubated for 20 min, pelleted and cooled, upon resuspension the short incubation protocol was followed. All incubations contained 1mM $CaCl_2$. 7.5% gels were run. Immunoblots were labelled with phospho-MAPK antibody (1:1000, NEB) and reported using ^{125}I-labelled protein A, and detected using phosphorimager spectroscopy (Molecular Dynamics). *P<0.05 compared to 100% 37°C controls (Student's paired t-test, Short Incubation n=3, Preincubation n=5). **A.** Phosphorimage of effects. **B. & C.** Quantification of ERK 1,2 phosphorylation levels.

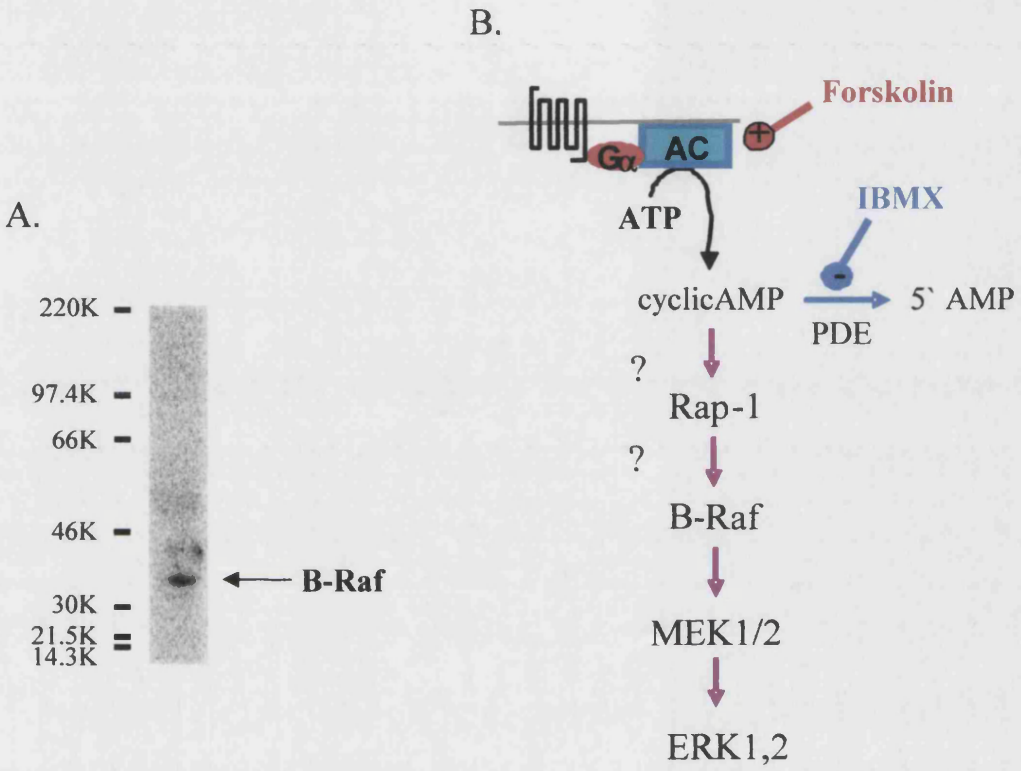


Figure 3.12 B-Raf Isoforms are Present in Cerebrocortical Nerve Terminals. Purified synaptosomes (100μg) were lysed and then separated using gel electrophoresis, before being transferred onto nitrocellulose membrane. The gels used were 10% polyacrylamide gels. The nitrocellulose membranes were then immunoblotted using a B-Raf-specific primary antibody (1:250, Upstate Biotech) and reported using ^{125}I-labelled protein A. Signal levels were detected using phosphorimager spectroscopy (Molecular Dynamics). **A.** Phosphorimage showing the presence of B-Raf isoforms in cerebrocortical nerve terminals. **B.** Possible pathway through which B-Raf could be involved in cAMP-mediated increases in ERK 1,2 activation in synaptosomes.

active in this system. Of course just identifying the presence of a component does not itself necessarily confirm its involvement. The latter objective indeed begs development of specific and cell-permeant reagents that can alter the activation of B-Raf.

The final experiments conducted here, examined the involvement of a protein phosphorylation substrate found downstream of PKA and ERK1,2 activation, synapsin I (Czernik et al., 1987; Jovanovic et al., 1996). Synapsin I has been shown to be involved in the tethering of synaptic vesicles to actin, to form a reserve pool kinetically distinct from the docked/primed vesicle pool lodged at the plasma membrane through SNARE proteins (Hilfiker et al., 1999). Furthermore, phosphorylation of synapsin I (and other members of the family), at various sites, can regulate its interactions with actin, resulting in an enhancement of glutamate release through an increase in the number of vesicles available for exocytosis (Jovanovic et al., 1996; Chi et al., 2001). Figure 3.13 shows that treatment of synaptosomes with forskolin/IBMX resulted in an increase in PKA-mediated phosphorylation of synapsin I at site 1, as well as an increase in the phosphorylation of sites 4 and 5, which are regulated by ERK1,2 (mean ± s.e.m. in % compared to 100% 37°C controls: PKA-dependent site 1 = 215 ± 36%; ERK 1,2-dependent sites 4/5 = 163 ± 12%). These results confirm that addition of forskolin/IBMX is indeed leading to PKA activation, if not PKA-mediated ERK1,2 activation, and that the observed increase in ERK1,2 phosphorylation can lead to synapsin I site 4/5 phosphorylation. These results imply that increased levels of cAMP, combined with A_1 receptor inhibition, could be leading to the enhancement of glutamate release, through a mechanism dependent on increased vesicle availability resulting from ERK1,2 and PKA stimulation of synapsin I phosphorylation.

It is interesting to note that synapsin site 2/3 phosphorylation levels appear to be decreasing upon stimulation of the nerve terminals with forskolin/IBMX (individual values of site 2/3 phosphorylation levels compared to 100% = 79.0% and 88.9%). Given that previous studies indicate that intracellular Ca^{2+} levels increase upon treatment with forskolin/IBMX, these results were somewhat surprising, as the increased Ca^{2+} should also result in CaMKII activation and, thereby increase phosphorylation of synapsin site 2/3 (Jovanovic et al., 2001). A role of the Ca^{2+}/CAM-dependent phosphatase, calcineurin, cannot be posited to explain the dephosphorylation, because the particular

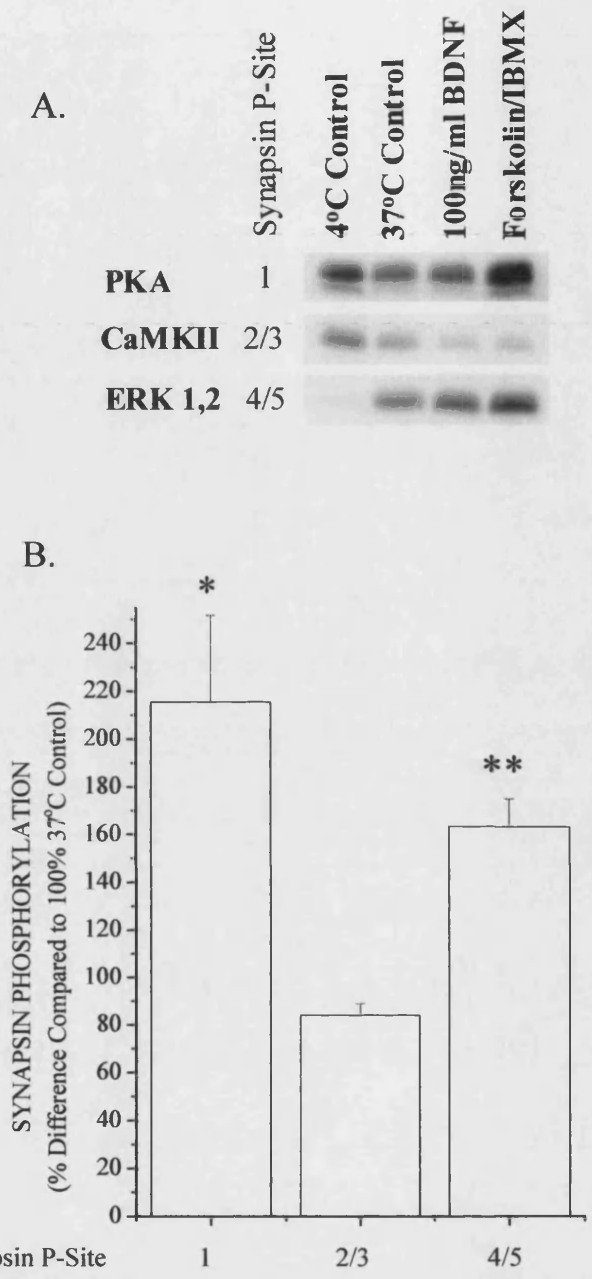


Figure 3.13 Forskolin/IBMX Regulates Synapsin I Phosphorylation at Multiple Sites. Synaptosomes were incubated at 37°C with 1mM $CaCl_2$ for 10min either in the absence (control) or presence of forskolin/IBMX (100mM/50mM) (+forskolin/IBMX), or BDNF (100ng/ml) (+BDNF). Immunoblots were labelled with antibodies specific for synapsin I phosphorylated at sites1, 2/3 or 4/5 (site 2/3 and site 4/5, 1:500; site 1, 1:200; J.J) and reported using ^{125}I-labelled protein A. Levels of ^{125}I labelling were detected and analysed using phosphorimager spectroscopy (Molecular Dynamics). *P<0.05, **P<0.01 compared to 100% 37°C controls (Student's paired t-test, site 1 n=3; site 2/3 n=2; site 4/5 n=9). **A.** Phosphorimage of synapsin I phosphorylation levels. **B.** Quantification of synapsin I phosphorylation levels (N.B. site 2/3 error bar is indicative of range).

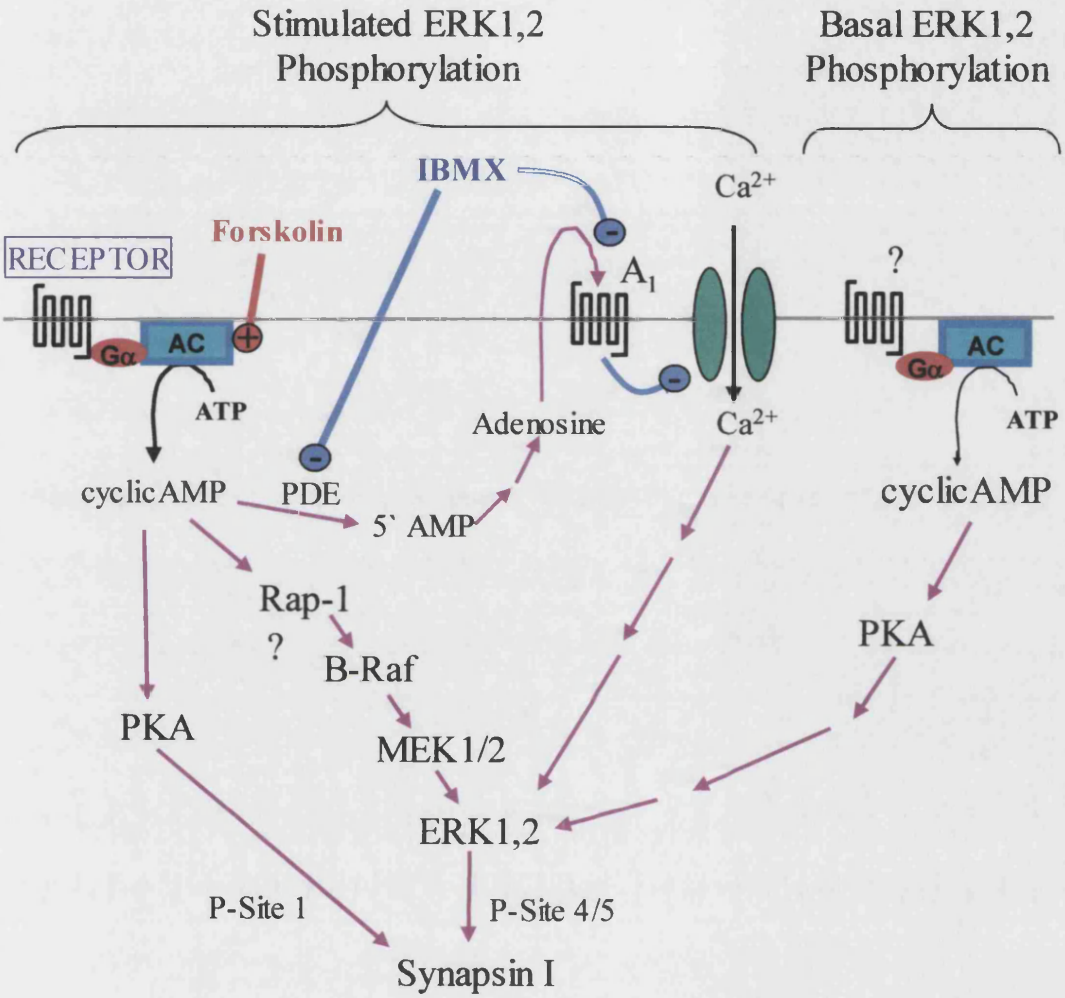


Figure 3.14 A Summary of the Possible cAMP/PKA Pathways Involved in Regulating ERK1,2 Phosphorylation Following Short Incubation Protocols. Further studies need to be conducted to confirm cAMP signalling through B-Raf to enhance ERK1,2 phosphorylation. Question marks denote components yet to be fully identified and groups of arrows denote tentative signalling pathways. Blue arrows indicate an inhibitory pathway, whereas pink arrows indicate a stimulatory effect. Abbreviations: AC = adenylate cyclase; ATP = adenosine triphosphate; cyclicAMP = cyclic adenosine monophosphate; PKA = protein kinase A; PDE = phosphodiesterase; 5'AMP = 5'-adenosine monophosphate; A_1 = inhibitory adenosine receptor type I; ERK1,2 = extracellular regulated protein kinase 1,2.

sites in question are not substrates for this phosphatase but rather protein phosphatase 2A (PP2A, (Jovanovic et al., 2001)). The mechanism leading to this observed dephosphorylation of site 2/3 in response to forskolin/IBMX therefore remains cryptic. Further experiments confirming the dephosphorylation and elucidating the signalling pathways therein need to address whether an increase in the activation of PP2A occurs directly as consequence of the application of a combination of forskolin/IBMX. It is also interesting to observe that BDNF appears to be decreasing the phosphorylation of site 2/3 of synapsin, as well as forskolin/IBMX. Though, again, this needs to be further investigated in order to draw any conclusions from it.

3.4 Summary of Results

- Increased intracellular levels of cAMP combined with inhibition of A_1 receptors enhances the levels of glutamate released from cerebrocortical nerve terminals by 4-AP.

- This enhancement of release can occur through a mechanism which is independent of Ca^{2+} channel regulation.

- Treatment of synaptosomes with IBMX enhances ERK1,2 phosphorylation levels. Combined treatment, with the adenylate cyclase activator forskolin, and with IBMX, appears to result in an additive increase in ERK1,2 phosphorylation levels.

- Basal levels of ERK1,2 phosphorylation may be partly dependent of PKA activation, whereas forskolin/IBMX stimulation of ERK1,2 phosphorylation may occur independently of PKA.

- Regulation of ERK1,2 phosphorylation levels by forskolin/IBMX can be bidirectional, depending on the incubation protocol used.

- Forskolin/IBMX increases synapsin I phosphorylation at sites mediated by PKA and at the sites regulated by ERK1,2 phosphorylation.

- B-Raf isoforms are present in cerebrocortical nerve terminals.

- Treatment of synaptosomes with the MEK inhibitor, U0126, enhances 4-AP-evoked glutamate release, possibly through increasing levels of intracellular Ca^{2+}.

3.5 Discussion

It was interesting to observe that the treatment of synaptosomes with IBMX resulted in a significant increase in ERK1,2 phosphorylation levels. As mentioned before, IBMX can act at two sites in nerve terminals. This compound can act as an inverse agonist at adenosine A_1 receptors, reducing activation through a direct inhibitory action on the receptor (Klinger et al., 2002). The inverse agonist property of IBMX has also implied a role for constitutive activity of the receptor in the regulation of cell function (Schutz and Freissmuth, 1992). In addition to this, IBMX can inhibit phosphodiesterase activity, resulting in increased levels of cAMP (Corbin and Francis, 2002). This action of IBMX could also reduce activation of adenosine receptors, as the phosphodiesterase occurs at the first stage of the metabolic pathway which produces adenosine from cAMP (Ribeiro et al., 2003).

Adenosine A_1 receptors have been shown to couple to Gi and Go G-proteins, resulting in the inhibition of adenylate cyclase activity and a decrease in intracellular levels of cAMP (Munshi et al., 1991). This means that ERK1,2 activation following treatment with IBMX could be mediated by cAMP, levels of which would have been enhanced not only by inhibition of phosphodiesterase activity, but also through the relieving of the adenylate cyclase inhibition. In contrast to this, studies in CHO cells have found that stimulation of all the subtypes of adenosine receptors resulted in the activation of the ERK1,2 signalling cascade (Schulte and Fredholm, 2000). This was believed to be through the activation of src kinases or PI3Ks, resulting in the downsteam activation of Ras (Schulte and Fredholm, 2002; Schulte and Fredholm, 2003). Whether this signalling occurs through $\beta\gamma$ subunits or through direct activation of $G\alpha i$, is a matter which is still under debate (Klinger et al., 2002). However, although A_1 receptor activation may be following this pathway in contributing to the basal levels of ERK1,2 phosphorylation observed in nerve terminals, it is unlikely to be mediating the increase in ERK1,2 activation following A_1 inhibition. Activation of A_1 adenosine receptors has been shown to decrease Ca^{2+} influx through presynaptic channels to decrease neurotransmitter release from nerve terminals (Park et al., 2001). It is also possible that the likely increase in Ca^{2+} levels following A_1 receptor inhibition mediated by IBMX,

could be stimulating increases in ERK1,2 phosphorylation levels through Ca^{2+}-dependent GEFs (Farnsworth et al., 1995).

The levels of ERK1,2 phosphorylation upon the application of both forskolin and IBMX are in agreement with a role for IBMX in adenosine receptor inhibition. The substantial increase in ERK1,2 activation is likely due to the combined influence of two pathways, rather than just one, seeing as either drug has significantly reduced effects on its own (see Figure 3.14). This effect on ERK1,2 activation could be of functional significance with regards to presynaptic plasticity and glutamate release. cAMP/PKA-dependent increases in glutamate release are transient at the Schaffer collateral-CA1 synapse, whereas they are long lasting at the mossy fibre-CA3 synapse. Inhibition of the A_1 receptor in the CA1 region was found to result in an unmasking of the cAMP effect, leading to forskolin-induced LTP at this synapse (Lu and Gean, 1999). Further work needs to be carried out to identify the exact mechanisms through which IBMX is mediating increases in ERK1,2 activity. This work could start with examining the effects of the application of specific phosphodiesterase inhibitors compared to the application of specific adenosine A_1 receptor antagonists. These experiments would help clarify the relative involvements of phosphodiesterase inhibition and A_1 inhibition on ERK1,2 phosphorylation levels.

The results presented here, suggesting that the forskolin/IBMX-mediated stimulation of ERK1,2 phosphorylation in cerebrocortical synaptosomes, lead to equivocal conclusions. On the one hand, parsimoniously it could be suggested that, given the known actions of these agents, ERK1,2 stimulation indicates the mechanistic involvement of PKA. On the other hand, in the absence of clear PKA inhibitor profiles for the effects seen, one could tentatively invoke that the activation of the MAP kinase cascade occurs independently of PKA activation. The latter conclusion would be contrary to studies examining forskolin/IBMX-mediated glutamate release in cerebrocortical synaptosomes, which found that both H-7, a mixed protein kinase inhibitor, and Rp-cAMPS, a PKA inhibitor, were able to inhibit the forskolin/IBMX-mediated enhancement of glutamate release (Wang and Sihra, 2003). There are potentially several reasons for the discrepancies noted. Firstly, the long incubation times (30 minutes before the application of the secretogogue, with forskolin and IBMX being added for 5 minutes and 2 minutes, respectively) may suggest that the lack of

effect of Rp-cAMPS in the current study may solely be due to the shorter incubation conditions, allowing insufficient time for the inhibitor to equilibrate into the synaptosomes in adequate quantities to significantly inhibit PKA. Arguing against this slow-permeability issue is the observation in my studies that both H-89 and KT-5720, manifest their effects, albeit somewhat cryptically, in decreasing basal ERK1,2 phosphorylation levels following just 10 minutes of incubation. However, while these data suggest that these inhibitors are entering the nerve terminal in sufficient quantity to elicit effects, the drugs were unable to inhibit forskolin/IBMX-induced enhancement of ERK1,2 activation. Thus, the possibility remains that these effects observed with KT-5720 and H-89 could be solely mediated by non-specific events and that inhibition of PKA does not occur to sufficient levels to elicit an inhibition in forskolin/IBMX-mediated stimulation. The effectiveness of H-89 and KT-5720-mediated inhibition of PKA could be tested under these conditions by using a phospho-specific antibody to examine the phosphorylation levels of site 1 on synapsin I, which is mediated by PKA (Czernik et al., 1987). If the phosphorylation of this site was attenuated by H-89 and KT-5720, then this would add evidence to the suggestion that forskolin/IBMX stimulation of ERK1,2 phosphorylation is occurring through a signalling pathway which is independent of PKA.

A second possible mechanism underlying the lack of correlation between the PKA-dependency of enhanced glutamate release and of ERK1,2 phosphorylation could lie within the use of indifferent inhibitors. For example, H-7 is able to inhibit PKA, PKC and PKG with similar potency, leading to the possibility of the involvement of non-specific effects in the observed inhibition of glutamate release (Davies et al., 2000). It would be interesting to examine the effects of the inhibitors that I have used, on basal and forskolin/IBMX-enhanced depolarisation-induced glutamate release. It would be interesting to discover whether basal inhibition of ERK1,2 could also lead to a basal inhibition of glutamate release. Activation of PKA has been shown to regulate glutamate release through a diverse array of different mechanisms (Lonart et al., 1998b). As a result, it remains possible that the effects of H-7 in inhibiting the forskolin/IBMX enhancement of glutamate release, occur through a PKA-dependent pathway which does not cross-talk with the ERK1,2 signalling cascade, or only does so under particular conditions.

The basal effects of the PKA inhibitors, H-89 and KT5720, in decreasing ERK1,2 activation raises the possibility that a tonically active, PKA-dependent, facilitation of ERK1,2 phosphorylation may be present in cerebrocortical nerve terminals. This is in contrast to the supposedly PKA-independent facilitatory effects of forskolin/IBMX treatment (see Figure 3.14). These results are not without precedent, as a recent study conducted by Morozov and collegues in PC12 cells suggested the existence of different pools of ERK1,2 which were subject to differential upstream regulation. Basal levels of ERK1,2 phosphorylation were found to be dependent on Rap-1, however, stimulated levels of ERK1,2 were found to be only partially dependent on Rap-1 (Morozov et al., 2003).

It is interesting that longer preincubations result in ERK1,2 inhibition upon application of forskolin/IBMX. The facile explanation, that this inhibition of ERK1,2 phosphorylation by forskolin/IBMX is attributed to an extended time in the presence of the compounds, is untenable given that the protocol used was such that the only change made in the conditions was to increase the preincubation time and not the drug application time. A more likely scenario, is that the inhibition could be due to PKA-mediated inhibition of Raf-1, or activation of a phosphatase (Dhillon et al., 2002; Usui et al., 1998). Further investigations will need to be carried out to substantiate this, however, it may also be that the 37°C control values increase following the preincubation protocol, leading to an apparent inhibition of ERK1,2 signalling, rather than an actual inhibition. This particular mechanism is thought unlikely in the current situation, as experiments pertaining to later chapters in this thesis were able to find an agonist-mediated stimulation of ERK1,2, albeit using a metabotropic glutamate receptor agonist (see Chapter 6). This inhibition of ERK1,2 phosphorylation by forskolin/IBMX is also not due to an extended time in the presence of the compounds, as the only change made in the conditions was to increase the preincubation time and not the drug application time. It is possible this effect is resulting from the cross-talk of signalling pathways, as activation of the Group III metabotropic glutamate receptor has been shown to attenuate forskolin-enhanced glutamate release from cerebrocortical nerve terminals (Millan et al., 2002). It would be interesting to correlate these results examining ERK1,2 phosphorylation levels with changes in glutamate release in this system. Elucidation of this mechanism could also help explain why treatment with forskolin elicits an excitatory response in some systems and an inhibitory response in

others. Treatment of cells with forksolin has been shown to have different effects, depending on the compartmentalisation and the coupling of the effector system (Erhardt et al., 1995), and it may be that forskolin/IBMX is acting through several different pathways to elicit its effects on ERK1,2 (see Figure 3.14).

The finding that forskolin/IBMX can increase the activation of ERK1,2, resulting in the downstream increase in phosphorylation of synapsin I site 4/5, at the very least, suggests a mechanism through which these drugs could be enhancing Ca^{2+} channel-independent glutamate release from cerebrocortical nerve terminals. The results in this chapter have highlighted the likelihood of the existence of at least two different signalling pathways occurring between the level of forskolin/IBMX stimulation and ERK1,2 phosphorylation (see Figure 3.14). This is most probably due to the plieotropic nature of the forskolin/IBMX-mediated stimulation, which could also complicate the further identification of the upstream signalling components. To address this, the next chapter in this thesis goes on to examine the downstream signalling of an adenylate cyclase-linked receptor, in order to see whether physiological stimulation of AC could be leading to regulation of the ERK1,2 signalling cascade.

Chapter 4

β-Adrenoceptor-Mediated Modulation of Presynaptic Function:
Cross-Talk of PKA and ERK 1,2 Signalling Cascades.

4.1 Introduction

β-adrenergic receptors are examples of Gs-linked GPCRs which are linked to adenylate cyclase, resulting in increased intracellular levels of cAMP and activation of PKA (De Blassi, 1990). Reports of an involvement of β-adrenoceptors in synaptic plasticity in brain date from as long ago as 1992, when it was found that noradrenaline could modulate the generation of long term potentiation (LTP) in the fimbria-CA3 pathway in a manner that was attenuated by treatment with a β-adrenoceptor antagonist (Katsuki et al., 1992). Since then, β-adrenoceptors have been found to modulate long term plasticity in the medial amygdala and the CA1 region of the hippocampus, with conflicted reports obtained in the lateral amygdala (Watanabe et al., 1996; Huang et al., 2000; Winder et al., 1999). As well as postsynaptic effects leading to the activation of PKA and the regulation of immediate early gene transcription, roles for β-adrenoceptors in the regulation of presynaptic function have also been identified (Roberson et al., 1999). Several slice preparations have reported an increase in synaptic transmission upon the activation of these receptors (Gereau and Conn, 1994; Huang et al., 1996). These results have been consolidated in studies using isolated nerve terminal preparations, which found an increase in glutamate release following treatment with the β-adrenoceptor agonist, isoproterenol (Herrero and Sanchez-Prieto, 1996; Wang et al., 2002).

Investigations into the mechanisms of isoproterenol-mediated enhancement of glutamate release from cerebrocortical nerve terminals have so far identified two possible downstream targets. The first study found the isoproterenol-mediated enhancement of glutamate release to be dependent on β_2-adrenergic receptor activation and to be dependent on the activation of PKA, which occurred following increases in intraterminal levels of cAMP. The observation that glutamate release was enhanced when 4-AP was used as the secretogogue, but not when KCl was used, led to the proposal that the main mechanism for this enhancement of release was mediated by a β-adrenoceptor-dependent increase in nerve terminal excitability. A role was also identified for Ca^{2+}, which was found to shift the dose/response curve for isoproterenol stimulation of cAMP production to the left (Herrero and Sanchez-Prieto, 1996). The more recent study using cerebrocortical synaptosomes found that treatment with

isoproterenol significantly increased basal intracellular levels of Ca^{2+} (Wang et al., 2002). This has been supported by studies investigating isoproterenol-enhancement of synaptic transmission in the amygdala, which found a requirement for increased Ca^{2+} influx through P-type Ca^{2+} channels (Huang et al., 1996; Huang et al., 1998). However, a study in cerebellar neurones has also found that isoproterenol treatment can result in an increase in the probability of synaptic vesicle release, in a manner independent of the regulation of Ca^{2+} channels or membrane excitability (Chen and Regehr, 1997).

As well as Gs-coupled activation of AC leading to increased intracellular concentrations of cAMP and activation of PKA, β-adrenoceptors have also been shown to signal to the ERK1,2 signalling cascade. This cross-talk of signalling cascades has been shown to have functional significance in the hippocampus CA1 region, where the induction of LTP by combined theta pulse and isoproterenol application was blocked by inhibition of ERK1,2 activation (Giovannini et al., 2001). Further studies in the hippocampal CA1 region have also implicated the ERK1,2 signalling cascade as the locus of convergence of multiple pathways in the regulation of LTP (Watabe et al., 2000). The mechanisms regulating ERK1,2 activation downstream of the β-adrenoceptors are still under debate and may be dependent on the location of the receptor. It has been suggested that the ERK1,2 activation, downstream of β-adrenergic receptor stimulation is dependent upon clathrin-mediated endocytosis of the receptor (Daaka et al., 1998; Lefkowitz, 1998). However, a study conducted in COS-7 cells using fluorescent-tagged receptors found that β_2-adrenoceptor endocytosis occurred independently of clathrin-mediated endocytosis and ERK activation (Pierce et al., 2000). There have also been suggestions that β-adrenoceptor mediated enhancement of ERK1,2 phosphorylation levels can occur both PKA-dependently, in HEK293 cells and the CA1 hippocampal region, and PKA–independently, in primary afferent nociceptor cells and COS-7 cells (Friedman et al., 2002; Roberson et al., 1999; Aley et al., 2001; Cao et al., 2000). This begs the question whether the forskolin/IBMX-mediated regulation of glutamate release and ERK1,2 phosphorylation examined in the previous chapter could have a physiological correlate representing signalling and cross-talk downstream of presynaptic β-adrenoceptor activation.

The above discussion has shown that isoproterenol can increase glutamate release in the cerebellum through a mechanism occurring independently of Ca^{2+}-channel or membrane potential regulation (Chen and Regehr, 1997). This chapter investigates whether β-adrenoceptor activation could be enhancing glutamate release from cerebrocortical nerve terminals through a similar mechanism, also occurring downstream of Ca^{2+} channel modulation. A possible locus for this enhancement has been suggested by studies demonstrating that a neurotrophin-linked ERK 1,2 cascade leading to synapsin I phosphorylation could be enhancing glutamate release, by increasing the availability of synaptic vesicles (Jovanovic et al., 2000). Recent evidence has suggested that stimulation of the ERK 1,2 signalling cascade can also occur downstream of β-adrenoceptor activation (Giovannini et al., 2001; Watabe et al., 2000). As a result of this, this chapter also examines whether the cAMP/PKA signalling cascade, more commonly associated with signalling downstream of β-adrenoceptor activation, could be involved in cross-talking with the ERK1,2 signalling cascade to elicit increases in synapsin I phosphorylation, as a possible mechanism for this Ca^{2+}-channel independent enhancement of release.

4.2 Method

Synaptosomes were prepared as described in section 2.1. (Sihra, 1997)

4.2.1 Glutamate Release

The standard glutamate release protocol was used, as described in section 2.2 (Nicholls and Sihra, 1986; Perkinton and Sihra, 1999). Ionomycin (5μM) was used as the secretogogue, with 10μM isoproterenol added 5 minutes prior to the addition of the secretogogue (Wang et al., 2002). All incubations were carried out in the presence of 1mM $CaCl_2$.

4.2.2 Standard Incubation for ERK 1,2 Phosphorylation/Activation

All standard incubations lasted for a total time of 10 minutes at a temperature of 37°C and contained 1mM $CaCl_2$. Pellets of synaptosomes were resuspended at 4°C to give a final synaptosomal concentration of 1mg/ml using HBM, with the HBM itself already containing 1mg/ml of BSA. Isoproterenol (100μM), propranolol (100μM), H-89 (10μM), KT-5720 (1μM), or Rp-cAMPS (100μM) was added immediately prior to the transfer of the synaptosomes to the 37°C incubation. The dose response experiments for isoproterenol utilised 1nM, 10nM, 100nM, 1μM, 10μM or 100μM final concentrations of the drug, which were added 5 minutes and 30 seconds prior to the termination of the 37°C incubation. All incubations were terminated using 5x SDS-PAGE STOP buffer. This incubation protocol for measuring ERK 1,2 phosphorylation has previously been described by Jovanovic et al., 2000 (Jovanovic et al., 2000).

4.2.3 Preincubation

Synaptosome pellets were resuspended at 4°C to a final synaptosomal concentration of 1mg/ml using HBM containing 1mg/ml of BSA. $CaCl_2$ (1mM) was added immediately prior to the transfer of the synaptosomes to the 37°C incubation. This preincubation lasted for 20 minutes, after which the synaptosomes were recentrifuged using a microcentrifuge (10,000 x g for 30 seconds). The supernatant was discarded and the pellet cooled back to 4°C for 5 minutes. The standard incubation protocol was then utilised, with 10μM of isoproterenol being added 6 minutes prior to the termination of the incubation.

Samples were processed for ERK1,2 and synapsin phosphorylation levels as described in the main methods in chapter 2 (sections 2.4.3 to 2.5) (Dai et al., 2001; Jovanovic et al., 1996)

4.2.4 Reagents

Isoproterenol: β-adrenoceptor agonist (4-[-1-hydroxy-2-[(1-methylethyl)amino]ethyl]-1,2-benzenediol hydrochloride).
Dissolved in water immediately prior to experimentation, to give the 100x concentrations of 100nM, 1μM, 10μM, 100μM, 1mM, and 10mM.

Propranolol: β-adrenoceptor antagonist ((RS)-1-[(1-methylethyl)amino]-3-(1-naphthalenyloxy)-2-propanol hydrochloride) ((Corrodi et al., 1963); (Leszkovszky and Tardos, 1965)).
Dissolved in water to a concentration of 10mM, immediately prior to experimentation.

H-89: PKA inhibitor acting at the catalytic site (N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride) (Chijiwa et al., 1990).
This was dissolved with DMSO to give a stock concentration of 10mM, which was further diluted with water prior to use to give a concentration of 1mM.

KT-5720: PKA inhibitor acting at the catalytic site ((9R,10S,12S)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3',2',1'-Kl]pyrolo[3,4-i][1,6]benxodiazocine-10-carboxylic acid, hexyl ester) (Kase et al., 1987); (Gadbois et al., 1992).
This was dissolved to a concentration of 1mM in DMSO and was further diluted before use to 100μM with water.

Rp-cAMPS: PKA inhibitor acting at the regulatory site (Rp-adenosine 3',5'-cyclic monophosphorothioate triethylammonium salt) (Botelho et al., 1988).
This was dissolved in water to a concentration of 10mM.

All the above drugs were purchased from the Sigma-Aldrich Company, Gillingham, Dorset.

Unless stated, all drugs were added using 100x stock solutions to minimise volume changes and only final concentrations are quoted in the text forthwith.

4.3 Results

Isoproterenol is a β-adrenoceptor agonist which has been shown to increase glutamate release from cerebrocortical nerve terminals (Herrero and Sanchez-Prieto, 1996; Wang et al., 2002). Previous experiments examining the mechanisms of isoproterenol-mediated enhancement of glutamate release have so far identified two pathways through which this could be occurring. The first suggested a role for increasing levels of membrane excitability, whereas the second suggested the involvement of presynaptic Ca^{2+} channels (Herrero and Sanchez-Prieto, 1996; Wang et al., 2002). The preliminary experiments conducted in this chapter investigated whether isoproterenol could still increase glutamate release from synaptosomes when Ca^{2+}-channel regulation was bypassed. Glutamate release was evoked using ionomycin (5μM), which increases the influx of Ca^{2+} into the nerve terminal independently of Ca^{2+}-channels. Figure 4.1 illustrates that isoproterenol (10μM) was still able to significantly enhance ionomycin-evoked glutamate release, thus suggesting the additional involvement of a mechanism occurring downstream of Ca^{2+} entry (mean ± s.e.m. cumulative glutamate release (nmol/mg) calculated from 4 independent experiments: control = 6.98 ± 1.4; isoproterenol = 11.18 ± 1.6).

ERK1,2 phosphorylation has now been shown to occur downstream of β-adrenoceptor signalling in several systems (Aley et al., 2001; Friedman et al., 2002; Roberson et al., 1999). The next series of experiments conducted in this chapter investigated whether stimulation with this agonist could also elicit increases in ERK1,2 activation in cerebrocortical synaptosomes. Figure 4.2 shows that treatment with isoproterenol can indeed stimulate ERK1,2 phosphorylation in a concentration-dependent manner, and can be saturated, as illustrated by the sigmoidal log-dose response curve. All the tested concentrations of the β-adrenoceptor agonist were able to significantly enhance basal ERK1,2 phosphorylation levels following 10 minutes of incubation with 5 mins 30 secs in the presence on the drug (mean ± s.e.m. of isoproterenol effect in % compared to 100% 37°C controls: 1nM = 121 ± 10; 10nM = 122 ± 10; 100nM = 131 ± 12; 1μM = 143 ± 11; 10μM = 149 ± 10; 100μM = 152 ± 12). As stated earlier, isoproterenol is a β-adrenergic receptor agonist, which implies the observed stimulation of ERK1,2 phosphorylation is occurring downstream of β-adrenoceptor activation. In order to

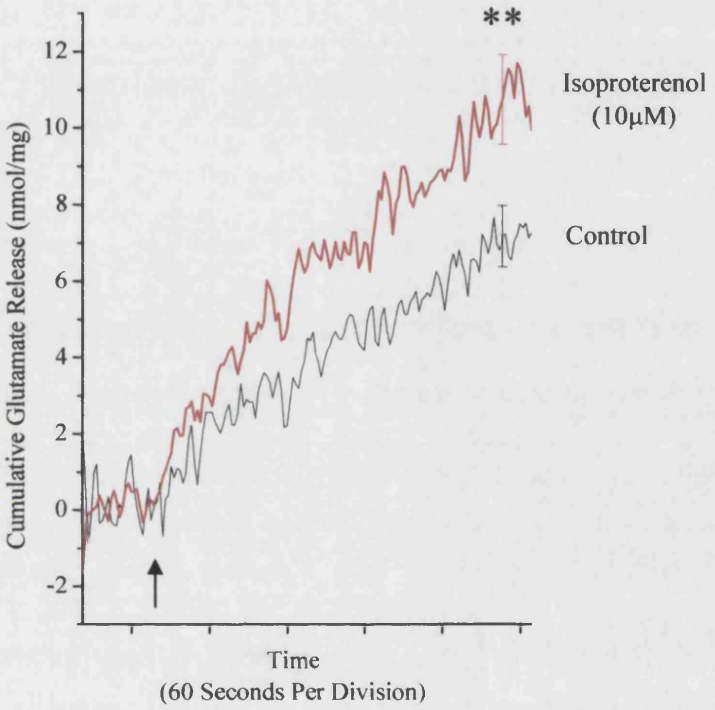


Figure 4.1 Isoproterenol Can Enhance Glutamate Release Independently of Ca^{2+} Channel Regulation. Synaptosomes were incubated in the presence of 1mM $CaCl_2$ as indicated in section 4.2.1. Glutamate release was evoked with the addition of ionomycin (5μM, arrow) in the absence (control), or presence (+isoproterenol) of isoproterenol (10μM). Isoproterenol was added 5mins prior to the addition of the secretogogue. The mean traces from four individual experiments, measuring cumulative release at 2sec intervals, are shown. Mean ± s.e.m. release was calculated at each time point, but the cumulative glutamate release values following 4mins 30secs of incubation with the secretogogue were used for statistical analysis **P<0.01; different from control (student's paired t-test; n=4).

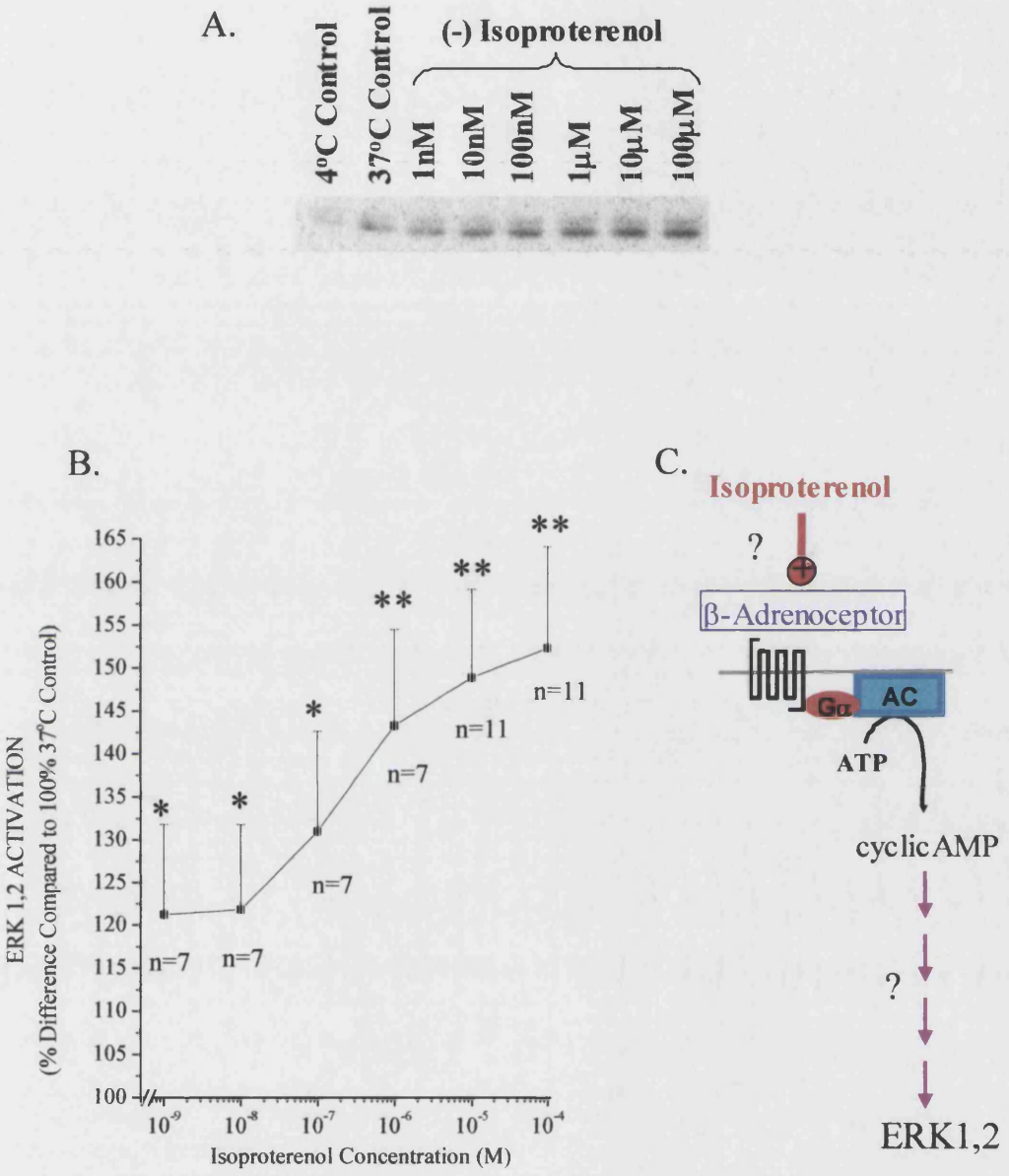


Figure 4.2 Isoproterenol Dose-Dependently Enhances ERK1,2 Phosphorylation. Synaptosomes were incubated for 10 minutes with 1mM $CaCl_2$ either in the absence (control) or presence (+isoproterenol) of varying concentrations of isoproterenol. Concentrations used were 1nM, 10nM, 100nM, 1μM, 10μM and 100μM. 7.5% polyacrylamide gels were run. Immunoblots were labelled using phospho-specific MAPK primary antibody (NEB, 1:1000) and probed using ^{125}I-labelled protein A. Levels of ^{125}I-labelling from several independent experiments were detected and analysed using phosphorimager spectroscopy (Molecular Dynamics). *P<0.05, **P<0.01 compared to 100% 37°C control (Student's paired t-test). **A.** Phosphorimage of effects on ERK1,2 phosphorylation. **B.** Quantification of ERK1,2 phosphorylation levels. **C.** Possible pathway involved (**?** denotes hypothetical pathway).

confirm this, the effects of the β-adrenoceptor antagonist, propranolol, on ERK1,2 phosphorylation in synaptosomes were examined.

Figure 4.3 demonstrates the levels of ERK1,2 phosphorylation obtained following 10 mins incubation in the presence of propranolol. As can be seen from the immunoblot and the graph, propranolol (100μM) was able to significantly decrease basal levels of ERK1,2 activation in this system (mean ± s.e.m. compared to 100% 37°C control: propranolol = 81.5 ± 2.8). These results imply that β-adrenoceptors are endogenously activated in purified cerebrocortical nerve terminals, and that their activation contributes to the basal phosphorylation levels of ERK1,2 found in this system. This observation agrees with previous studies which have identified inverse agonist properties for propranolol, and also adds evidence to the physiological nature of this pathway in synaptosomes (Chidiac et al., 1994; Varma et al., 1999).

In order to investigate whether β-adrenoceptor inhibition could also attenuate isoproterenol-mediated increases in ERK1,2 phosphorylation, the next experiments looked at the levels of ERK1,2 activation in synaptosomes treated with isoproterenol and propranolol. The results from these experiments are displayed in Figure 4.4. As can be seen from the graph and immunoblot, propranolol was still able to inhibit basal levels of ERK1,2 activation, as well as competitively attenuate the isoproterenol-induced increases in ERK1,2 phosphorylation (mean ± s.e.m. compared to 100% 37°C control: propranolol (100μM) = 80.7 ± 4.8; isoproterenol (100μM) = 124 ± 5; isoproterenol/propranolol = 87.1 ± 6.0). This confirms the hypothesis that the isoproterenol-mediated increase in ERK1,2 phosphorylation in cerebrocortical synaptosomes is occurring downstream of β-adrenoceptor activation.

The results obtained so far in this chapter have been in agreement with the results obtained in other systems showing that activation of β-adrenoceptors can lead to stimulation of ERK1,2 phosphorylation (Berkeley and Levey, 2003; Storm and Khawaja, 1999; Schmitt and Stork, 2000). The pathways involved in mediating this stimulation have been further elucidated in several systems. For example, in HEK293 cells, β2-adrenoceptors have been shown to signal via Src to bring about the activation of the Rap-1/B-Raf/MEK/ERK signalling cascade, occurring following a PKA-

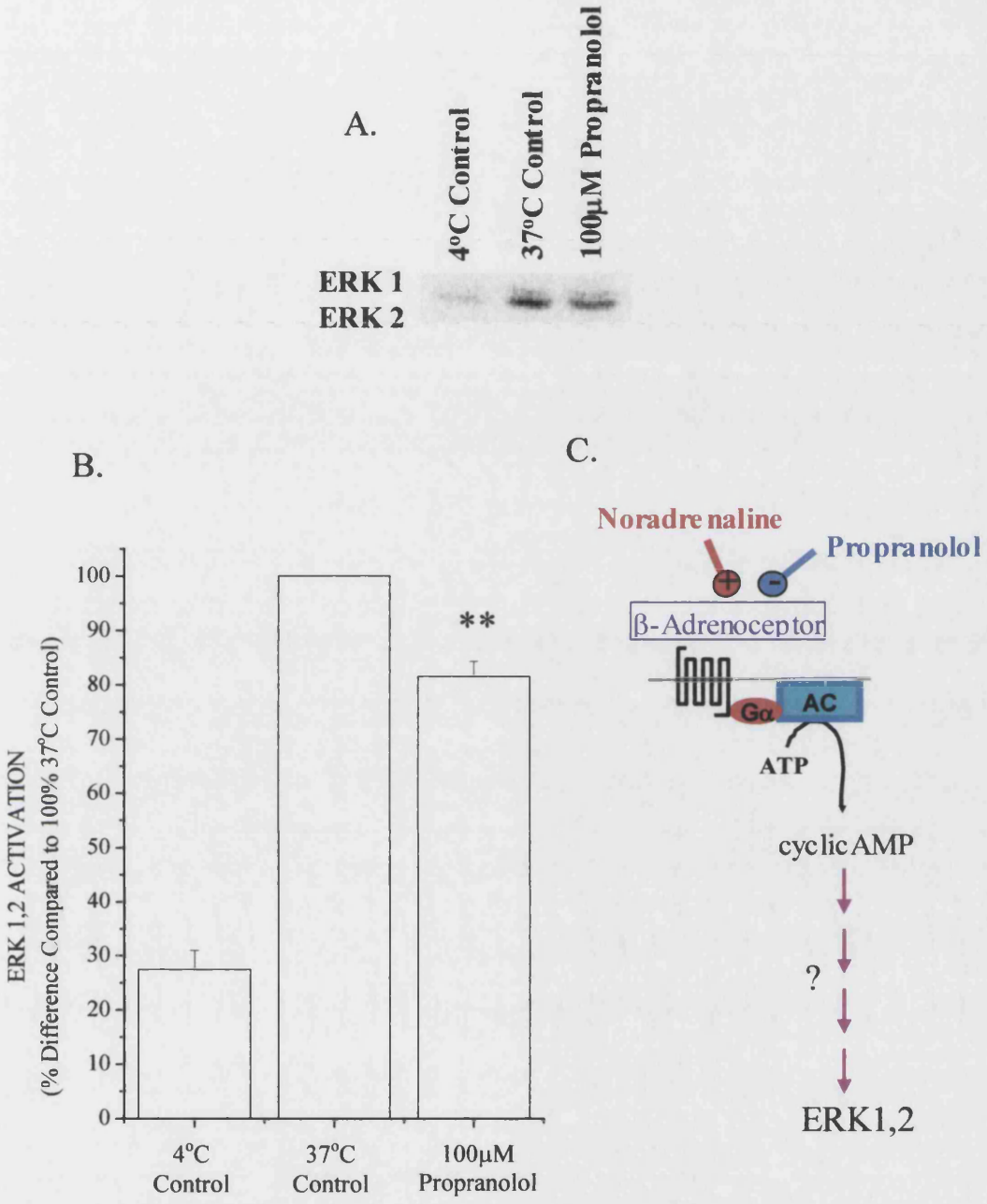


Figure 4.3 Propranolol Significantly Inhibits Basal Levels of ERK 1,2 Activation. Synaptosomes were incubated with 1mM $CaCl_2$ for 10 minutes either in the absence (control), or presence (+propranolol) of 100µM propranolol. 7.5% polyacrylamide gels were used. Immunoblots were labelled with phospho-specific MAPK primary antibody (NEB, 1:1000) and reported using ^{125}I-labelled protein A. Levels of ^{125}I-labelling were detected and analysed from several separate experiments using phosphorimager spectroscopy (Molecular Dynamics). **P<0.01 compared to 100% 37°C control (Student's paired t-test, n=10). **A.** Phosphorimage of the effects of propranolol on ERK1,2 phosphorylation. **B.** Quantification of ERK1,2 phosphorylation levels. **C.** Suggested pathway involved in mediating these effects on ERK 1,2 activation (**?** indicates hypothetical pathways).

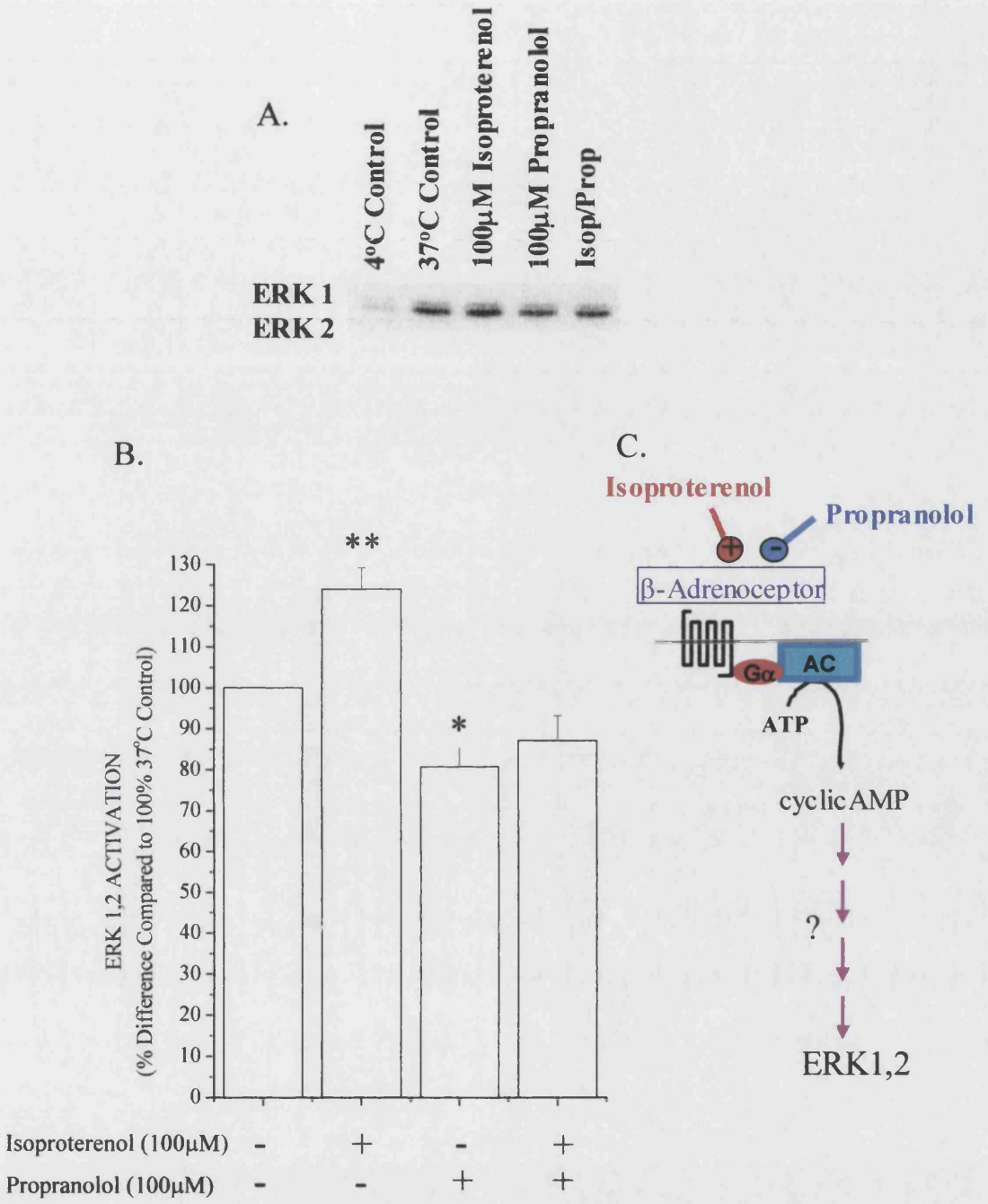


Figure 4.4 Isoproterenol-Mediated Stimulation of ERK 1,2 Phosphorylation is Dependent on β-Adrenoceptor Activation. Synaptosomes were incubated at 37°C for 10 minutes with 1mM $CaCl_2$ either in the absence (control) or presence of 100µM isoproterenol (+isoproterenol) and/or 100µM propranolol (+propranolol). 7.5% polyacrylamide gels were run. A Phospho-specific MAPK primary antibody (NEB, 1:1000) was used to label immunoblots which were probed with ^{125}I-labelled protein A. Levels of ^{125}I were detected and analysed using phosphorimager spectroscopy (Molecular Dynamics). **P<0.01, *P<0.05 compared to 100% 37°C control (ANOVA followed by Duncan's *post hoc* analysis, isop n=16, prop and isop/prop n=5). **A.** Immunoblot showing ERK1,2 phosphorylation levels. **B.** Quantification of effects. **C.** Proposed pathway involved in these effects (**?** denotes hypothetical pathways).

mediated phosphorylation of Src (Schmitt and Stork, 2002; Friedman et al., 2002). A PKA-dependency has also been observed in the β-adrenoceptor-mediated enhancement of ERK1,2 phosphorylation in the CA1 region of the hippocampus (Roberson et al., 1999). However, several studies have also found signalling to ERK1,2 downstream of β-adrenoceptor activation to occur in a manner independent of PKA, albeit in COS-7 cells and primary nociceptor afferents (Cao et al., 2000; Aley et al., 2001). In order to elucidate the pathway involved in this system, the next experiments conducted in this chapter investigated the PKA-dependency of the β-adrenoceptor-mediated enhancement of ERK1,2 activation in cerebrocortical synaptosomes.

Figure 4.5 illustrates the effects of the PKA inhibitor, H-89, on isoproterenol stimulated levels of ERK1,2 phosphorylation. As can been seen from the immunoblot in Figure 4.5A and from Figure 4.5B, H-89 was able to attenuate the enhancement of ERK1,2 phosphorylation by isoproterenol. The ERK1,2 phosphorylation levels in the presence of H-89 alone and those in the presence of H-89 and isoproterenol were found to be no different from each other, however, they were both found to be significantly lower than control levels (mean ± s.e.m. in % compared to 100% 37°C control: isoproterenol = 124 ± 5; H-89 = 60 ± 1; isoproterenol/H-89 = 62 ± 9). These results suggest that the signalling to ERK1,2 downstream of β-adrenoceptor activation in cerebrocortical nerve terminals is dependent upon the activation of PKA (Figure 4.5C). However, as discussed in the previous chapter, H-89 is not highly specific for PKA, so it remains possible that the observed attenuation of ERK1,2 activation could be occurring through a mechanism which is PKA-independent. In order to confirm a role for PKA in this signalling pathway in synaptosomes, the effects of a second PKA inhibitor were examined.

The effects of the second PKA inhibitor, KT-5720, on isoproterenol-enhanced levels of ERK1,2 phosphorylation are shown in figure 4.6. KT-5720 was also found to attenuate β-adrenoceptor-mediated increases in ERK1,2 activation and, as with H-89, both the ERK1,2 phosphorylation levels with KT-5720, whether in the absence and presence of isoproterenol, were found to be significantly lower than control levels (mean ± s.e.m. in % compared to 100% 37°C control: KT-5720 = 78 ± 6; KT-5720/isoproterenol = 79 ± 9; isoproterenol = 124 ± 5). These results are in complete agreement with those obtained

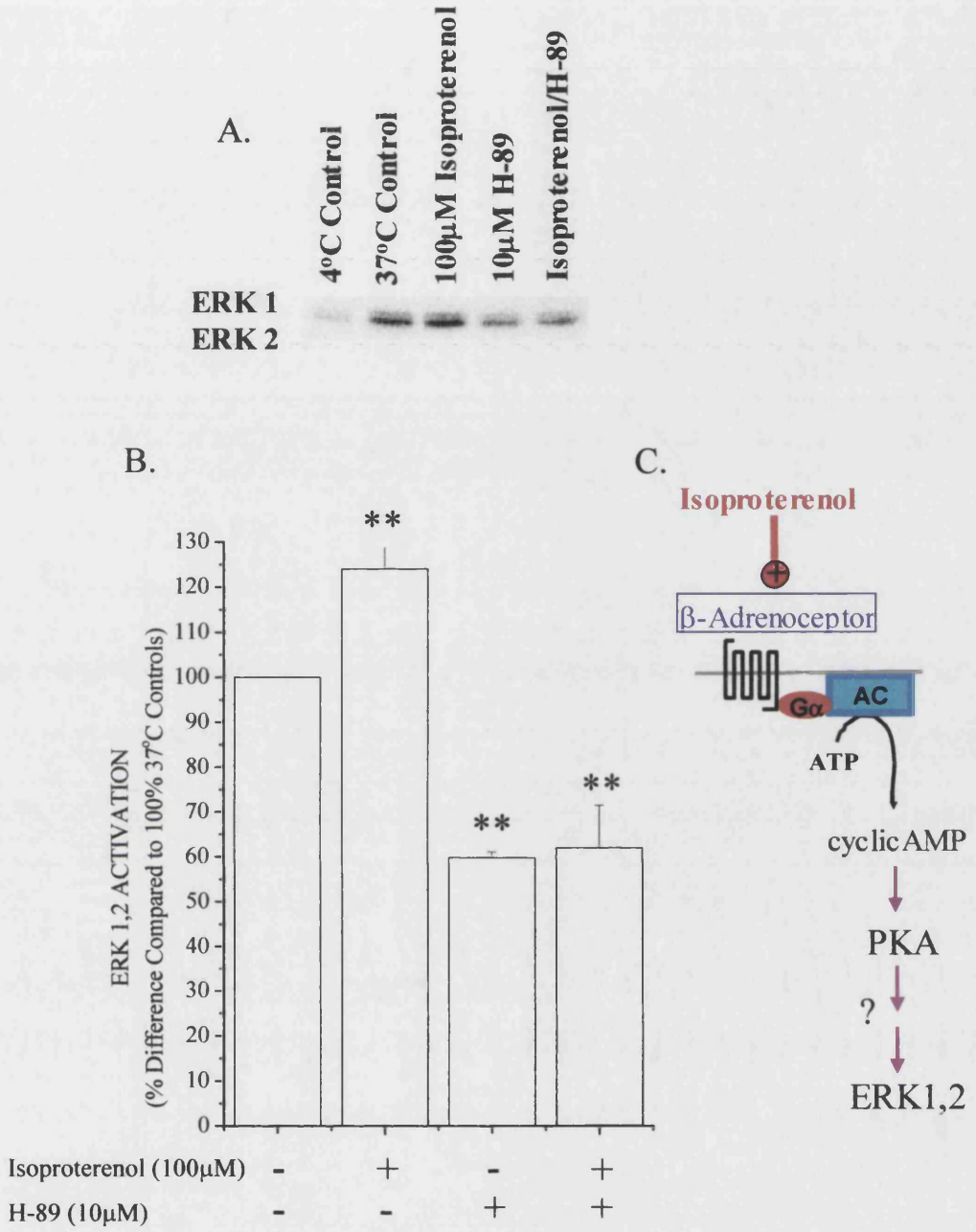


Figure 4.5 β-Adrenoceptor-Mediated Stimulation of ERK 1,2 May Be Dependent on PKA. Synaptosomes were incubated with 1mM $CaCl_2$ for 10 mins at 37°C either in the absence (control) or presence of 100µM isoproterenol (+isoproterenol) and/or 10µM H-89. 7.5% polyacrylamide gels were run. Immunoblots were labelled with phospho-specific MAPK primary antibody (NEB, 1:1000) followed by secondary labelling with ^{125}I-bound protein A. Signal levels of ^{125}I from several separate experiments were detected and analysed using phosphorimager spectroscopy (Molecular Dynamics). **P<0.01 (ANOVA followed by Duncan's *post hoc* analysis, isop n=16, H-89 and isop/H-89 n=6). **A.** Phosphorimage of ERK1,2 phosphorylation. **B.** Quantification of ERK1,2 effects. **C.** A suggested pathway mediating isoproterenol stimulation of ERK1,2 activation (**?** = hypothetical pathway).

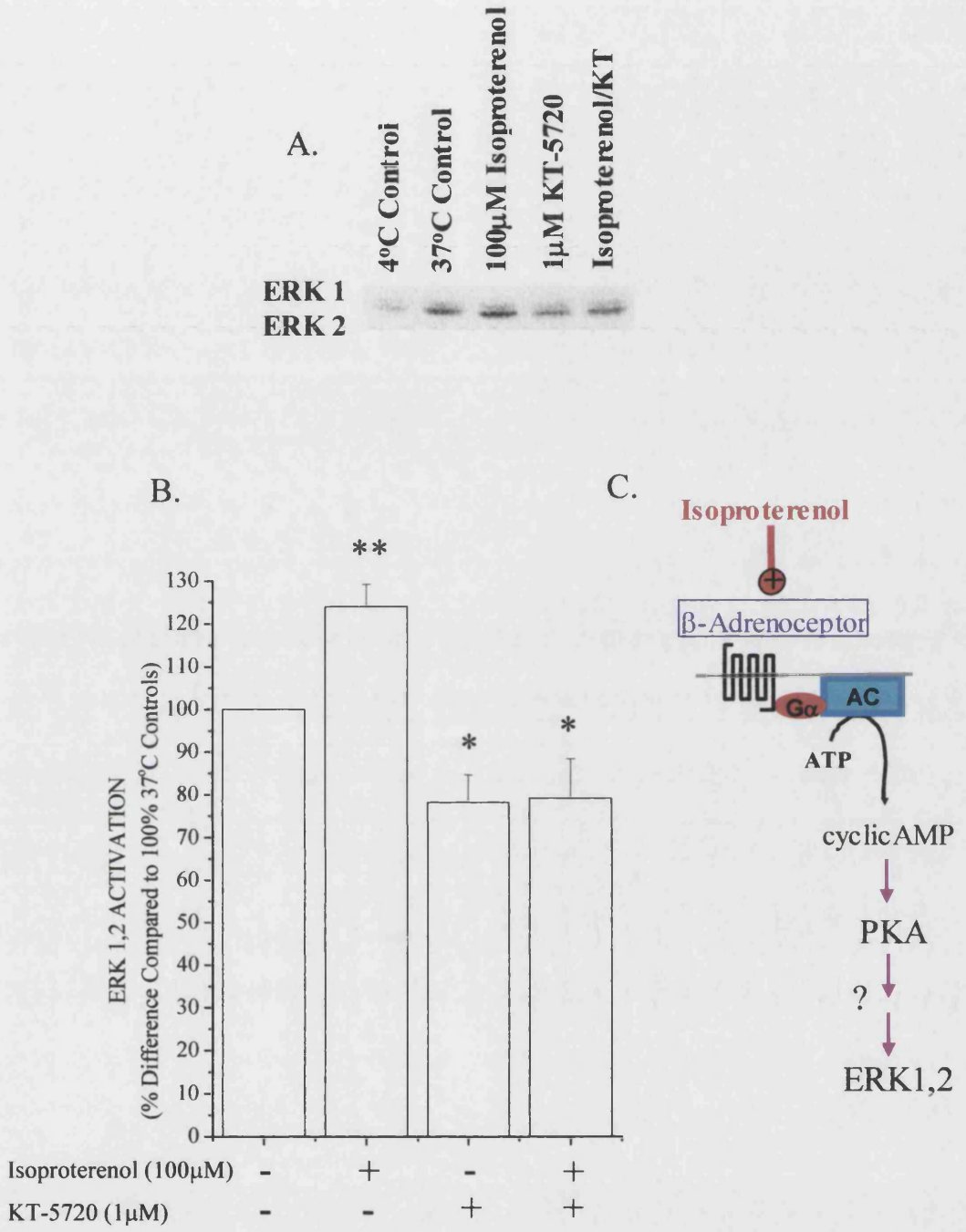


Figure 4.6 KT-5720 Also Inhibits Isoproterenol-Mediated Stimulation of ERK 1,2. Synaptosomes were incubated at 37°C for 10 mins with 1mM $CaCl_2$ either in the absence (control) or presence of 100µM isoproterenol (+isoproterenol) and/or 1µM KT-5720 (+KT-5720). 7.5% Polyacrylamide gels were used. Immunoblots were labelled using a phospho-specific MAPK primary antibody (NEB, 1:1000) and reported using ^{125}I-labelled protein A. Signal levels from several separate experiments were detected and analysed using phosphorimager spectroscopy (Molecular Dynamics). **P<0.01, *P<0.05 compared to 100% 37°C control (ANOVA followed by Duncan's *post hoc* analysis, isop n=16, KT-5720 and isop/KT n=5). **A.** Example of an immunoblot. **B.** Quantification of ERK1,2 phosphorylation levels. **C.** Possible pathway mediating these effects (**?** = hypothetical pathway).

with H-89, and imply a role for PKA in regulating the signalling between β-adrenoceptors and ERK1,2 in cerebrocortical nerve terminals.

The final experiments conducted in this chapter to examine the role of PKA, used an inhibitor of the regulatory subunit of PKA, Rp-cAMPS. In the previous chapter, this inhibitor was found not to be active against the forskolin/IBMX-mediated enhancement of ERK1,2 phosphorylation following the 10 minute incubation protocol. This was thought likely to be due to low levels of the inhibitor crossing the plasma membrane, however, it could also have been due to displacement of the drug by the high levels of cAMP produced, or to PKA-independency of the pathway. Activation of β-adrenoceptors by isoproterenol should also result in enhanced cAMP production, but the levels of the second messenger would be expected to be lower, and thus less likely to displace Rp-cAMPS. The previous experiments have also suggested that the β-adrenoceptor-mediated enhancement in ERK1,2 activation is likely to be PKA-dependent. Notwithstanding, Figure 4.7 shows that Rp-cAMPS was also unable to inhibit isoproterenol-mediated enhancement of ERK1,2 activation. This experiment confirms the original hypothesis, that this incubation protocol is not allowing Rp-cAMPS to accumulate in synaptosomes in sufficient quantity to inhibit PKA activation (mean ± s.e.m. in % compared to 100% 37°C control: isoproterenol = 124 ± 5; Rp-cAMPS = 100 ± 13; Rp-cAMPS/isoproterenol = 124 ± 6).

The results obtained so far suggest that the β-adrenoceptor-mediated enhancement of ERK1,2 phosphorylation is PKA-dependent. This is in contrast to the results obtained in the previous chapter, which found the majority of the forkolin/IBMX-mediated enhancement of ERK1,2 phosphorylation might be occurring independently of PKA. Combined, these results suggest the presence of more than one cAMP-mediated pathway able to regulate ERK1,2 activity in cerebrocortical nerve terminals. The next experiments conducted in this chapter investigated whether the β-adrenoceptor/PKA/ERK1,2 pathway was able to undergo a time-dependent switch from facilitation of ERK1,2, to inhibition, as observed with the forskolin/IBMX mediated pathway.

Synaptosomes were preincubated for 20 minutes at 37°C then re-pelleted and cooled

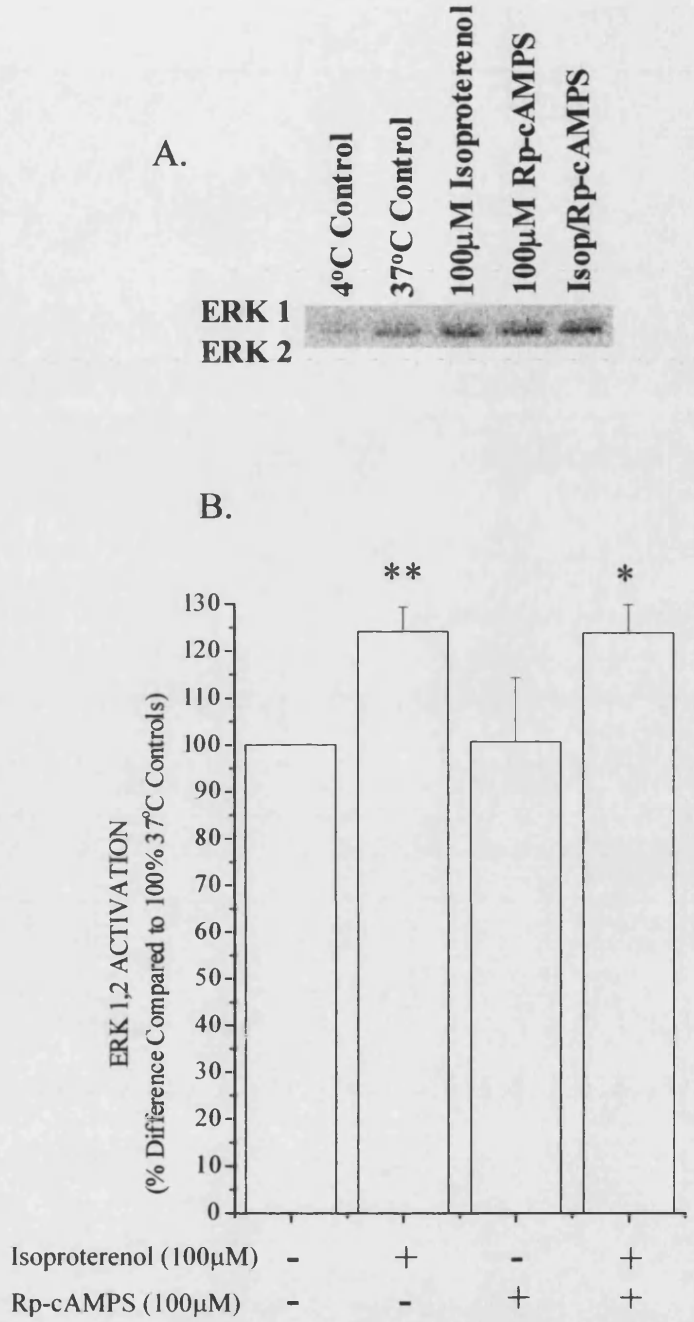


Figure 4.7 Rp-cAMPS Does Not Inhibit Isoproterenol-Mediated Stimulation of ERK 1,2 Phosphorylation. 37°C incubation of synaptosomes with 1mM $CaCl_2$ lasted for 10 mins either in the absence (control) or presence of 100μM Rp-cAMPS (+Rp-cAMPS) and/or 100μM isoproterenol (+isoproterenol). 7.5% polyacrylamide gels were run. Immunoblots were labelled with a phospho-specific MAPK antibody (NEB, 1:1000) and probed using protein A labelled with ^{125}I. Levels of ^{125}I-labelling from several separate experiments were detected and analysed using phosphorimager spectroscopy (Molecular Dynamics). **P<0.01, *P<0.05 compared to 100% 37°C control (ANOVA with Duncan's *post hoc* analysis, isop n=16, Rp-cAMPS and isop/Rp n=5). **A.** Phosphorimage of effects. **B.** Quantification of ERK1,2 phosphorylation levels.

back to 4°C before undergoing the standard 10 minute incubation protocol. Isoproterenol (10μM) was added for a total of 6 minutes. The results from these experiments are shown in figure 4.8 and, as can be seen from the immunoblot (Figure 4.8A) and the graph (Figure 4.8B), isoproterenol was still able to significantly enhance ERK1,2 phosphorylation levels following this preincubation protocol (mean ± s.e.m. in % compared to 100% 37°C control: isoproterenol = 125 ± 6). This suggests that the β-adrenoceptor signalling pathway is differentially regulated compared to the forskolin/IBMX signalling pathway, and that positive signalling through the β-adrenoceptor does not undergo endogenous desensitisation following this incubation protocol.

So far, this chapter has identified a signalling pathway through which β-adrenoceptor activation leads to a PKA-dependent increase in ERK1,2 phosphorylation. The next set of experiments proceed to investigate the signalling occurring downstream of ERK1,2 activation. Previous studies have identified synapsin I as a synaptic vesicle tethering protein which can be phosphorylated by multiple protein kinases. The phosphorylation of this protein by PKA or ERK1,2 has been shown to decrease the tethering of synaptic vesicles and to increase their availability for release, thus presenting a possible mechanism for increasing glutamate release from nerve terminals (Chi et al., 2001; Chi et al., 2003). Figure 4.9 shows examples of immunoblots labelled with phospho-specific antibodies for the sites of synapsin I phosphorylated by PKA or by ERK1,2. As can be seen from the blots, treatment of synaptosomes with isoproterenol led to increases in synapsin I phosphorylation by both PKA and ERK1,2. Further experiments will need to be conducted to absolutely confirm these results, but the data as they are, suggest that the component of β-adrenoceptor-mediated glutamate release occurring downstream of Ca^{2+} channel regulation, could be occurring through a mechanism involving enhanced synapsin I phosphorylation and increased synaptic vesicle availability. If this is so, then it shares a mechanism with the neurotrophin-mediated enhancement of glutamate release that has been described in cerebrocortical nerve terminals (Jovanovic et al., 2000). This immediately begs the question as to whether the β-adrenoceptor-mediated and neurotrophin-mediated enhancements are occurring within the same or different populations of ERK1,2.

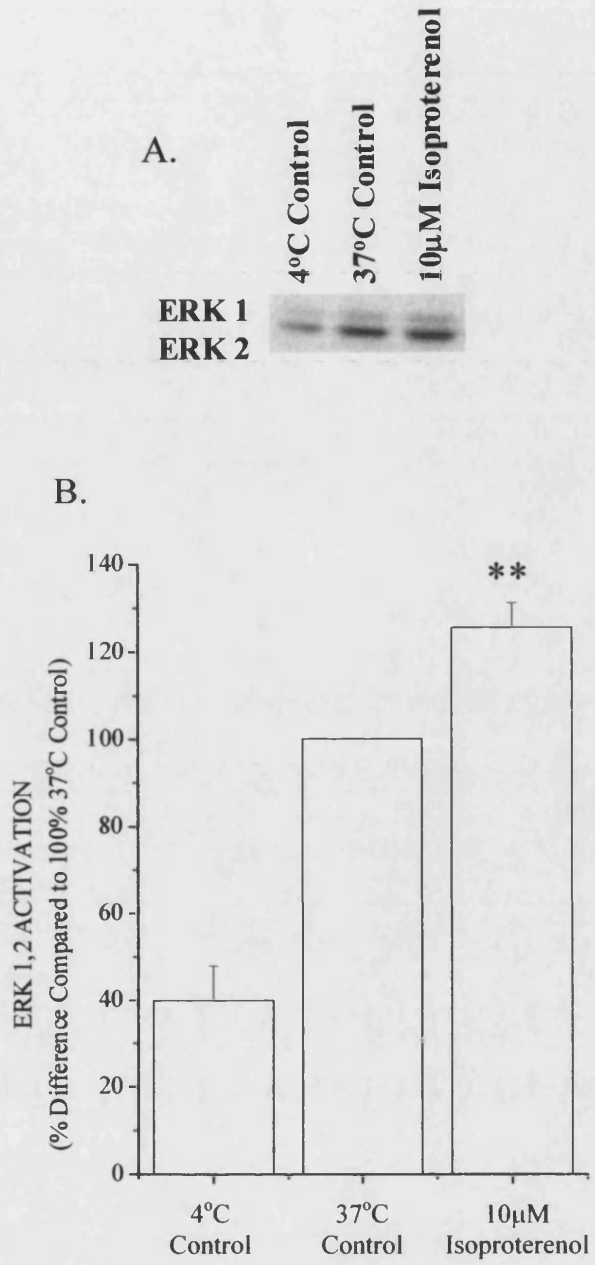


Figure 4.8 Isoproterenol is Still Able to Enhance ERK1,2 Phosphorylation Levels in Preincubated Synaptosomes. Synaptosomes were preincubated with 1mM $CaCl_2$, as described in section 4.2.3. A 10min 37°C incubation followed, with either water (control) or 10μM isoproterenol (+isoproterenol) added 6mins prior to the termination of the reaction. Gels containing 7.5% polyacylamide were run. Immunoblots were labelled with phospho-specific MAPK antibody (NEB, 1:1000) and probed using ^{125}I-labelled protein A. Levels of labelling from several separate experiments were detected and analysed using phosphorimager spectroscopy (Molecular Dynamics). **P<0.01 compared to 100% 37°C control (ANOVA followed by Duncan's *post hoc* analysis, n=5). **A.** Phosphorimage of effects. **B.** Quantification of ERK1,2 phosphorylation levels.

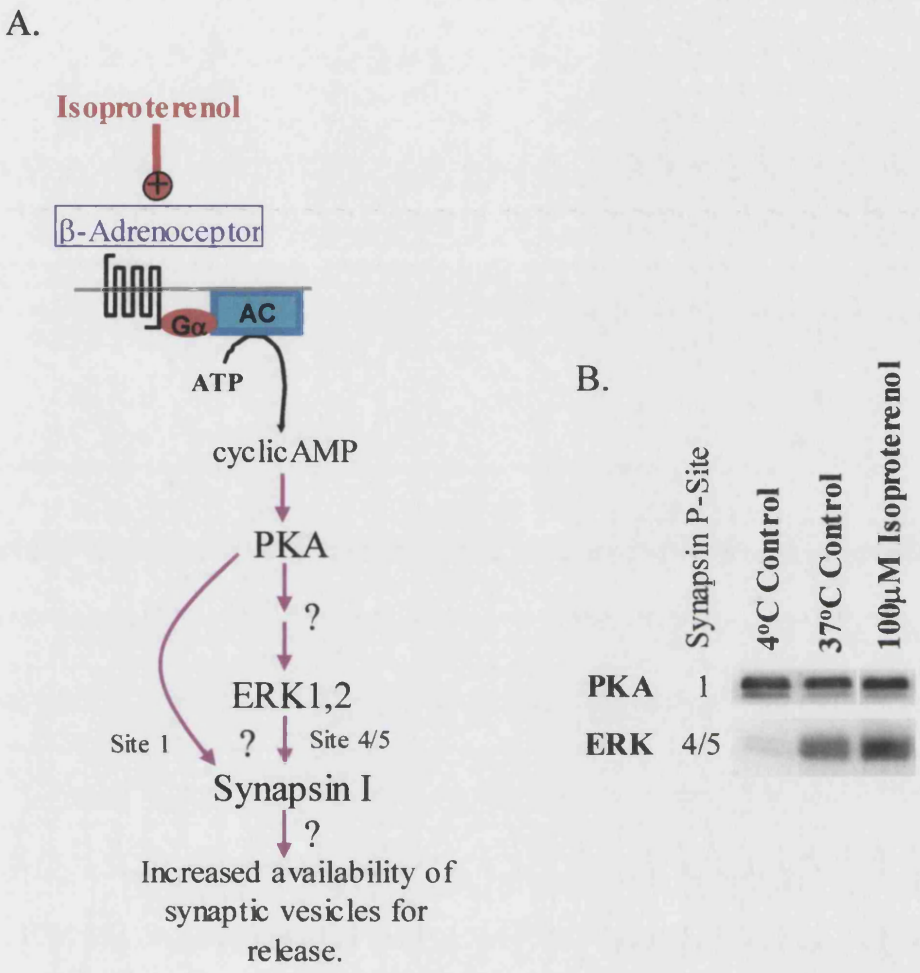


Figure 4.9 Stimulation of Synaptosomes with Isoproterenol May Be Leading to Increased Phosphorylation of Synapsin I at Two Different Sites. Synaptosomes were incubated at 37°C for 10 minutes with 1mM $CaCl_2$ either in the absence (control) or presence (+isoproterenol) of 100μM isoproterenol. 7.5% polyacrylamide gels were run. Immunoblots were labelled with phospho-specific primary antibodies either for phosphorylation site 1 (1:200, J.J) or site 4/5 (1:500, J.J.) of synapsin I. Blots were probed using ^{125}I-labelled protein A, with levels of labelling being detected using phosphorimager spectroscopy (Molecular Dynamics). **A.** A suggested pathway for the Ca^{2+} channel-independent regulation of glutamate release by β-adrenoceptors (**?** = hypothetical pathways). **B.** Examples of immunoblots showing increased levels of synapsin I site 1 and site 4/5 phosphorylation with isoproterenol.

The final experiments conducted in this chapter investigated whether the β-adrenoceptor and neurotrophin receptor signalling to ERK1,2 could be occurring within the same nerve terminals. Synaptosomes were incubated for 10 minutes with BDNF added for 6 minutes, to stimulate TrkB receptors, and isoproterenol added for 5 minutes and 30 seconds, to stimulate β-adrenoceptors. Figure 4.10 A and B illustrate the effects of coapplication of BDNF (50ng/ml) and isoproterenol (10μM) on ERK1,2 phosphorylation levels. Both BDNF and isoproterenol were found to significantly enhance ERK1,2 activation when applied alone, but not when applied together (mean ± s.e.m. in % compared to 100% 37°C control: BDNF (50ng/ml) = 175 ± 29; isoproterenol (10μM) = 174 ± 11; BDNF/isoproterenol = 142 ± 9). This could imply that these two signalling pathways are interacting to limit ERK1,2 activation, however, no significant differences were found between the effects of isoproterenol and BDNF alone, compared to their combined effects. Therefore, further experiments would need to be conducted to elucidate on any interacting properties of these signalling cascades, as only a small number of repeats were obtained on this occasion.

Figure 4.10 also describes the results obtained with this protocol when a higher concentration of BDNF (100ng/ml) was used (see Figures 4.10 C and D). These results show a similar pattern of ERK1,2 phosphorylation to that described with the lower, 50ng/ml, BDNF concentration. Both BDNF and isoproterenol were found to significantly enhance ERK1,2 activation when applied alone, but not when added in combination (mean ± s.e.m. in % compared to 100% 37°C control: BDNF (100ng/ml) = 185 ± 30; isoproterenol (10μM) = 162 ± 14; BDNF/isoproterenol = 127 ± 10). On this occasion the level of ERK1,2 response in the presence of BDNF alone was also found to be significantly different from the level obtained in the presence of BDNF and isoproterenol. This suggests that the activation of β-adrenoceptors in cerebrocortical nerve terminals may be having an attenuating effect on neurotrophin-mediated ERK1,2 activation when these concentrations of agonists are used. However, further experiments would also need to be carried in order to absolutely confirm the results obtained here.

The final combination of BDNF and isoproterenol concentrations used were 100ng/ml and 100μM, respectively. Figure 4.11 illustrates the results obtained with these

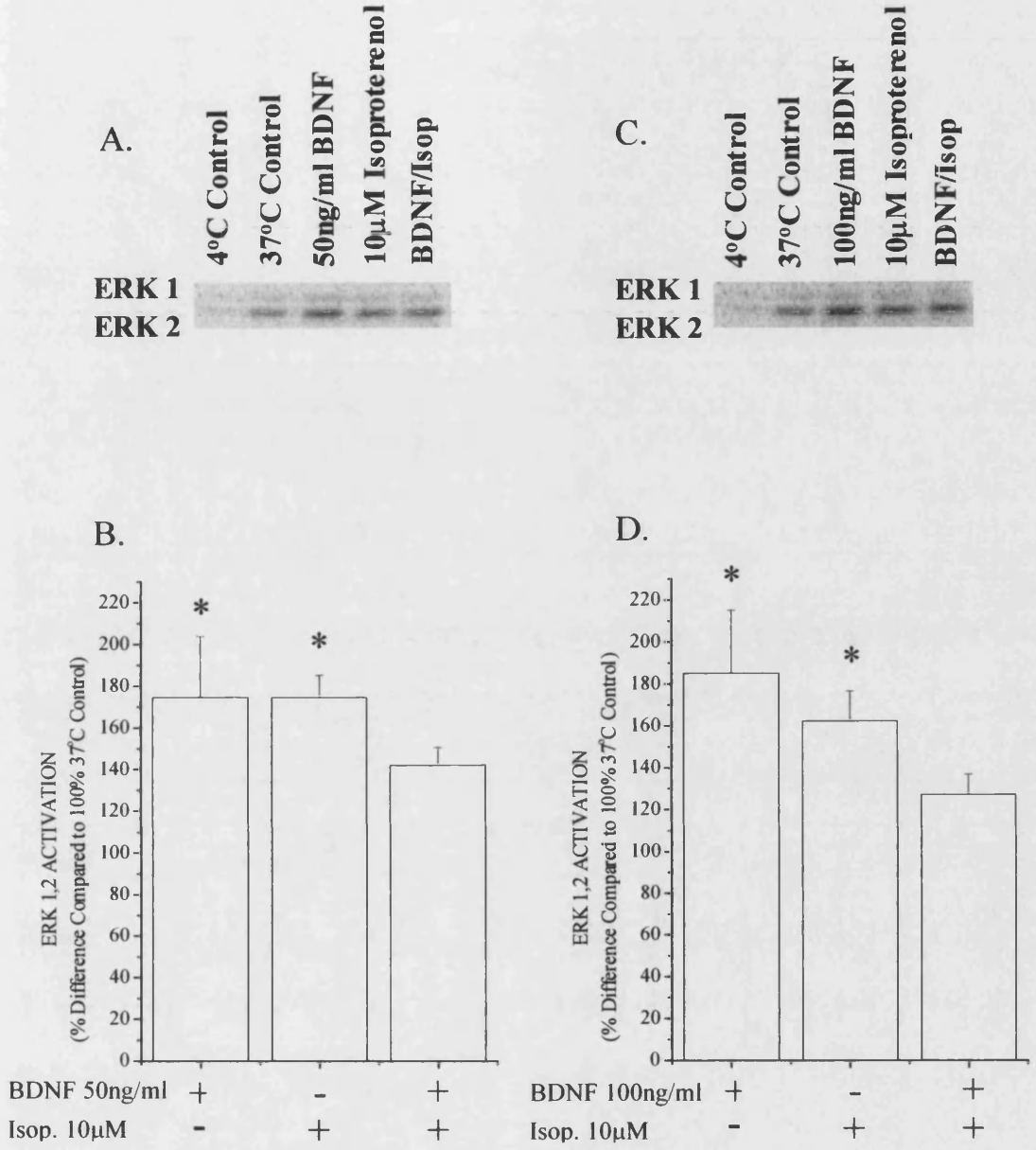


Figure 4.10 Isoproterenol and BDNF-Mediated Increases in ERK 1,2 Phosphorylation are not Additive. Synaptosomes were incubated for 10 minutes with 1mM $CaCl_2$ either in the absence (control) or presence of BDNF (+BDNF) and/or isoproterenol (+isop.). BDNF (50 or 100ng/ml) was added 6mins prior, and isoproterenol (10μM) 5mins 30s prior to the termination of the reactions. 7.5% polyacrylamide gels were run. Immunoblots were labelled with phospho-ERK primary antibody (1:1000, NEB) and probed using ^{125}I-labelled protein A, levels of which were detected and analysed with phosphorimager spectroscopy (Molecular Dynamics). *P<0.05 compared to 100% 37°C control (ANOVA followed by Duncan's *post hoc* analysis, B (50ng/ml BDNF) n=3; D (100ng/ml BDNF) n=4). **A&B.** Phosphorimages of isoproterenol and BDNF effects. **C&D.** Quantification of ERK1,2 phosphorylation levels.

concentrations of agonists. On this occasion both BDNF and isoproterenol in isolation, as well as the two agonists in combination, were able to significantly enhance ERK1,2 phosphorylation levels (mean ± s.e.m. in % compared to 100% 37°C control: BDNF (100ng/ml) = 203 ± 34; isoproterenol (100μM) = 167 ± 19; BDNF/isoproterenol = 181 ± 7). No significant differences were found between the levels of ERK1,2 activation with the individual agonists or when they were coapplied. The lack of additivity of BDNF and isoproterenol treatment, as well as the possible attenuating effect of β-adrenoceptor activation on neurotrophin-mediated signalling, implies the involvement of the same pool of ERK1 and ERK2 in the regulation of these effects. This suggests that these receptor-linked signalling pathways are present within the same nerve terminals (as illustrated in Figure 4.12), adding evidence to the hypothesis that isoproterenol may be utilising parts of the same downstream pathway as BDNF to enhance glutamate release from cerebrocortical nerve terminals. Further investigation is required to confirm this hypothesis and the level of interaction of the two pathways also remains to be determined.

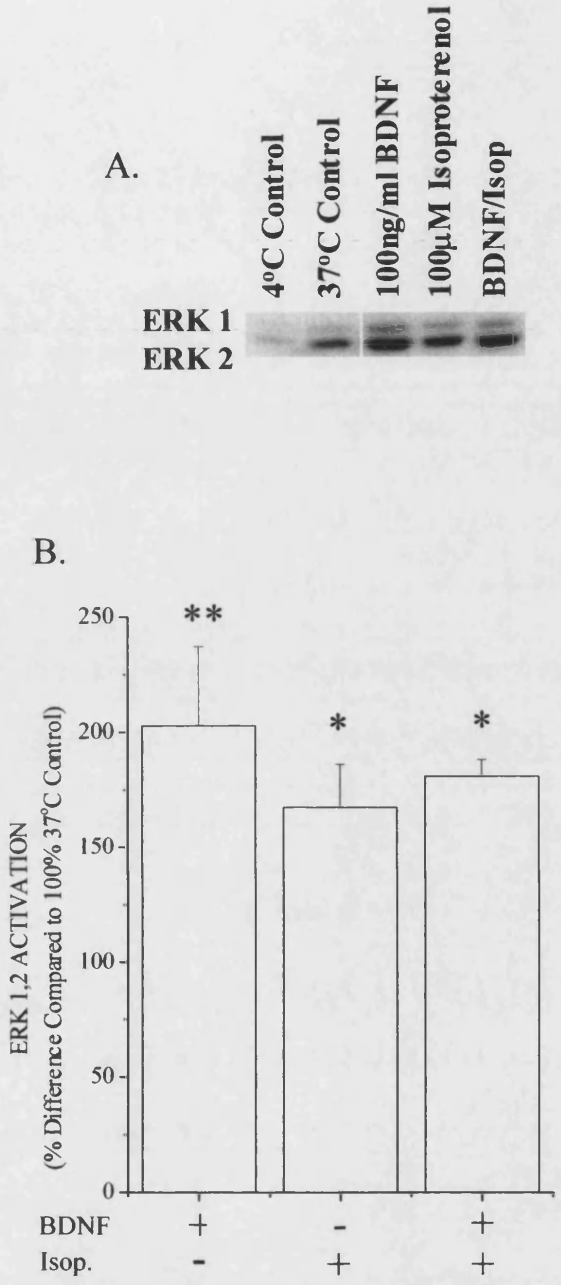


Figure 4.11 Combined Effects of Isoproterenol and BDNF on ERK 1,2 Activation. Synaptosomes were incubated with 1mM $CaCl_2$ for 10 mins in the presence of 100ng/ml BDNF (+BDNF) added 6 mins prior to the termination of the incubation, and/or 100µM isoproterenol (+isop.) added 5 mins 30 sec prior to the end of the incubation. Gels containing 7.5% polyacylamide were run. Immunoblots were labelled with phospho-specific MAPK primary antibody (1:1000, NEB) and reported using ^{125}I-labelled protein A. ^{125}I-labelling was quantified using phosphorimager spectroscopy (Molecular Dynamics). $^{*}P>0.05$, $^{**}P>0.01$ (ANOVA with Duncan's *post hoc* analysis, n=3). **A.** Phosphorimage of isoproterenol and BDNF effects. **B.** Quantification of isoproterenol and BDNF effects.

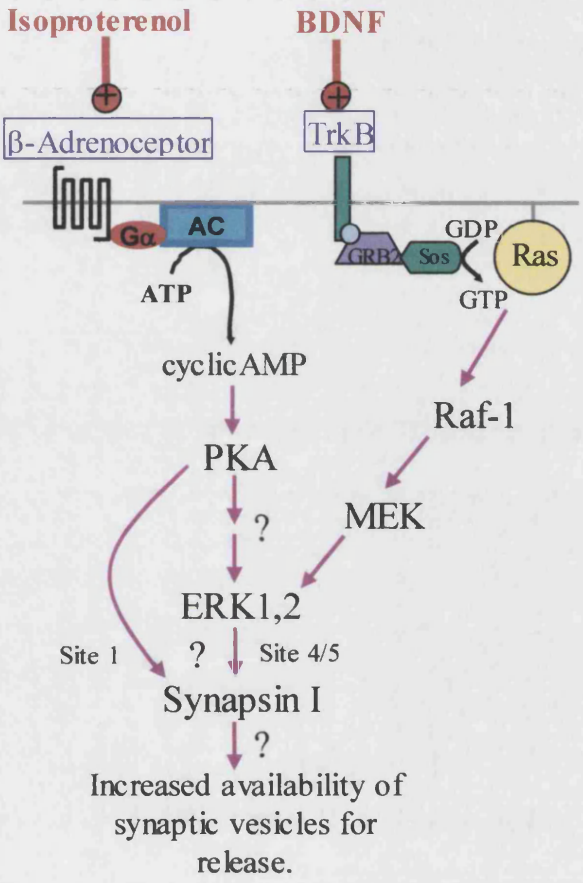


Figure 4.12 A Summary Diagram Illustrating a Possible Role for ERK 1,2 in the Cross-Talk of Neurotrophin and Adrenergic Receptor Signalling Pathways in the Regulation of Glutamate Release. BDNF stimulates the TrkB receptor resulting in phosphorylation of the receptor and binding of the adaptor protein GRB2. This, in turn, binds the Ras guanine nucleotide exchange factor (Sos), resulting in the exchange of Ras-GDP for Ras-GTP and activation of the Ras/Raf/MEK/ERK signalling cascade. Isoproterenol stimulates the β-adrenoceptor GPCR leading to activation of PKA and ERK1,2. The exact mechanism for the PKA-dependency of ERK1,2 activation remains to be determined, as well as the level of interaction of the two signalling pathways. Abbreviations: AC = adenylate cyclase, cyclicAMP = cyclic adenosine monophosphate, ATP = adenosine triphosphate, PKA = protein kinase A, ERK1,2 = extracellular-regulated protein kinase 1 and 2, MEK = mitogen activated protein kinase kinase. (**?** Indicate hypothetical pathways).

4.4 Summary of Results

- β-adrenoceptor-mediated enhancement of depolarisation-induced glutamate release can be manifested in a mechanism occurring downstream of Ca^{2+} entry in cerebrocortical nerve terminals.

- Activation of β-adrenoceptors leads to a dose-dependent enhancement of ERK1,2 phosphorylation in cerebrocortical nerve terminals.

- Stimulation of ERK1,2 phosphorylation downstream of β-adrenoceptor activation occurs through a signalling cascade which is dependent on PKA.

- Endogenous stimulation of β-adrenoceptors appears to partially account for the basal levels of ERK1,2 activation observed in cerebrocortical nerve terminals.

- Evidence suggests that β-adrenoceptor activation may be leading to the enhancement of PKA- and ERK1,2-dependent phosphorylation of synapsin I, thus providing a mechanism for Ca^{2+} channel independent increases in glutamate release from synaptosomes treated with isoproterenol.

- β-adrenoceptor and TrkB mediated signalling to ERK1,2 appears to occur within the same nerve terminals.

- These results, combined with those obtained in the previous chapter, indicate the existence of a pathway for ERK1,2 activation following forskolin/IBMX treatment that displays differential characteristics to the β-adrenoceptor/PKA/ERK1,2 pathway described here.

4.5 Discussion

The β-adrenoceptor has been shown to be a Gs-coupled GPCR, whose activation leads to the stimulation of adenylate cyclase and increased intracellular levels of cAMP which, in turn, leads to enhanced PKA activation (De Blassi, 1990). The PKA inhibitors, H-89 and KT-5720, were both shown to attenuate β-adrenoceptor-mediated increases in ERK1,2 phosphorylation, which naturally leads to the conclusion that the signalling pathway occurring between the β-adrenoceptor and ERK1,2, in cerebrocortical synaptosomes, is dependent on PKA. These results are in contrast to those obtained following the enhancement of intracellular cAMP levels and inhibition of adenosine A_1 receptors with forskolin/IBMX, which indicated that the stimulation of ERK1,2 phosphorylation might occur independently of PKA based on inhibitor profile. It is possible, considering the non-specific effects of these inhibitors described in the previous chapter, that the attenuation of β-adrenoceptor signalling to ERK1,2 is mediated through the inhibition of another kinase, and not PKA. However, several studies have identified a role for PKA in regulating the signalling occurring between β-adrenoceptors and ERK1,2, such as in the CA1 region of the hippocampus, and in HEK293 cells (Roberson et al., 1999; Schmitt and Stork, 2002). A number of factors also point towards separate pathways mediating the effects of β-adrenoceptor signalling and the signalling occurring following treatment with forskolin/IBMX in cerebrocortical nerve terminals. These include the PKA-dependency of β-adrenoceptor signalling versus the PKA-independency of forskolin/IBMX signalling to ERK, as well as the persistent stimulatory effect on ERK1,2 phosphorylation of isoproterenol versus the switch from facilitation to inhibition by forskolin/IBMX.

The regulation of ERK1,2 phosphorylation by different cAMP-mediated pathways is not without precedent, as a number of reports have identified a role for direct cAMP activation of the ERK1,2 signalling cascade through stimulation of a Rap1-GEF, such as Epac (de Rooij et al., 1998; Lin et al., 2003). This means that the forskolin/IBMX-mediated increases in ERK1,2 phosphorylation could be occurring through cAMP-dependent but PKA-independent signalling pathway. If this was found to be so, then it would suggest the existence of two different cAMP-mediated ERK1,2 activation pathways in nerve terminals. However, it is also possible that the differences found

between ERK1,2 activation following β-adrenoceptor stimulation and treatment with forskolin/IBMX are mediated by adenosine A1 receptor inhibition, rather than enhancement of cAMP levels. It would be interesting to further investigate these differences in regulation of ERK1,2 phosphorylation by isoproterenol and forskolin/IBMX in nerve terminals, and in particular, to clarify the cAMP-dependency of the forskolin/IBMX effect.

The results showing a propranolol-mediated inhibition of basal ERK1,2 activation levels, combined with those obtained with the PKA inhibitors, H-89 and KT5720, adds weight to the proposal from the previous chapter, that a PKA-dependent basal phosphorylation of ERK1,2 occurs in nerve terminals. These results also suggest that the β-adrenergic receptor is providing a locus for at least part of the basal level of ERK1,2 phosphorylation observed in synaptosomes. Studies conducted in other systems have found that propranolol, as well as being a β-adrenoceptor inverse agonist, can also have partial agonist responses in stimulating the ERK1,2 signalling cascade, which could suggest a role for this compound as a dual efficacy ligand (Azzi et al., 2003; Baker et al., 2003). However, propranolol was found to significantly decrease ERK1,2 phosphorylation in cerebrocortical synatosomes, rather than to elicit any increase, suggesting this is not occurring here.

As mentioned before, β-adrenoceptor-mediated increases in ERK1,2 phosphorylation have been shown to be dependent on PKA in several different systems, nevertheless, the exact mechanisms involved are still under debate. In PC12 cells, for example, PKA has been shown to directly phosphorylate Rap1, leading to increased GTP-binding and activation of the Rap1/B-Raf/MEK/ERK1,2 signalling cascade (Vossler et al., 1997). This is in contrast to more recent studies in cortical neurones which have identified an increase in Ras-GTP levels following PKA stimulation, leading to activation of the Ras/Raf-1/MEK/ERK1,2 cascade (Ambrosini et al., 2000). There is also evidence to suggest that PKA can directly phosphorylate and activate Src, leading to recruitment and activation of Rap-1 in HEK293 cells (Schmitt and Stork, 2002). However, also of note is that PKA has been shown to phosphorylate the β-adrenoceptor resulting in the switching of the coupling from Gs to Gi/o and consequent activation of the ERK1,2 signalling cascade through βγ subunits (Zamah et al., 2002; Laroche-Joubert et al.,

2003). Though this mechanism may be dependent on the cell type studied, as the dominant mechanism for β-adrenoceptor activation of ERK1,2 in HEK293 cells did not require PKA phosphorylation of the receptor but did require the activation of a Src family member (Friedman et al., 2002). Further experiments would need to be conducted to clarify the mechanism for PKA-dependent ERK1,2 activation in cerebrocortical synaptosomes. It would be possible to determine the Src-dependency of PKA signalling to ERK1,2, downstream of β-adrenoceptor activation in cerebrocortical synaptosomes by using the selective Src family kinase inhibitor, SU6656 (Blake et al., 2000). Several studies have demonstrated that the same GPCR has the ability to activate ERK1,2 via multiple signalling pathways and the cellular context within which a receptor is expressed can determine the mechanism of GPCR-mediated ERK activation (Della Rocca et al., 1999; Pierce et al., 2000). This means that until further experiments are conducted, the mechanism determining the PKA-dependency of β-adrenoceptor-mediated ERK1,2 activation can at best, only be speculation.

The combined activation of TrkB receptors (with BDNF) and β-adrenoceptors (with isoproterenol) did not result in an addition of the ERK1,2 phosphorylation levels in cerebrocortical nerve terminals. This suggests that these two receptors are involved in regulating the same pool of ERK1 and 2. Studies conducted in COS-7 cells have identified a mechanism through which β-adrenoceptors are able to 'transactivate' neurotrophin receptors, albeit epidermal growth factor receptors (EGFR) (Pierce et al., 2000; Maudsley et al., 2000). It was found that activation of β-adrenoceptors could lead to endocytosis of the EGFR and stimulation of the ERK1,2 signalling cascade. It is possible that the stimulation of synaptosomes with isoproterenol is leading to β-adrenoceptor-mediated 'transactivation' of the TrkB receptor, which would explain how both receptors could be stimulating the same pool of ERK1 and 2. However, a role for PKA in this signalling pathway has yet to be defined, although Src activation has been shown to be required, so it is possible that PKA is involved in phosphorylating and activating Src (Maudsley et al., 2000). It should be noted that 'transactivation' of TrkB receptors by β-adrenoceptors and PKA-mediated phosphorylation of Src is yet to be shown in cerebrocortical synaptosomes, and so this mechanism for activation of ERK1,2 remains speculative until further experiments can be conducted.

In a study conducted in the striatum, β-adrenoceptor-mediated enhancement of glutamate release was found to occur through a cAMP/PKA upregulation of kainate receptors (Dohovics et al., 2003a; Dohovics et al., 2003b). Although this mechanism cannot be ruled out for the enhancement, by β-adrenoceptors, of glutamate release from cerebrocortical nerve terminals, the stimulation of ERK1,2, combined with the observed phosphorylation of the downstream protein, synapsin I, at least implies the presence of an alternative mechanism for the regulation of glutamate release in this system. The phosphorylation of synapsin I on sites mediated by both PKA and ERK1,2 opens the way for an involvement of β-adrenoceptor signalling in the regulation synaptic vesicle availability. Increased phosphorylation of synapsin I at these sites has been shown to decrease the tethering of synaptic vesicles, resulting in an increase in the number of vesicles available for release (Chi et al., 2001; Chi et al., 2003). This hypothesis could be used to explain the ability of isoproterenol to enhance glutamate release independently of Ca^{2+} channel modulation in cerebrocortical nerve terminals, as well as independently of the regulation of membrane excitability and Ca^{2+} influx in cerebellar cells (Chen and Regehr, 1997) (see Figure 4.12). However, only examples of isoproterenol-mediated signalling to synapsin I are shown in Figure 4.9, and so further experiments will need to be conducted to prove the role of synapsin I downstream of β-adrenoceptor signalling in presynaptic nerve terminals.

The results obtained in this chapter have described an ability of two major presynaptic signalling cascades, PKA and ERK1,2, to cross-talk with each other downstream of GPCR activation, resulting in a possible functional involvement in the regulation of glutamate release. The next chapter in this thesis goes on to investigate whether another major presynaptic signalling cascade, the PKC cascade, could also be able to cross-talk with ERK1,2 in presynaptic cerebrocortical nerve terminals.

Chapter 5

Phorbol Ester-Mediated Modulation of Presynaptic Function:
Cross-Talk of PKC and ERK 1,2 Signalling Cascades.

5.1 Introduction

Protein kinase C (PKC) has long been known to enhance the release of various neurotransmitters from neurones, including noradrenaline (Allgaier et al., 1986), acetylcholine (Allgaier et al., 1988), glutamate (Barrie et al., 1991), GABA (Bartmann et al., 1989), dopamine (Nichols et al., 1987), and serotonin (Feuerstein et al., 1987). It is commonly agreed that depolarisation of nerve terminals followed by the inevitable influx of Ca^{2+} ions leads to the activation of PKC (Wang et al., 1988). However, the mechanisms through which PKC is able to modulate release are still under debate. There are currently believed to be four possible ways through which PKC could mediate its effects on increasing neurotransmitter release. These are through: regulation of Ca^{2+} channels, regulation of K^+ channels, interactions with the exocytotic machinery, or by regulating the size of the readily releasable pool of synaptic vesicles.

Exocytosis of synaptic vesicles can be triggered by calcium influx through N-, P/Q-, and R-type calcium channels (Heidelberger et al., 1994; Dunlap et al., 1995; Wu et al., 1998). Increases in exocytosis can be mediated by increasing the Ca^{2+} influx through these channels. PKC has been shown to enhance synaptic transmission mediated by Q-type channels in hippocampal neurones (Wheeler et al., 1994). N- and P/Q-type channels are also reputed to be necessary for the enhancement of glutamate release resulting from PKC activation in cerebrocortical nerve terminals (Vazquez and Sanchez-Prieto, 1997). It has since been shown that these channels can undergo a shift to an easily activated state following direct PKC phosphorylation, representing an established PKC-dependent mechanism for increasing neurotransmitter release (Hamid et al., 1999; Herlitze et al., 2001).

As well as phosphorylating Ca^{2+} channels to increase neurotransmitter release, PKC could also be modulating K^+ channel function. K^+ channels, in nerve terminals, are responsible for the hyperpolarisation phase of the action potential and are involved in regulating the general excitability of the membrane. Inhibitory effects on these channels by PKC would result in an increased propensity towards depolarisation of the plasma membrane. This could increase the probability of Ca^{2+} channel opening and of vesicular release. A study using guinea pig cerebrocortical synaptosomes suggested

phorbol ester stimulation of glutamate release was due to PKC mediated inhibition of 4-AP resistant K^+ channels (Barrie et al., 1991). There has also been a study using cultured rat cerebellar granule cells where it was suggested that PKC was stimulating [^{3}H]-D-Aspartate release through inhibition of a dendrotoxin-1-sensitive K^+ channel (Cousin et al., 1999). It should be noted however, that neither of these studies looked for direct phosphorylation of the K^+ channels by PKC, making it impossible to rule out other factors in their regulation. Further studies are needed to identify a specific location for PKC-dependent K^+ channel modulation in nerve terminals.

The third mechanism for PKC-mediated enhancement of neurotransmitter release is through interactions with the exocytotic machinery. PKC has recently been shown to phosphorylate Munc-18 in adrenal chromaffin cells. This phosphorylation results in the decreased affinity of Munc-18 for syntaxin 1A, allowing progression from the primary SNARE conformation to the binary conformation. However, this phosphorylation served only to modulate single vesicle release kinetics and did not affect the number of exocytotic events that phorbol esters are also able to regulate (Barclay et al., 2003). This suggests the involvement of additional pathways in phorbol ester mediated increases in release.

The fourth and, currently, final mechanism through which PKC could increase neurotransmitter release is by regulating the interactions of small synaptic vesicles with the actin cytoskeleton. The vesicles are tethered away from the plasma membrane, to the actin cytoskeleton, to form the reserve pool of synaptic vesicles. Their interactions to the cytoskeleton can be regulated through phosphorylation, resulting in an increase in the number of vesicles available for release. Recent evidence has suggested that PKC could be involved in regulating the tethering of synaptic vesicles to the actin cytoskeleton. Work on glutamate release from retinal bipolar cells has highlighted a possible role for PKC modulation of vesicle recruitment. Phorbol ester treatment increased glutamate release from the second, slow component of vesicles, suggesting that PKC was working to increase the pool size of the slow component in these nerve terminals (Minami et al., 1998; Berglund et al., 2002). PKC regulation of vesicle availability has also been shown to have a role in enhancing noradrenalin release from isolated nerve terminals (Walaas, 1999) and, work on hippocampal slices suggested a

modulatory involvement of PKC in the disassembly dynamics of synaptic vesicles from the actin cytoskeleton (Pasantes-Morales et al., 2002).

Synapsin I is a vesicular protein that binds synaptic vesicles to the actin cytoskeleton in glutamatergic cerebrocortical nerve terminals. Phosphorylation of synapsin I by kinases such as ERK 1,2 has been shown to reduce tethering by synapsin I, resulting in an increase in glutamate release from these nerve terminals (Jovanovic et al., 2000). PKC has not been shown to directly regulate synapsin I phosphorylation but it is possible that it interacts with, and activates, kinases upstream of ERK 1,2 and synapsin I phosphorylation.

PKC activation has been shown to upregulate ERK 1,2 phosphorylation in many different systems including PC12 cells (Brodie et al., 1999), SH-SY5Y neuroblastoma cells (Olsson et al., 2000), HEK 293 cells (Kawasaki et al., 1998), cultured astrocytes (Neary et al., 1999; Zhao and Brinton, 2003), and CA1 hippocampal neurones (Roberson et al., 1999). The exact location for PKC crosstalk with the ERK 1,2 pathway is still yet to be ascertained, but recent studies have identified a Raf kinase inhibitory protein (RKIP) that is phosphorylated directly by PKC. This releases RKIP from Raf-1, enabling signalling to continue down the ERK 1,2 pathway (Corbit et al., 2003). There are also reports of PKC pathway interactions at the level of Ras signalling. More recent evidence, however, suggests that signalling through Ras could be mediated by a PKC-independent DAG stimulation of RasGEFs (Lorenzo et al., 2000).

The evidence considered here suggests that, as well as modulating neurotransmitter release through the phosphorylation of Ca^{2+} channels and regulation of K^{+} channels, PKC could also be acting through an alternative mechanism to increase vesicular availability. This chapter aims to investigate whether PKC could be stimulating ERK 1,2 phosphorylation in cerebrocortical nerve terminals, and whether this could lead to downstream synapsin I phosphorylation. If so, this could provide a basis for PKC modulation of glutamate release through increasing the availability of synaptic vesicles in cerebrocortical nerve terminals. This would not only suggest an alternative mechanism for PKC modulation of glutamate release downstream of Ca^{2+} entry, but would also imply that distinct receptor-mediated signalling pathways coupled to protein

phosphorylation, could crosstalk with each other to produce a common mechanism for the modulation of glutamate release.

5.2 Methods

Synaptosomes were prepared as described in section 2.1 (Sihra, 1997).

5.2.1. Glutamate Release

The standard release protocol was followed as described in section 2.2 (Nicholls and Sihra, 1986; Perkinton and Sihra, 1999). PDBu (1μM) was added 1 minute prior to the addition of ionomycin 5μM.

5.2.2. Time Course of ERK 1,2 Phosphorylation/Activation

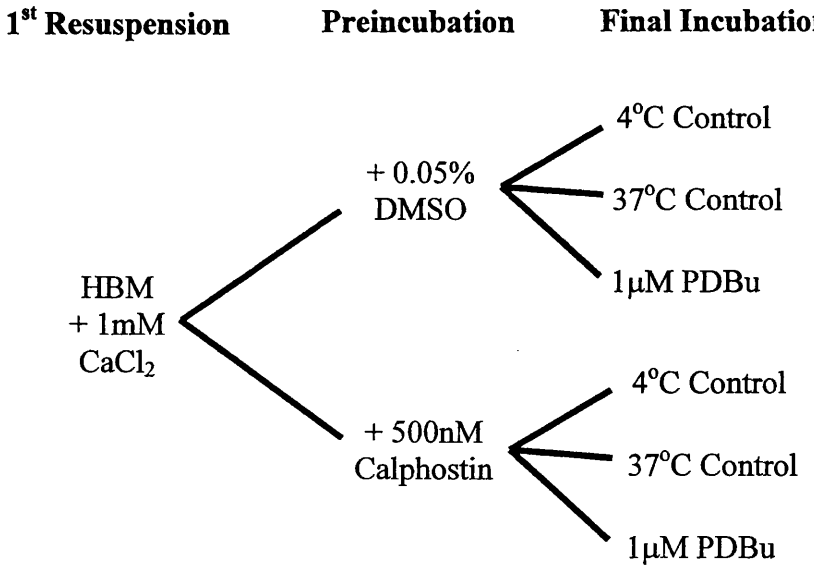
The synaptosomes were resuspended at 4°C to a final synaptosomal concentration of 1mg/ml using HBM containing BSA. $CaCl_2$ was added, to give a final concentration of 1mM, immediately prior to splitting the synaptosomes into two test tubes primed with solutions of either 0.1% DMSO or 1μM PDBu (final concentrations given). 100μg samples of the synaptosomes were then simultaneously taken from each test tube and added to small Eppendorf tubes containing 25μl of 5X SDS-PAGE STOP buffer to terminate any reactions. These 4°C samples were taken to check for similar low levels of basal ERK 1,2 phosphorylation. The two test tubes were then immediately put to incubate at 37°C with stirring. Further 100μg samples were simultaneously taken from each tube following 1, 3, 6, 10, and 15 minutes of incubation. These were also added to small Eppendorf tubes containing 25μl of 5X SDS-PAGE STOP buffer in order to terminate any reactions.

5.2.3. Preincubation

HBM containing BSA was used to resuspend synaptosomes to a final synaptosomal concentration of 1mg/ml whilst being kept at 4°C. $CaCl_2$ (1mM final concentration) was added before immediately splitting the synaptosomes into two tubes and putting them to incubate at 37°C with stirring. Following 10 minutes of incubation, 500nM calphostin C was added to one tube, with 0.05% DMSO being added to a control tube. These additions were carried out in the dark to reduce degradation of calphostin C before it could enter the nerve terminals. The light was reinstated after a further 5 minutes of incubation to allow activation of the calphostin C that had entered the synaptosomes (Bruns et al., 1991). The incubations were continued for a further 20

minutes before the contents of the two tubes were pelleted using a 30 second pulse at 10,000 x g using a microcentrifuge. The supernatants were removed from the pellets, to aid with cooling, and transferred to separate tubes. Both the pellets and the supernatants were cooled for 10 minutes at 4°C to return ERK 1,2 phosphorylation back to basal levels. Once cooled the pellets were carefully resuspended with the kept supernatants before being split into three tubes per pellet, which had also been cooled to 4°C. Two tubes from each condition, control and + calphostin C, were then incubated at 37°C for a further 10 minutes. PDBu (1μM) was added to one of these tubes from each condition, 1 minute prior to the termination of the incubation, with a final concentration of 0.1% DMSO being added to the other tube as the vehicle control. The remaining tubes were kept at 4°C until the end of the experiment (see Scheme 5.2). Reactions were terminated through addition of 5X SDS-PAGE STOP buffer.

Scheme 5.2: Sample division, preincubation and incubation protocol.



5.2.4. Standard Incubation

Synaptosomes were resuspended at 4°C using HBM with BSA to a final synaptosomal concentration of 1mg/ml. A final concentration of 1mM $CaCl_2$ was added immediately

prior to splitting the resuspension into individual sample tubes. The sample tubes contained solutions which would give final concentrations of either 500nM calphostin C, 1μM Ro-32-0432, 0.1% DMSO, or 0.05% DMSO upon addition of the synaptosomes. The sample tubes were then incubated at 37°C for 10 minutes with either 0.1% DMSO or 1μM PDBu being added 1 minute prior to the termination of the incubation. One sample tube was always kept at 4°C for the duration of the experiment to act as a 4°C control. Reactions were terminated by addition of 5X SDS-PAGE STOP buffer to each of the sample tubes. This incubation protocol for measuring ERK 1,2 phosphorylation has previously been described by Jovanovic et al., 2000 (Jovanovic et al., 2000).

Samples were processed for MAPK and synapsin phosphorylation levels as described in the main methods in chapter 2.4 (Dai et al., 2001; Jovanovic et al., 1996).

5.2.5. Reagents

PDBu: Analogue of diacylglycerol that is a potent stimulator of conventional and novel forms of PKC (phorbol 12,13-dibutyrate) (Arcoleo and Weinstein, 1985; Majewski and Iannazzo, 1998).
Dissolved in DMSO to give a stock concentration of 10mM. The stock solutions were diluted to 1mM with DMSO and then on to 100μM with water just before use. This ensured the DMSO concentration in the presence of synaptosomes was not more than 0.1%.

Calphostin C: PKC inhibitor acting at the regulatory site, from Cladosporium cladosporioides (UCN-1028c) (Kobayashi et al., 1989).
Maintained in a 1mM 100% DMSO stock solution in the dark. It was diluted, with water, to a concentration of 50μM immediately before use.

Ro-32-0432 hydrochloride: PKC inhibitor which binds to the catalytic site in the C3 domain (Bisindoylmaleimide XI; 2-(8-[(Dimethylamino)methyl]-6,7,8,9-tetrehydropyrido[1,2-a]indol-3-yl)-3-(1-methylindol-3-yl)maleimide) (Wilkinson et al., 1993).

Dissolved to a stock solution of 1mM in 100% DMSO. Immediately prior to use, the stock concentration was diluted to 100μM using water.

All the above reagents were purchased from the Sigma-Aldrich Company, Gillingham, Dorset.

Unless stated, all drugs were added using 100x stock solutions to minimise volume changes and only final concentrations are listed.

5.3 Results

The first experiment described in this chapter investigated the ability of the phorbol ester, PDBu, to enhance ionomycin-elicited glutamate release from cerebrocortical nerve terminals. PDBu was used as it is a potent stimulator of all the currently identified nerve terminal PKC isoforms. It also has a more rapid rate of activation than other phorbol esters, which should limit the problems encountered with dephosphorylation when using the slower forms of phorbol ester (Majewski and Iannazzo, 1998). As can be seen from Figure 5.1, PDBu significantly increased glutamate release from nerve terminals when ionomycin is used as the secretogogue (mean ± s.e.m. accumulated release in nmol/mg: control = 7.21 ± 0.6, PDBu = 14.48 ± 1.8). This suggests that, as well as regulating Ca^{2+} channels to increase glutamate release; PKC can act via an alternative pathway, which is downstream of Ca^{2+} entry. The next step in this chapter was to investigate whether ERK 1,2 may be involved in this pathway. One way of addressing this would be to see if MEK inhibitors could attenuate the PDBu-enhancement of glutamate release. However, as discussed previously in Chapter 3, it was not possible to use MEK inhibitors with release protocols based on fluorescent readouts. Instead, I looked at ERK 1,2 phosphorylation levels following the activation of PKC with phorbol ester treatment. To do this I used an antibody which detects only the dually phosphorylated, activated, forms of ERK 1 and ERK 2, obtained from New England Biolabs (as described in Section 2.4.4).

Synaptosomes were incubated in the presence and absence of PDBu and immunoblotted for phosphorylated ERK 1,2. Figure 5.2 suggests that PDBu can enhance ERK 1,2 phosphorylation levels and that this possibly occurs in a time-dependent manner. Figure 5.3 confirms that PDBu can significantly enhance ERK 1,2 phosphorylation levels in cerebrocortical nerve terminals (mean ± s.e.m. %: 1 min = 117 ± 6, 10 min = 126 ± 8 compared to 100% control levels). The total incubation time for the experiments in Figure 5.3 was 10 minutes, with the times stated on the graph being the length of incubation in the presence of PDBu.

I have shown that treatment of nerve terminals with PDBu can increase ERK 1,2 phosphorylation levels, which is indicative of ERK 1,2 activation. Phorbol esters can

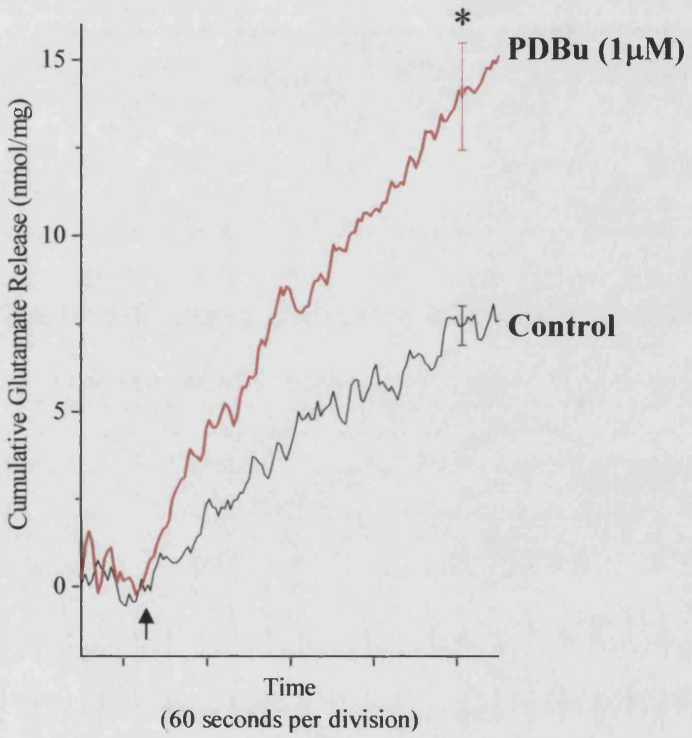


Figure 5.1 Effect of Phorbol Ester on Ionomycin-Elicited Glutamate Release. Synaptosomes were incubated in the presence of 1mM $CaCl_2$ as indicated in section 5.2.1. Glutamate release was elicited with the addition of ionomycin (5μM, arrow) in the absence (control), or presence (+PDBu) of 1μM PDBu, added 1 min prior to the secretogogue. The mean traces from four independent experiments, measuring accumulative release at 2 sec intervals, are shown. Mean ± s.e.m. release was calculated at each time point, but the cumulative glutamate release values following 4 mins of incubation with the secretogogue were used for statistical analysis *$P<0.05$; different from control (Student's paired t-test; n=4).

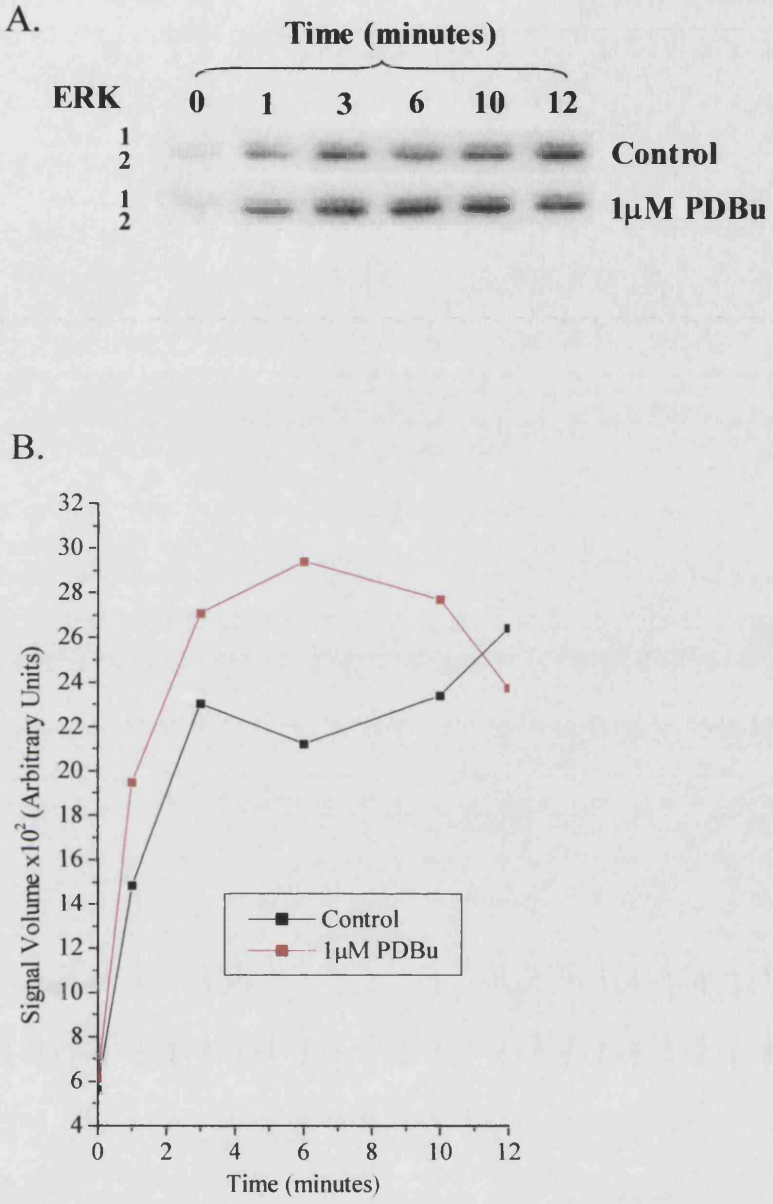


Figure 5.2 PDBu Stimulation of ERK 1 and ERK 2 Phosphorylation/Activation. Synaptosomes were incubated in the presence of 1mM $CaCl_2$ as indicated in section 5.2.2. Immunoblot analysis of synaptosomes incubated in the absence (control) or presence (+PDBu) of 1μM PDBu for 1, 3, 6, 10, or 12 mins. 7.5% polyacrylamide gels were used. Immunoblots were probed with a 1:1000 dilution of primary antibody (NEB) and ^{125}I-Protein A used as the reporter. ^{125}I-labelling was assessed by Phosphorimager spectroscopy (Molecular Dynamics). **A.** Representative phosphorimage of time-course. **B.** Representative quantification of ERK 1,2 phosphorylation (n=1).

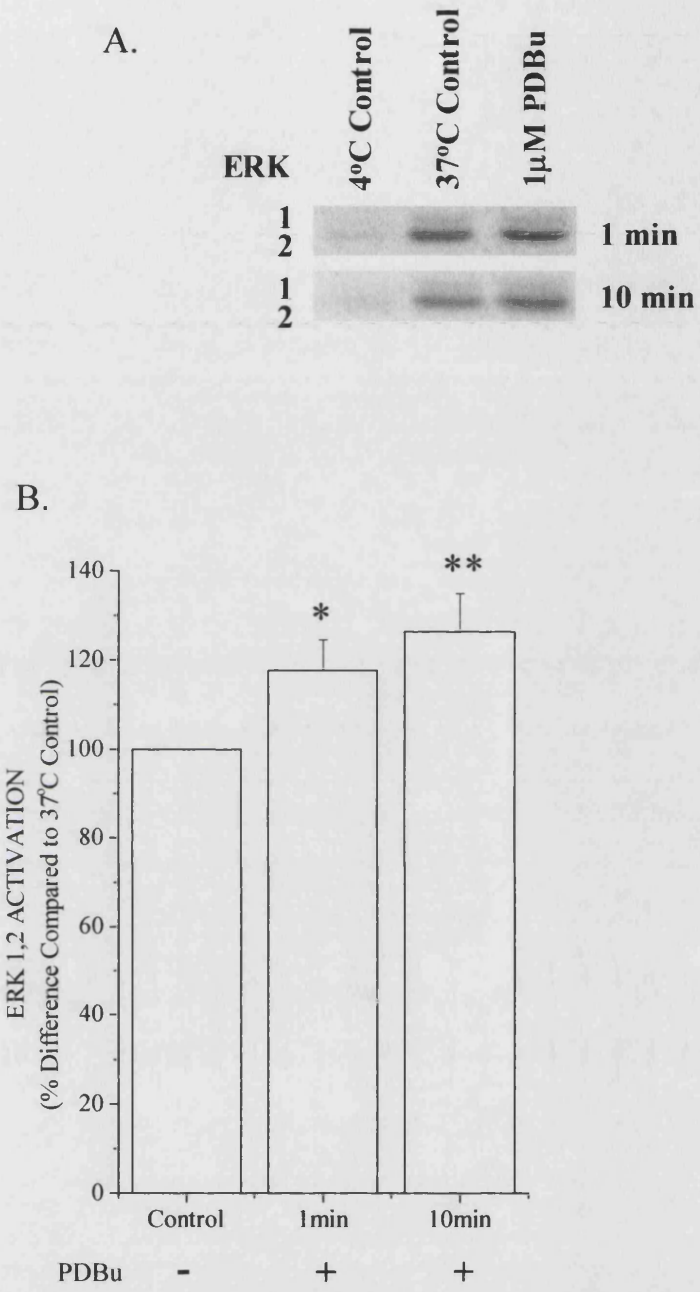


Figure 5.3 Significant Enhancement of ERK 1,2 Phosphorylation by PDBu. Synaptosomes were incubated with 1mM $CaCl_2$ for 10 mins, either in the absence (control) or presence (+PDBu) of 1μM PDBu. 1min and 10min denote the time in the presence of PDBu, as described in section 5.2.4. Gels containing 7.5% polyacrylamide were used. Immunoblots probed with phospho-MAPK antibody (1:1000, NEB) and ^{125}I-Protein A and analysed using phosphorimager spectroscopy (Molecular Dynamics) to assess the ^{125}I-labelling. Mean ± s.e.m. ERK 1,2 phosphorylation levels obtained from separate experiments individually normalised to 100% 37°C control levels. *P<0.05, **P<0.01 (Student's paired t-test, 1min n=4; 10 min n=11). **A.** Phosphorimage of PDBu effect. **B.** Quantification of ERK 1,2 phosphorylation levels. **C.** Proposed pathways leading to ERK 1,2 phosphorylation.

strongly activate PKC but may not necessarily function exclusively to enhance the activity of only this kinase (Lorenzo et al., 2000). In order to confirm whether PDBu could enhance ERK 1,2 phosphorylation in a PKC-dependent manner, the following experiments investigated whether a PKC inhibitor could attenuate PDBu-enhanced ERK 1,2 phosphorylation. Calphostin C, like phorbol esters, binds to the DAG binding site on novel and conventional PKC isoforms (Kobayashi et al., 1989). As nerve terminals are currently believed to only contain novel and conventional forms of PKC, calphostin C should inhibit all of them. The synaptosomes were preincubated at 37°C for 20 minutes with calphostin C before being pelleted, cooled back to 4°C, and resuspended ready for a standard 10 minute incubation. This protocol was used as it has been suggested that longer incubation times with calphostin C would be required in order to obtain complete inhibition of PKC. Figure 5.4 shows the effects of this "long" incubation with calphostin C on basal ERK 1,2 phosphorylation levels in cerebrocortical nerve terminals. The graph shows a significant increase in 4°C ERK 1,2 phosphorylation levels in the presence of the inhibitor when compared to control (mean ± s.e.m. compared to 100% 37°C control: 4°C control = 33 ± 4%, 4°C calphostin C = 77 ± 13%). Given that the protocol involves a preincubation step in the presence of the inhibitor followed by a cooling step, these data suggest that calphostin C is causing either a stimulation of a phosphorylating component of the ERK 1,2 cascade or an inhibition of a dephosphorylating component.

Figure 5.5 examines the effect of calphostin C on PDBu-enhanced ERK 1,2 phosphorylation levels. Data was analysed by comparing the sample containing PDBu to the DMSO control (100%), and the sample containing PDBu and calphostin C to the calphostin C control (100%). Given the effects of calphostin C on its own, this normalization was done to identify any effects of PDBu in isolation from the effect of calphostin C. While calphostin C appeared to suppress the effects of PDBu in some experiments, overall analysis found no significant differences between the control samples and those containing PDBu using an ANOVA (mean ± s.e.m. in %: PDBu = 139 ± 12, PDBu with calphostin C = 115 ± 30). The evident variability could, in part, be introduced by calphostin C altering the basal levels of ERK 1,2 phosphorylation, by either upregulating an activity stimulating the ERK 1,2 cascade, or by down-regulating the dephosphorylation of ERK 1,2 itself under these conditions. Notably, PDBu is able

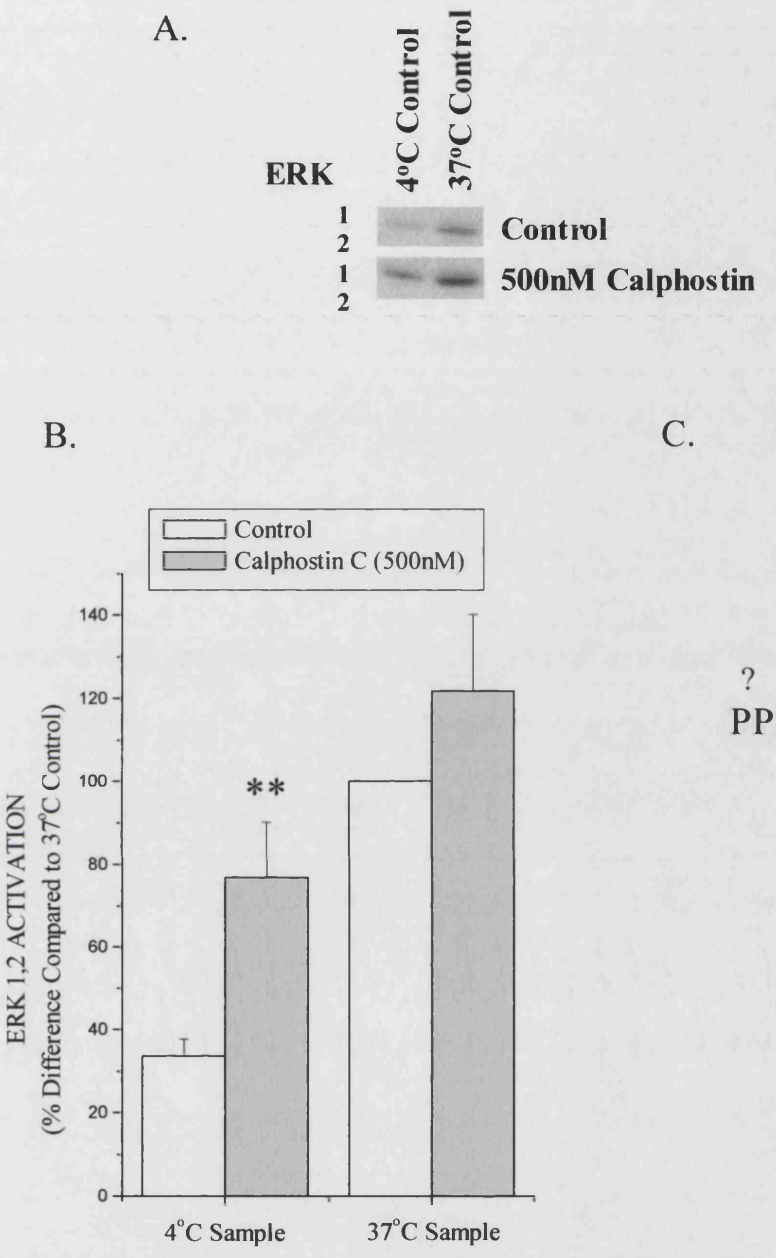


Figure 5.4 Stimulation of Basal ERK 1,2 Phosphorylation with Prolonged Exposure to Calphostin C. Synaptosomes were preincubated with 1mM $CaCl_2$ for 30 mins, with 20 mins in the absence (control) or presence (+Calphostin) of calphostin C, as described in section 5.2.3. 7.5% polyacrylamide gels were run. Immunoblots labelled with 1:1000 phospho-MAPK primary antibody (NEB) were reported using ^{125}I-Protein A and quantified with phosphorimager spectroscopy (Molecular Dynamics). Mean ± s.e.m is from 10 independent experiments, each of which had previously been normalised to its own 100% 37°C control. **P<0.01 different from 4°C control (Student's paired t-test, n=10). **A.** Phosphorimage of calphostin C effect. **B.** Quantification of ERK 1,2 phosphorylation levels. **C.** Proposed pathways leading to ERK 1,2 phosphorylation.

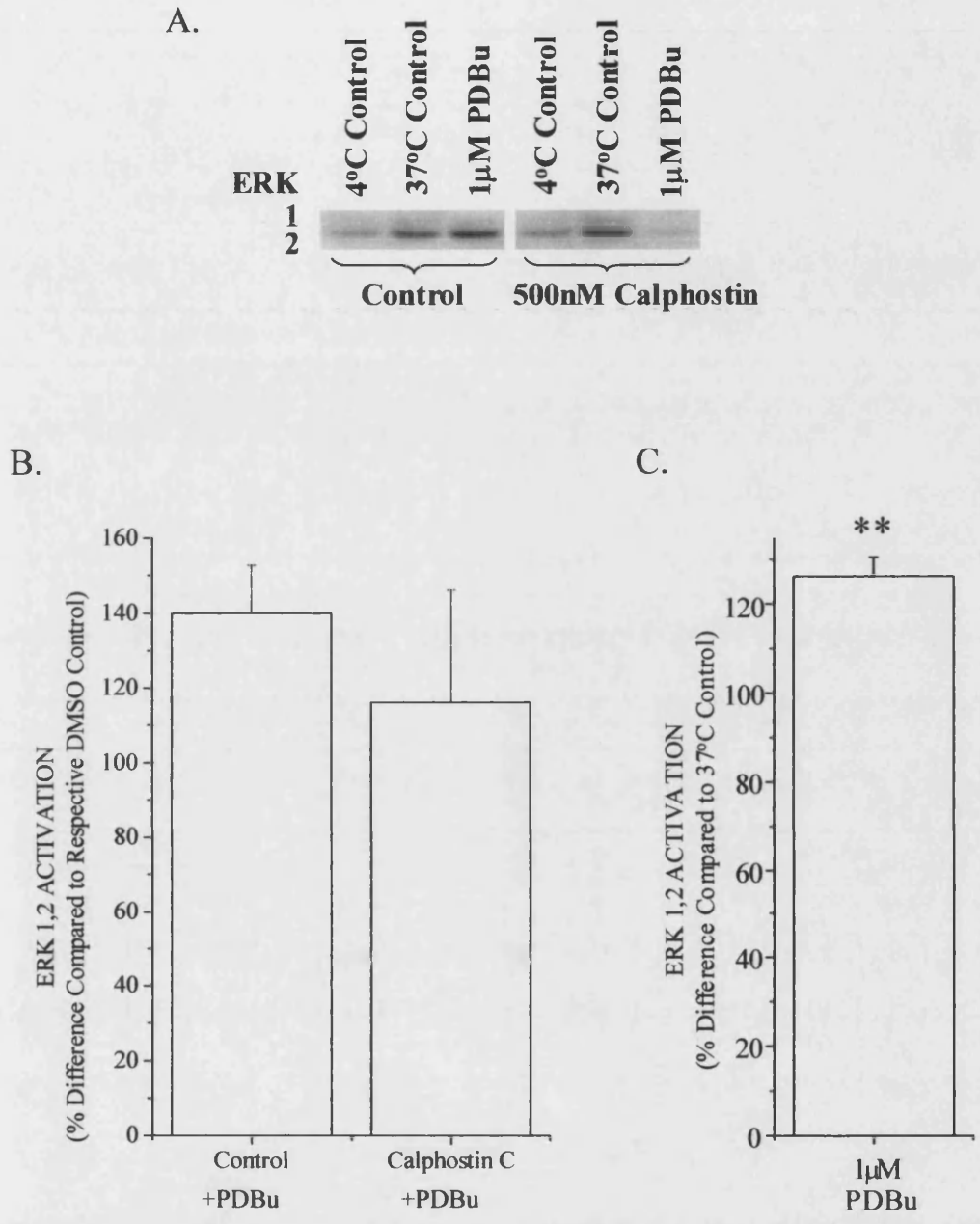


Figure 5.5 Effect of Prolonged Calphostin C Exposure on PDBu-Stimulated ERK 1,2 Phosphorylation. Synaptosomes were preincubated as described in section 5.2.3 followed by a 10 min incubation with 1 min in the presence (+PDBu) or absence (control) of PDBu. ERK 1,2 phosphorylation levels were detected using phospho-MAPK primary antibody (1:1000, NEB), reported with ^{125}I-Protein A, and measured using phosphorimager spectroscopy (Molecular Dynamics). Mean ± s.e.m. from 6 independent experiments, individually normalised to their own relevant 37°C control. **A.** Phosphorimage of combined effects of calphostin C and PDBu. **B.** Quantification of combined effects (no significant differences, ANOVA). **C.** Quantification of PDBu effect following long preincubation protocol, **P<0.01 (Student's paired t-test, n=9).

to significantly increase ERK 1,2 phosphorylation levels on its own under these conditions (mean ± s.e.m. in % compared to 100% 37°C control: PDBu = 126 ± 4). The increase in ERK 1,2 phosphorylation levels with calphostin C alone may be as a result of effects introduced or enhanced by the long incubation protocol. The next experiments conducted in this chapter used the inhibitor with the shorter, standard 10 minute protocol, in order to try and minimise the potentially non-specific effects of the drug produced by the long incubation.

Calphostin C (500nM) was added to the synaptosomes at 4°C and then incubated for 10 minutes at 37°C. As can be seen from Figure 5.6, the inhibitor significantly reduces ERK 1,2 phosphorylation levels (mean ± s.e.m. compared to 100% 37°C control = 73 ± 7%). Figure 5.7 shows the effect of calphostin C on PDBu-enhanced ERK 1,2 phosphorylation. The effect of PDBu on its own was found to be significantly reduced compared to the effect of PDBu with calphostin C (mean ± s.e.m. compared to 37°C control: PDBu = 117 ± 6%, PDBu with calphostin C = 85 ± 6%, calphostin C = 81 ± 9%). This suggests that calphostin C is inhibiting PDBu-stimulated ERK 1,2 phosphorylation, which also suggests PDBu is acting through PKC to mediate the observed increases in ERK 1,2 phosphorylation levels. However, phorbol esters activate novel and conventional forms of PKCs by interacting with the C1 domain at the site of DAG binding (Hurley et al., 1997). This means their actions may not simply be limited to PKC isoforms as they are able to bind other, unrelated proteins with a typical C1 domain (Hurley et al., 1997). These include proteins such as Ras-GRP, a guanine nucleotide exchange factor, which is able to activate the ERK signalling cascade (Lorenzo et al., 2001). As calphostin C works through the same binding site to inhibit PKC, it could be possible it is inhibiting "non-specific" binding to C1 domains in these other proteins, rather than just inhibiting PKC isoforms. This could also mean that effects of calphostin C on ERK 1,2 phosphorylation are mediated through such effects. In order to try and address this possibility, a second PKC inhibitor was used, which utilises an alternative binding site.

Ro-32-0432 was chosen as the alternative PKC inhibitor as it is known to block all the isoforms found in nerve terminals and does not bind to the C1 domain. Ro-32-0432 acts through binding to the ATP site in the C3 catalytic domain, thus preventing

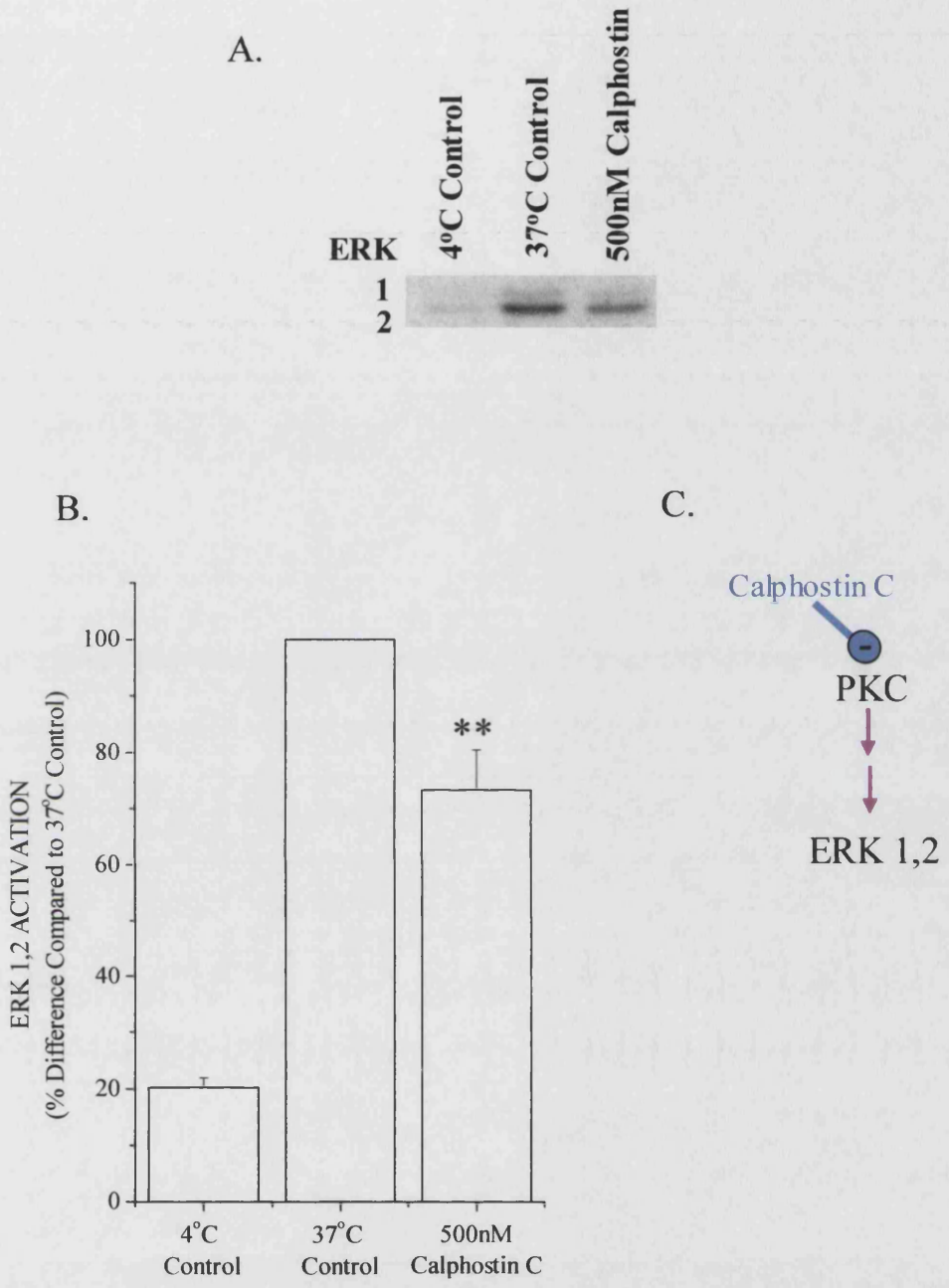


Figure 5.6 Short Incubation with Calphostin C Decreases ERK 1,2 Phosphorylation. Synaptosomes were incubated for 10 min with 1mM $CaCl_2$ either in the absence (control) or presence (+Calphostin) of calphostin C. 7.5% polyacrylamide gels were used. Immunoblots were probed with 1:1000 dilution of the phospho-MAPK primary antibody (NEB) and ^{125}I-Protein A used as a reporter. ^{125}I-labelling was assessed by phosphorimager spectroscopy (Molecular Dynamics). Mean ± s.e.m. was obtained from 8 independent experiments with each experiment being normalised to its own 100% 37°C value, **P<0.01 (Student's paired t-test, n=8). **A.** Phosphorimage of calphostin C effect. **B.** Quantification of calphostin C effect. **C.** Proposed pathways involved.

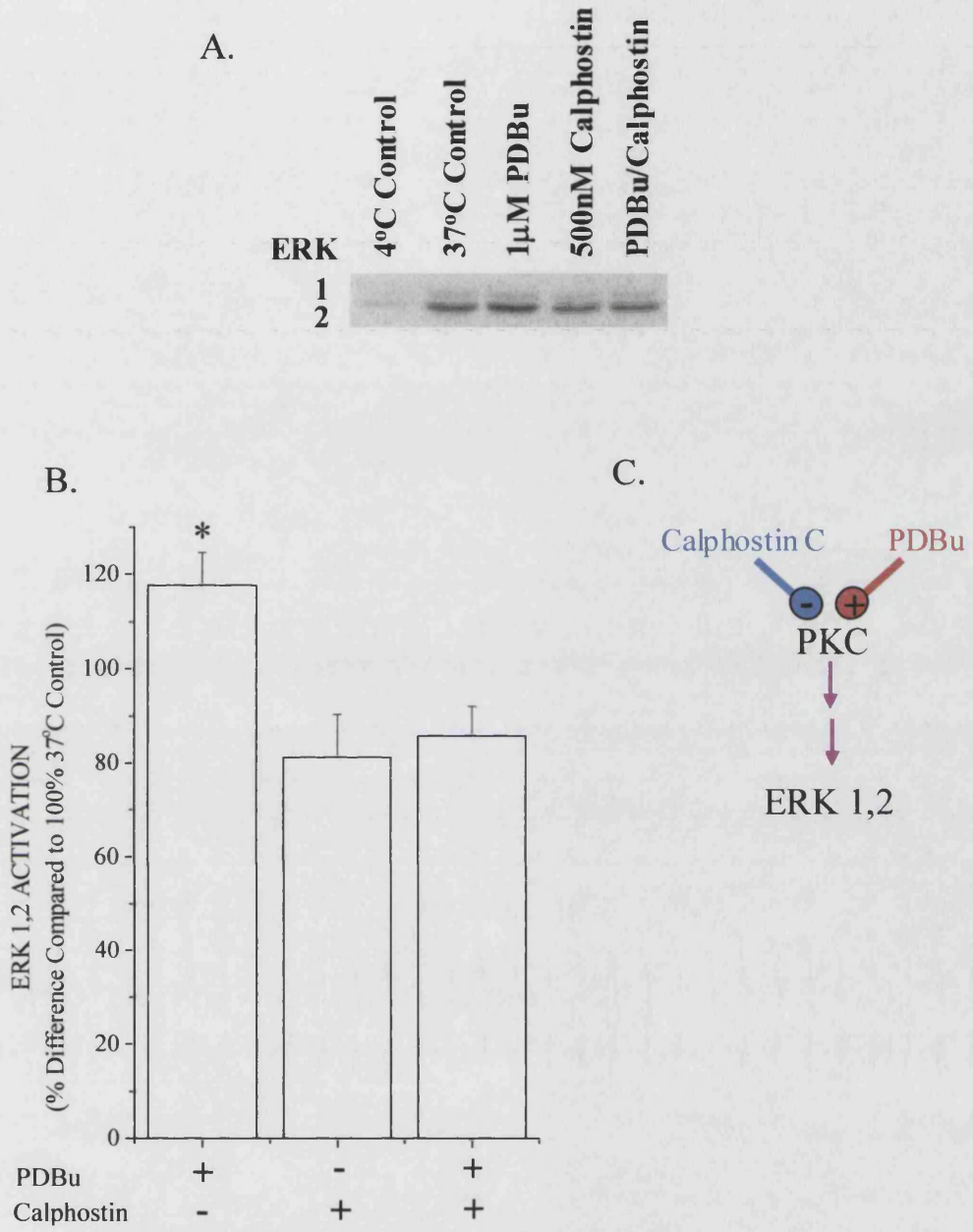


Figure 5.7 Calphostin C Attenuates PDBu-Enhanced ERK 1,2 Phosphorylation. Synaptosomes were incubated for 10 min with 1mM $CaCl_2$ in the presence (+calphostin) or absence (Control) of calphostin C. PDBu was added 1 min prior to the termination of the experiment, as described in section 5.2.4. Gels containing 7.5% polyacrylamide were used. Immunoblots were labelled with phospho-MAPK primary antibody (1:1000, NEB) and reported using ^{125}I-Protein A. ^{125}I-levels were detected using phosphorimager spectroscopy and normalised to 100% individual 37°C controls, *P<0.05 (ANOVA with Duncan's *post hoc* analysis, n=4). **A.** Phosphorimage of calphostin C effect. **B.** Quantification of calphostin C and PDBu effects. **C.** Proposed pathway mediating the calphostin C and PDBu effects.

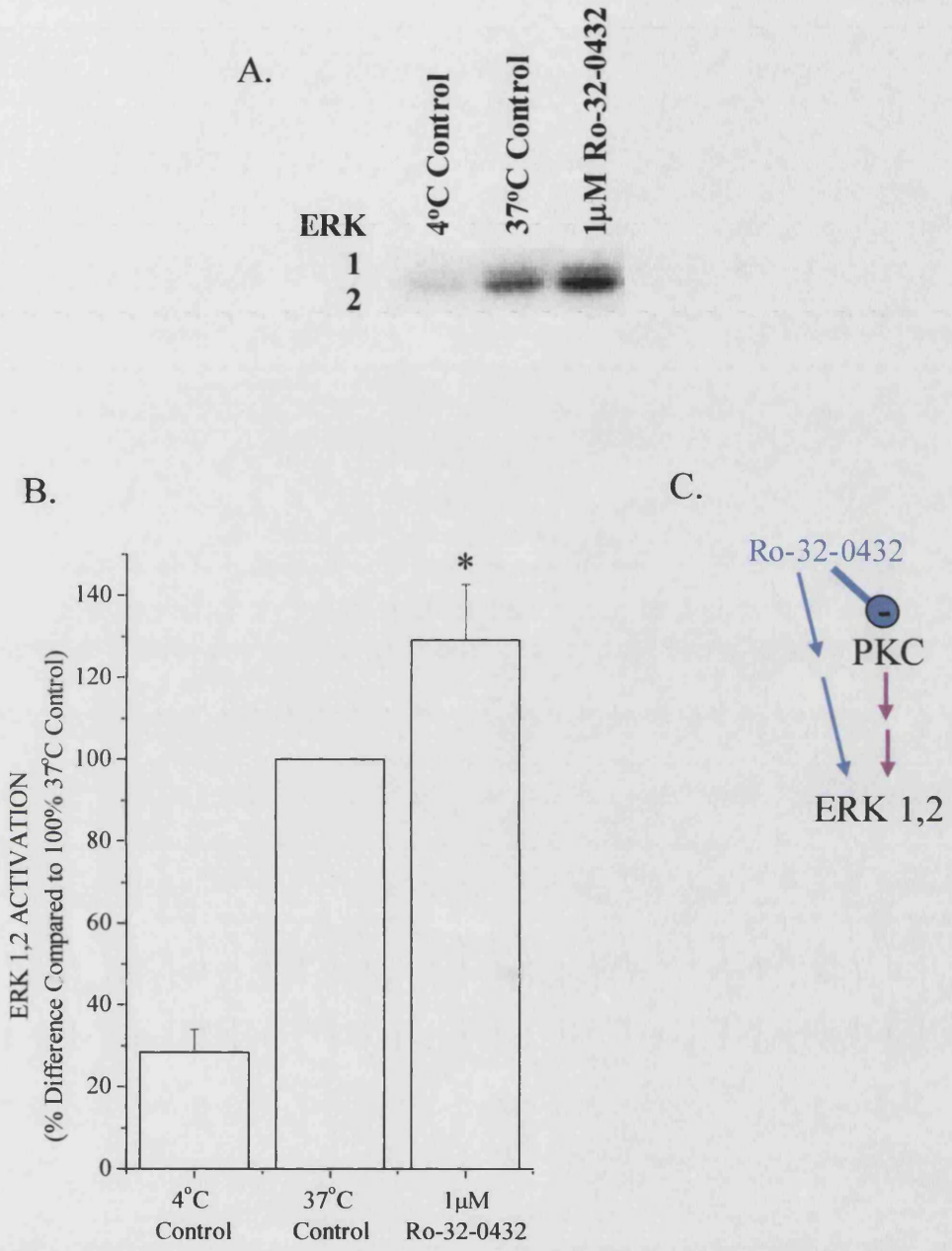


Figure 5.8 Ro-32-0432 Stimulates ERK 1 and 2 Phosphorylation/Activation. Synaptosomes were incubated for 10 mins with 1mM $CaCl_2$ either in the absence (control) or presence (+Ro-32-0432) of Ro32-0432. ERK 1,2 phosphorylation levels were detected through immunoblotting with phospho-MAPK primary antibody and ^{125}I-Protein A as the reporter. ^{125}I-signal levels were ascertained using phosphorimager spectroscopy. Data was taken from 6 independent experiments and normalised to individual 100% 37°C control levels, *P<0.05 (Student's paired t-test, n=6). **A.** Phosphorimage of Ro-32-0432 effect. **B.** Quantification of Ro-32-0432 effect. **C.** Possible pathways involved in mediating Ro-32-0432 effect.

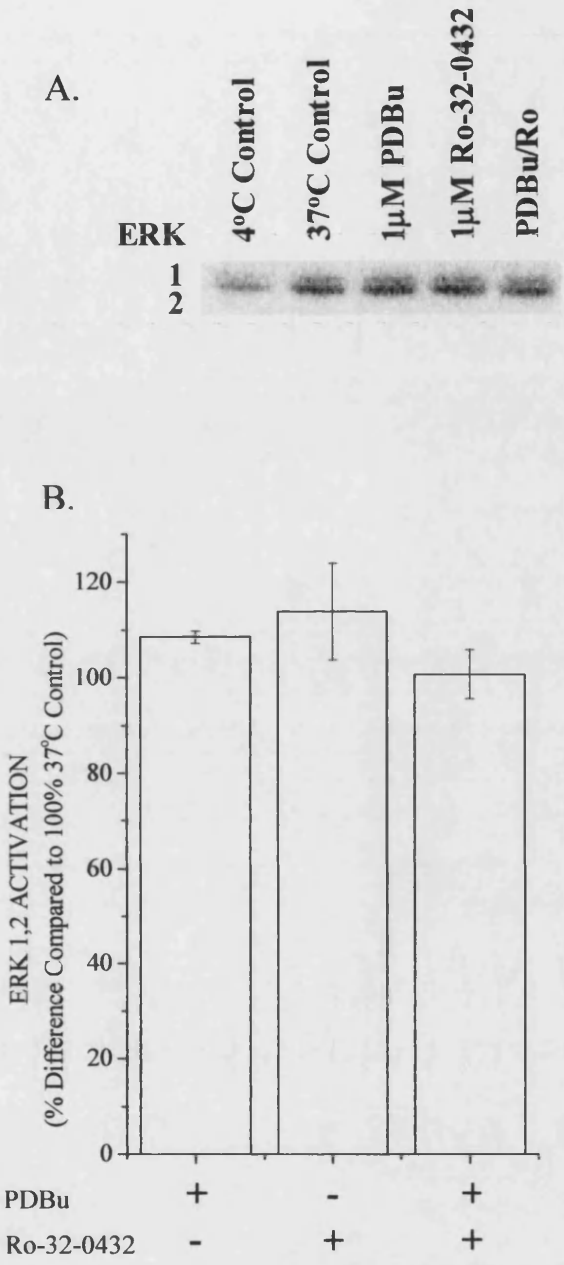


Figure 5.9 ERK 1,2 Phosphorylation Levels with Ro-32-0432 and PDBu. Synaptosomes were incubated for 10 mins with 1mM $CaCl_2$ either in the presence (+Ro-32-0432) or absence (control) of Ro-32-0432. PDBu was added to those samples labelled +PDBu, 1 min prior to the termination of the experiment. 7.5% polyacrylamide gels were used. Immunoblots were labelled with phospho-MAPK primary antibody (1:1000, NEB) and reported using ^{125}I-Protein A. ^{125}I-labelling was quantified using phosphorimager spectroscopy (Molecular Dynamics) with individual values normalised to 100% 37°C control samples. No significance was found between conditions (ANOVA, n=4). **A.** Phosphorimage of PDBu and Ro-32-0432 effects. **B.** Quantification of PDBu and Ro-32-0432 effects.

activation of PKC (Birchall et al., 1994). Figure 5.8 shows the results obtained from incubating synaptosomes in the presence of 1μM Ro-32-0432 for 10 minutes. As can be seen from the graph, Ro-32-0432 was found to significantly increase basal ERK 1,2 phosphorylation levels on its own (mean ± s.e.m. in % compared to 100% control: 129 ± 13). It is not known whether this is a PKC-dependent or non-specific effect. Figure 5.9 shows the combined effects of 1μM PDBu and 1μM Ro-32-0432 on ERK 1,2 phosphorylation in cerebrocortical nerve terminals. No significant differences were found between the samples, although there is a suggestion that Ro-32-0432 may be inhibiting PDBu increases in ERK 1,2 phosphorylation levels (mean ± s.e.m. in % compared to 100% control: PDBu = 108 ± 1, Ro-32-0432 = 113 ± 10, PDBu/Ro-32-0432 = 100 ± 5). Looking for Ro-32-0432-mediated attenuation of the stimulatory effect of PDBu on ERK 1,2 phosphorylation is complicated by the comparable effects of PDBu and Ro-32-0432 when presented alone. However, the intrinsic stimulatory effect of Ro-32-0432 is peculiar to the ERK 1,2 pathway, because direct assay of PKC activity using an intrasynaptosomal PKC substrate has demonstrated a clear inhibitory effect of the compound on PKC-dependent phosphorylation (Coffey et al., 1993).

The final experiments in this chapter examined the effects of PDBu on synapsin site 4/5 phosphorylation. Synapsin is a presynaptic nerve terminal protein which is regulated by phosphorylation and is involved in tethering synaptic vesicles to the presynaptic actin cytoskeleton. ERK 1,2 is known to be able to phosphorylate synapsin on site 4 and 5, representing a possible mechanism for the regulation of glutamate release through greater availability of glutamate containing vesicles. Synapsin site 4/5 phosphorylation was examined to see if this could be a possible alternative mechanism for PDBu-enhanced release at cerebrocortical nerve terminals. The experiment in Figure 5.10 shows that PDBu significantly enhanced synapsin site 4/5 phosphorylation levels (mean ± s.e.m. compared to 100% 37°C control values: 161 ± 6%), supporting the possibility that synapsin phosphorylation may, in part, contribute to the PDBu-mediated enhancement of glutamate release.

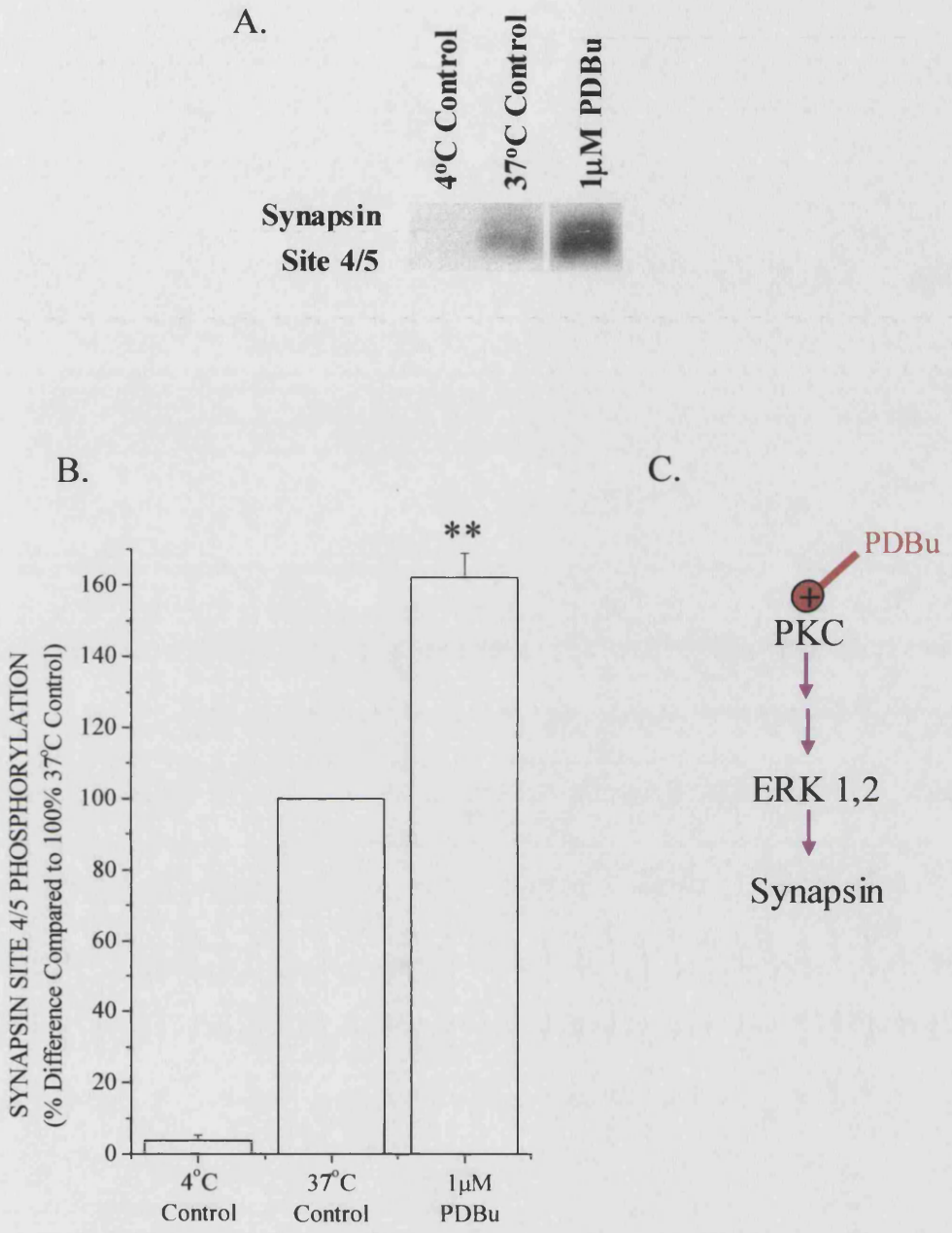


Figure 5.10 PDBu Stimulates Synapsin Site 4/5 Phosphorylation.
Synaptosomes were incubated for 10 mins with 1mM $CaCl_2$ with the final minute being either in the presence (+PDBu) or absence (control) of PDBu. 7.5% polyacrylamide gels were used. Immunoblots were labelled with site 4/5 phospho-synapsin primary antibody (1:500, J.J.), with ^{125}I-Protein A used as the reporter. ^{125}I-levels were detected with phosphorimager spectroscopy. Data was obtained from 5 independent experiments and normalised to their individual 100% 37°C control values, **P<0.05 (Student's paired t-test, n=5). **A.** Phosphorimage of PDBu effect. **B.** Quantification of PDBu effect. **C.** Proposed pathway for PDBu effect.

5.4. Summary of Results

- 1μM PDBu can significantly enhance glutamate release from cerebrocortical nerve terminals independently of calcium channel regulation.

- A 30 minute total incubation with 20 minutes of calphostin C results in a paradoxical increase in levels of ERK 1,2 phosphorylation in cerebrocortical nerve terminals.

- A 10 minute incubation with the PKC inhibitor, calphostin C, significantly reduces basal ERK 1,2 phosphorylation and prevents PDBu-induced increases in ERK 1,2 phosphorylation.

- A 10 minute incubation with the PKC inhibitor, Ro-32-0432, significantly increases basal levels of ERK 1,2 phosphorylation but the effects on PDBu enhanced ERK 1,2 phosphorylation levels remain unclear.

- Different PKC inhibitors, Ro-32-0432 and calphostin C, have opposing effects on ERK 1,2 phosphorylation levels following 10 minutes of incubation.

- A 10 minute total incubation time with 1 minute of PDBu produces an enhancement of ERK 1,2 phosphorylation, as well as increased site 4/5 synapsin phosphorylation; this representing a mechanism for a PDBu-mediated increase in glutamate release which is independent of calcium channel regulation. This mechanism seems likely to be PKC-dependent, but remains to be conclusively established through the further use of PKC inhibitors which lack the complicating features of calphostin C and Ro-32-0432, possibly contingent to their targeting of conserved C1 and ATP binding domains.

5.5 Discussion

The results have clearly shown that the phorbol ester, PDBu, is able to increase ERK 1,2 and synapsin site 4/5 phosphorylation levels in cerebrocortical nerve terminals. Confirmation that the increase in ERK 1,2 and synapsin I phosphorylation is mediating the increase in glutamate release has not been obtained on this occasion due to difficulties with MEK inhibitors. A precedence for this kind of regulation, however, has been observed in cerebrocortical synaptosomes following exposure to BDNF (Jovanovic et al., 2000). This makes it a likely candidate for involvement on this occasion, especially considering that non-specific effects of phorbol ester on synaptic vesicle fusion are unlikely to prove a major mechanism under these conditions (Cousin and Robinson, 2000; Coffey et al., 1993). Final confirmation that PDBu is utilising the ERK/synapsin I pathway to modulate glutamate release could be obtained by using an HPLC instrument. This would allow measurements of the PDBu-enhanced glutamate release in the presence and absence of the MEK inhibitor, PD098059, to be made.

This chapter has, unfortunately, not been able to prove conclusively whether the PDBu enhancement of ERK 1,2 phosphorylation is PKC dependent or not. This is mainly due to the PKC inhibitors being able to regulate basal levels of ERK 1,2 phosphorylation on their own. The longer, 20 minute, incubation with calphostin C resulted in a decrease in ERK 1,2 dephosphorylation. Previous studies have shown that this particular inhibitor can also modulate certain protein phosphatases in other systems. Calphostin C has been shown to inhibit the induction of MAPK phosphatase 1 (MKP1) in a ERK 1,2-independent manner (Stawowy et al., 2003), but this particular phosphatase has only been located in the nucleus of cells. The prominent cytosolic dual specificity phosphatase is believed to be MKP3 but this is only thought to be transcribed upon ERK 1,2 activation (Camps et al., 2000), and no link with calphostin C has yet been identified. The more likely explanation for calphostin C mediated decreases in ERK 1,2 dephosphorylation is through inhibition of a PKC-dependent pathway. Protein phosphatase 2A (PP2A) is a family of protein phosphatases, some of which can undergo rapid activation and dephosphorylate ERK 1,2 without the need for transcription (Alessi et al., 1995). PP2A has also been found in nerve cytosol (Sontag, 2001) and has been implicated in regulating neurotransmitter release (Sistiaga and Sanchez-Prieto, 2000b).

However, further experiments using phosphatase inhibitors, such as okadaic acid, would be needed to confirm any involvement of PP2A in the dephosphorylation of MAPK under these conditions.

The short, 10 minute incubation, with calphostin C elicited a decrease in basal ERK 1,2 phosphorylation levels. Calphostin C has been found to block PDBu binding of a RasGEF known as RasGRP through its C1 binding domain (Lorenzo et al., 2000). It is possible that the observed inhibition of MAPK phosphorylation levels is due to a non-specific binding of C1 domains. However, immunostaining for RasGRP in the striatum and hippocampus has found it solely localised to the soma and dendritic tree (Pierret et al., 2001; Pierret et al., 2002). There has not yet been a study conducted looking at distribution in the cortex but it seems likely that RasGRP is not a synaptosomal protein. This does not rule out the possibility that calphostin C could be acting through this pathway, but it does make it more unlikely.

Previous studies have shown that inhibition of PKC by Ro-32-0432 can be overcome by strong activation, such as with PDBu, which could explain why variable levels of ERK 1,2 phosphorylation are obtained when using this inhibitor. Also of note, is that recent studies have been using this compound as an inhibitor of GRK5 (G-protein receptor kinase), rather than of PKC (Aiyar et al., 2000). This raises the possibility that the observed basal effects of Ro-32-0432 on ERK 1,2 phosphorylation levels could be mediated through the inhibition of GRK5. Further experiments would be needed to elucidate on any role of GRK5 in the regulation of ERK 1,2 signalling, but this nevertheless highlights the lack of PKC specificity encountered with this particular inhibitor.

One way of trying to confirm the role of PKC in the PDBu-enhancement of ERK 1,2 phosphorylation would be to use other inhibitors of PKC. This is not without its own complications as specific inhibitors of PKC that can easily transverse plasma membranes are not readily available. For example, chelerythrine is known to inhibit some PKC isoforms found in nerve terminals but it has also been to shown stimulate some PKC isoforms and to have non-specific effects on the MAPK family of proteins (Lee et al., 1998; Yu et al., 2000). The PKC inhibitor peptide is a very specific inhibitor of PKC but it does not readily transverse plasma membranes and so even in the

myristolated form would require long preincubation times (Eichholtz et al., 1993). This can be effective in cell cultures but, as can be seen from the previous chapters in this thesis, long incubation times with synaptosomes can result in added complications and the running down of signalling pathways.

In conclusion, stimulation of cerebrocortical synaptosomes with PDBu results in increased ionomycin-elicited glutamate release, increased ERK 1,2 phosphorylation, and increased synapsin I phosphorylation at the ERK 1,2 sites. The data suggests that this could be occurring through a PKC-dependent mechanism but further experiments need to be conducted in order to prove this. The diagram below shows a schematic of the possible mechanisms involved if PKC mediates regulation of ERK 1,2 phosphorylation in cerebrocortical nerve terminals.

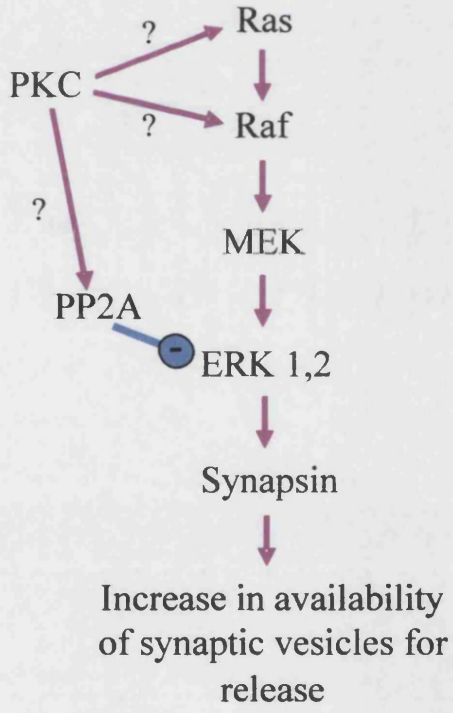


Figure 5.11 A schematic diagram illustrating a possible mechanism for PKC-mediated ERK 1,2 phosphorylation in cerebrocortical nerve terminals.

The non-specific effects of PDBu combined with the non-specific effects of the PKC inhibitors have made it very difficult to prove PKC-dependency of increased ERK 1,2 phosphorylation levels. Another way to address this issue would be to use activators of cell surface receptors that are known to link to PKC-dependent signalling cascades. Not only would these be more specific activators of downstream PKC pathways, but they would also be more likely to highlight endogenous cross-talking between signalling pathways. The following chapter investigates whether stimulation of cell surface receptors can lead to PKC-dependent increases in ERK 1,2 phosphorylation levels in cerebrocortical nerve terminals.

Chapter 6

Signalling Downstream of Group I Metabotropic Glutamate Receptors: Cross-Talk with ERK 1,2.

6.1 Introduction

Group I metabotropic glutamate receptors (mGluRs) have long been established as regulators of neurotransmission at post-synaptic terminals (Review, Alagarsamy et al., 2001). The presence of these receptors at presynaptic terminals, however, has been more controversial. Most localisation studies using antibodies raised against the C-terminal region of mGluR1 and 5 have only found these receptors in post-synaptic locations (Baude et al., 1993; Shigemoto et al., 1993; Petralia et al., 1997; Shigemoto et al., 1997), with a particular emphasis on perisynaptic and extrasynaptic areas. There are only a limited number of studies that have found these receptors in a presynaptic location. For example, Hubert et al., (Hubert et al., 2001) have seen light labelling of striatal axons terminals with an antibody to mGluR1a in the substantia nigra pars reticulata. Despite the limited immunohistochemical evidence for presynaptic Group I mGluRs, there is growing pharmacological evidence suggesting a role for these receptors in regulating neurotransmitter release from nerve terminals.

The first evidence suggesting that mGluRs may play a role in facilitating neurotransmitter release came from a study by Herrero et al in 1992. They used (1S,3R)-ACPD, a non-specific mGluR agonist, to enhance 4-AP elicited glutamate release from rat cerebrocortical synaptosomes. Arachidonic acid (AA) was required for the enhancement of release by (1S,3R)-ACPD, suggesting the presence of more than one pathway acting in concert to facilitate release (Herrero et al., 1992). A number of other groups have also found a requirement for polyunsaturated fatty acids (PUFAs) in the enhancement of release by (1S,3R)-ACPD from rat hippocampal synaptosomes and cortical slices (McGahon and Lynch, 1994; Lombardi et al., 1996). Upon subsequent investigation of the mGluR pathway involved in enhancing release from cerebrocortical nerve terminals, it was found that the regulation of release occurred upstream of Ca^{2+} channels or Ca^{2+}-secretion coupling. (1S,3R)-ACPD stimulated increases in DAG production were observed and the AA pathway was found to act downstream of DAG production in the synergistic activation of PKC (Coffey et al., 1994a: Herrero et al., 1994; Vazquez et al., 1994). This data in itself suggests the activation of PLC-coupled Group I mGluRs but the subsequent availability of more specific Group I mGluR

agonists, such as (3,5)-dihydroxyphenylglycine (DHPG), allowed confirmation of this (Herrero et al., 1998).

More recent studies have also found a role for Group I mGluRs in the regulation of neurotransmitter release that has not been limited to the cerebrocortex, nor to glutamatergic neurones. mGluR1 and 5 have been found to work in conjunction with each other to enhance KCl-elicited acetylcholine release from striatal synaptosomes (Marti et al., 2001). In the periaqueductal grey stimulation of mGluR5 is believed to be responsible for the increase in glutamate and GABA in the dialysates of freely moving rats (de Novellis et al., 2003). Electrically stimulated efflux of [^{3}H]-D-aspartate from rat forebrain slices was also found to be enhanced by stimulation of mGluR5 (Thomas et al., 2000). Regulatory Group I mGluRs have subsequently been found in the rat spinal cord where DHPG increased glutamate release and spontaneous nociceptive behaviour, both of which were blocked by MPEP, a mGluR5 antagonist (Lorrain et al., 2002).

Most of the work looking at pathways downstream of receptor activation have linked Group I mGluRs to PLC via Gq or Gi/o proteins. This leads to PIP_2 hydrolysis and production of IP_3 and DAG. DAG, in concert with an increase in intracellular calcium concentration, following voltage-dependent Ca^{2+} influx or IP_3-stimulated release from intracellular stores, activates conventional isoforms of PKC (Kishimoto et al., 1980;Sugiyama et al., 1987;Murphy and Miller, 1988; Manzoni et al., 1990; Pin et al., 1992). PKC can in turn phosphorylate mGluR1 and 5 in a negative feedback loop (Gereau and Heinemann, 1998; Francesconi and Duvoisin, 2000). Studies on nociception have also linked Group I mGluRs to ERK 1,2 signalling pathways (Karim et al., 2001).

Recent research into nociception in mice has identified an ERK 1,2-dependent pathway in dorsal horn neurones that can lead to enhanced pain sensitivity. Stimulation of mGluR1 and 5 by DHPG resulted in an increased phosphorylation of ERK 1,2, which could be partially inhibited by the application of CPCCOEt or MPEP. Notably, application of both antagonists together failed to have an additive effect on the reduction of ERK 1,2 phosphorylation, suggesting an overlap of roles for these two receptors when functioning postsynaptically (Karim et al., 2001). More postsynaptic

effects of DHPG-regulated ERK 1,2 phosphorylation have been found in the striatum. Acute administration of amphetamine is known to lead to increases in glutamate release in the striatum (McGinty, 1999). The glutamate goes on to activate Group I mGluRs which, in turn, lead to stimulation of ERK 1,2 phosphorylation and activation of the transcription factors Elk-1 and CREB (Choe and Wang, 2001a; Choe et al., 2002). Signalling downstream of Group I mGluR activation and upstream of ERK 1,2 phosphorylation was later found to be dependent on PKC stimulation. Interestingly, the pathway between PKC and increased ERK 1,2 phosphorylation was also shown to be sensitive to NMDA receptor inhibition and CaMKII phosphorylation (Choe and Wang, 2001b, Choe and Wang, 2002). This evidence suggests a role for group I mGluR signalling through ERK 1,2 in neuronal plasticity. A recent study examining long term potentiation in the dentate gyrus of adult rats has linked the requirement of AA in the (1S,3R)-ACPD enhancement of glutamate release to the ERK 1,2 pathway. It was found that AA and (1S,3R)-ACPD stimulated ERK 1,2 phosphorylation and that the MEK inhibitor, PD98059, inhibited the interaction between AA and (1S,3R)-ACPD on glutamate release (McGahon et al., 1999). Cultured rat cortical glia have also been found to possess a pathway whereby activation of mGluR5 by DHPG leads to the phosphorylation of ERK 1,2 through a PKC-independent mechanism (Peavy and Conn, 1998).

In the last chapter it was demonstrated that PDBu could act via PKC to stimulate ERK 1,2 and synapsin phosphorylation and increase glutamate release from cerebrocortical nerve terminals. Given the recent evidence suggesting that Group I mGluRs can regulate ERK 1,2 signalling cascades, this chapter investigates whether Group I mGluRs are the start of a receptor-linked signalling cascade which can utilise PKC to regulate downstream ERK 1,2 and synapsin phosphorylation. It also examines the relevance of this pathway as a possible common mechanism for regulation of glutamate release from cerebrocortical nerve terminals, linking neurotrophin and GPCR-mediated signalling pathways.

6.2 Methods

Synaptosomes were prepared as stated in section 2.1 (Sihra, 1997).

6.2.1 Standard Incubation for ERK 1,2 Phosphorylation/Activation

Synaptosomes were kept at 4°C and resuspended using HBM with BSA, to a final synaptosomal concentration of 1mg/ml. A final concentration of 1mM $CaCl_2$ was added immediately prior to splitting the resuspension into individual sample tubes. All incubations were at 37°C for 10 minutes, with drugs requiring 10 minute time points being added at 4°C, immediately before transferring the sample to the 37°C water bath. All other time points refer to the length of time, prior to the termination of the reaction, for which the drug was added to the sample. For the concentration-response experiments final concentrations of 100nM, 1μM, 10μM, 100μM and 1mM DHPG were used, added either 1 minute, 10 minutes, or 5 minutes 30 seconds prior to the termination of the reactions. The Ro-32-0432, MPEP and synergism experiments also used this protocol where 1μM Ro-32-0432 was added for 10 minutes, 10μM MPEP added for 2 minutes and 50, 100 or 200ng/ml BDNF added for 6 minutes. One sample tube was always kept at 4°C for the duration of the experiment to act as a 4°C control. Reactions were terminated using 5x SDS-PAGE STOP buffer. This incubation protocol for measuring ERK 1,2 phosphorylation has previously been described by Jovanovic et al., 2000 (Jovanovic et al., 2000).

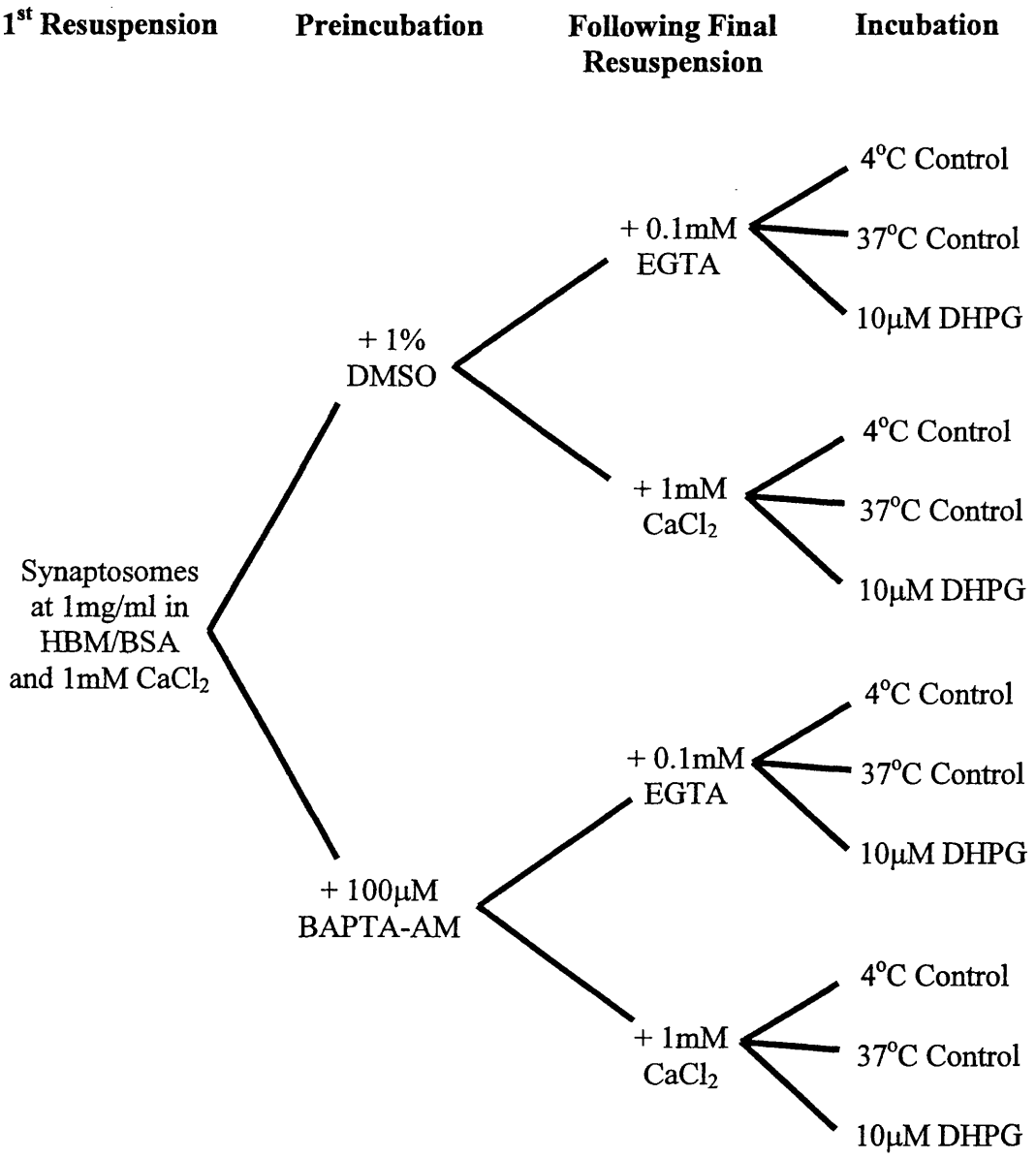
6.2.2 Preincubation

The same preincubation protocol was used for calphostin C as described in section 5.2.3, with DHPG (10μM) being added 1 minute prior to the final termination of the reactions with 5x SDS-PAGE STOP buffer.

Experiments using BAPTA-AM utilised the same preincubation protocol as for calphostin C except that a final concentration of 100μM BAPTA-AM was added instead of calphostin C. Following preincubation, the synaptosomes were spun using a 30 second pulse of centrifugation at 5,000g and the supernatants removed and discarded. The pellets were then resuspended using ice-cold HBM/BSA and recentrifuged as before. This process was repeated once more to remove any remaining external

BAPTA-AM from the medium. Following washing, the synaptosome pellets were left to cool at 4°C for a further 5 minutes before being resuspended in HBM/BSA to a concentration of 1mg/ml for a final time. The synaptosomes were then divided up as shown in Scheme 6.2.

Scheme 6.2: Sample division, preincubation and incubation protocol.



The concentrations listed are all final concentrations.

Following addition of calcium or EGTA, the synaptosomes were split into their incubation tubes and the 37°C control and DHPG put to incubate at 37°C for 10 minutes. DHPG was added 1 minute prior to the termination of the reaction by 5x SDS-PAGE STOP buffer. Control samples (4°C) were terminated without any incubation. Samples were processed for MAPK and synapsin phosphorylation levels as described in the main methods in Chapter 2.4.4 (Dai et al., 2001; Jovanovic et al., 1996).

6.2.3 Glutamate Release

The standard glutamate release protocol was followed, as described in section 2.2 (Nicholls and Sihra, 1986); (Perkinton and Sihra, 1999). DHPG (1μM) was added 1 minute prior to the addition of the secretogogue, 4-AP (1mM), and PDBu (1μM) was added 30 seconds prior to the secretogogue (Coffey et al., 1993; Herrero et al., 1998).

6.2.4 Reagents

DHPG: Group I metabotropic glutamate receptor agonist ((R,S)-3,5-Dihydroxyphenylglycine) (Ito et al., 1992)
Dissolved using water immediately prior to experimentation, to give 100x solutions of 10mM, 1mM, 100μM, 10μM, 1μM, 100nM or 10mM.

Calphostin C: PKC inhibitor acting at the regulatory site, from Cladosporium cladosporioides (UCN-1028c) (Kobayashi et al., 1989).
Maintained in a 1mM 100% DMSO stock solution in the dark. It was diluted, with water, to a concentration of 50μM immediately before use.

Ro-32-0432 hydrochloride: PKC inhibitor which binds to the catalytic site in the C3 domain (Bisindoylmaleimide XI; 2-(8-[(Dimethylamino)methyl]-6,7,8,9-tetrehydropyrido[1,2-a]indol-3-yl)-3-(1-methylindol-3-yl)maleimide) (Wilkinson et al., 1993).
Dissolved to a stock solution of 1mM in 100% DMSO. Immediately prior to use, the stock concentration was diluted to 100μM using water.

MPEP: mGluR5 non-competitive antagonist (6-Methyl-2-(phenylethynyl)pyridine) (Gasparini et al., 1999)

Dissolved in water to a concentration of 5mM. When final concentrations of 10μM were used, the stock was further diluted with water to give a concentration of 1mM MPEP.

BAPTA-AM: Cell permeable rapid Ca^{2+} chelator (1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester)) (Tsien, 1980; Lew et al., 1982))
Maintained in a 32.69mM 100% DMSO stock solution and was diluted to 10mM with DMSO, immediately prior to use.

All the above reagents were purchased from the Sigma-Aldrich Company, Gillingham, Dorset, with the exception of BAPTA-AM, which was obtained from Calbiochem, Beeston, Nottingham.

Unless stated, all drugs were added using 100x stock solutions to minimise volume changes and final concentrations are quoted in the text forthwith.

6.3 Results

The first experiment conducted in this chapter investigated whether stimulation of Group I mGluRs could result in an increase in levels of ERK 1 and ERK 2 phosphorylation in cerebrocortical nerve terminals. An agonist selective for Group I mGluRs, DHPG, was added to the synaptosomes for 1 minute at varying concentrations (Ito et al., 1992). Group I mGluRs are known to be desensitised through phosphorylation by PKC in a negative feedback loop (Gereau and Heinemann , 1998; Francesconi and Duvoisin, 2000). For this reason, a 1 minute time point was used in an attempt to observe any effects on ERK 1,2 phosphorylation before any desensitisation of the receptors (Herrero et al., 1998). Varying concentrations of DHPG were used to investigate the dose dependency of the effect. Figure 6.1 shows that stimulation of Group I mGluRs can result in increases in phosphorylation of ERK 1 and ERK 2 in cerebrocortical nerve terminals (mean ± s.e.m. % change from 37°C Control: 100nM = 190 ± 20%, 1μM = 142 ± 11%, 10μM = 129 ± 11%). The graph also shows that DHPG does not have a differential effect on the two ERKs (mean ± s.e.m. values for 1μM DHPG: p42 = 141 ± 16% and p44 =154 ± 19%). As a result of this, all subsequent data has been represented as combined (1 + 2) ERK values unless differences were identified.

The next experiment investigated the concentration response effects of DHPG on ERK 1,2 phosphorylation over longer incubation times. The longer incubation times were used to examine the possible desensitisation properties of the receptor/s and to elucidate on the appropriateness of different incubation protocols for subsequent studies. Figure 6.2A and B shows that DHPG can increase ERK 1,2 phosphorylation levels when added 5 minutes and 30 seconds prior to the termination of the incubation with a total length of 10 minutes. These data also show that the magnitude of ERK 1,2 phosphorylation does not decrease with increasing DHPG concentration as might be expected with receptor desensitisation. Instead, the ERK 1,2 response increases from 123 ± 11% to 180 ± 33% upon addition of 0.1 and 1nM DHPG respectively. With 1nM DHPG, the response appears to have reached a maximum and continues at this level to the highest concentration tried of 100μM (mean ± s.e.m. in %: 10nM = 158 ± 11, 100nM = 149 ± 12, 1μM = 157 ± 13, 10μM = 159 ± 17, 100μM = 166 ± 25).

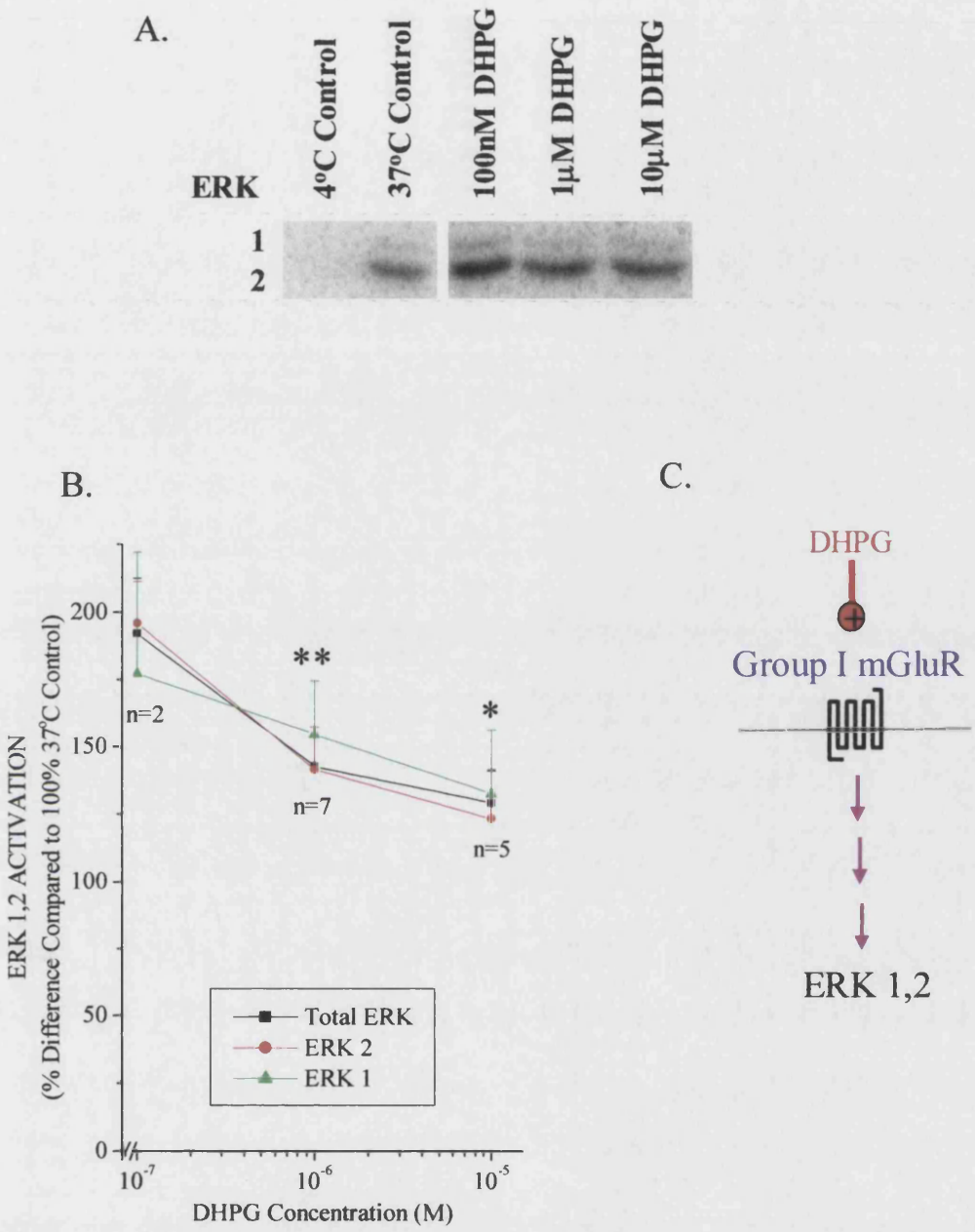


Figure 6.1 DHPG Stimulation of ERK 1 AND ERK 2 Activation.
Synaptosomes were incubated for 10 mins in the presence of 1mM $CaCl_2$ as indicated in section 6.2.1, with the final minute being in the absence (control) or presence (+DHPG) of 100nM, 1µM, or 10µM DHPG. 7.5% polyacrylamide gels were used. Immunoblots were probed using a 1:1000 dilution of phospho-MAPK antibody (NEB) with ^{125}I-Protein A used as the reporter. ^{125}I-labelling was assessed by Phosphorimager spectroscopy (Molecular Dynamics). Mean ± s.e.m. ERK 1,2 phosphorylation levels obtained from separate experiments were individually normalised to 100% 37°C control levels. ** P<0.01, *P<0.05 (Students paired t-test). **A.** Phosphorimage of dose response. **B.** Quantification of ERK 1,2 phosphorylation. **C.** Proposed pathway leading to ERK 1,2 phosphorylation/activation.

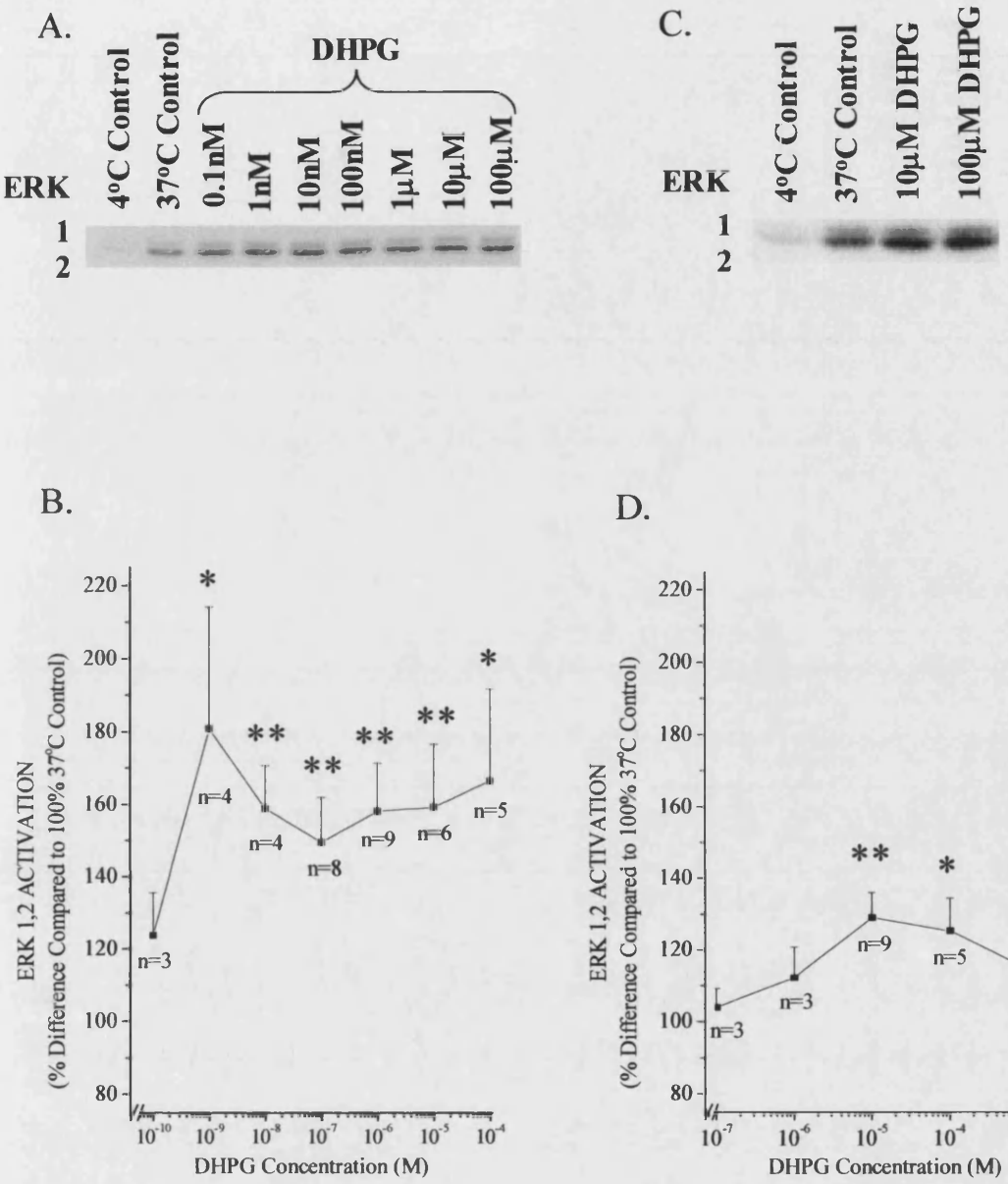


Figure 6.2 Concentration-Responses of DHPG Stimulation of ERK 1,2 Activation. Synaptosomes were incubated for 10 min with 1mM $CaCl_2$ in the presence (+DHPG) or absence (control) of varying concentrations of DHPG. DHPG was added for either 5min 30sec (**A and B**) or 10 min (**C and D**) prior to the end of the incubation, as described in section 6.2.1. 7.5% polyacrylamide gels were run. Samples were immunoblotted using phospho-MAPK primary antibody (NEB) and ^{125}I-Protein A as the reporter. Phosphoimages were analysed using spectroscopy (Molecular Dynamics) with mean ± s.e.m. obtained from several separate experiments each normalised to their own 100% 37°C control values. ** P<0.01, *P<0.05 (Students paired t-test). **A. and C.** Phosphorimages of concentration-responses. **B. and D.** Quantification of ERK 1,2 phosphorylation.

Figure 6.2C and D examines the effects of DHPG when added to synaptosomes still in their un-incubated state. The cerebrocortical nerve terminals were kept at 4°C until required, when DHPG was added immediately before commencing the 37°C incubation. The reactions were terminated following 10 minutes of incubation. Under these conditions, maximal increases in ERK 1,2 phosphorylation were observed when using 10μM DHPG (mean ± s.e.m.: 100nM = 103 ± 5%, 1μM = 112 ± 8%, 10μM = 129 ± 6%, 100μM = 125 ± 9%). The response also appeared to decrease at higher concentrations of DHPG, suggesting a possible desensitisation of the pathway (mean ± s.e.m.: 1mM DHPG = 114 ± 6% compared to control levels of 100%).

The dose response data obtained for 100nM, 1μM and 10μM DHPG has also been represented as time courses, as shown in Figure 6.3A. The results show that the increases in ERK 1,2 phosphorylation levels following 10 minutes incubation with DHPG were significantly smaller than those obtained following 5 mins 30 sec incubation with DHPG. This suggests that the levels of ERK 1,2 phosphorylation obtained when synaptosomes are treated with DHPG are sensitive to lengthening incubation times in the presence of the drug. It is also possible that the timing of addition of DHPG (before or after commencing 37°C incubation) may be a factor in determining the levels of ERK 1,2 phosphorylation obtained. Having carried out all these concentration-response and time course experiments, it was now possible to identify the appropriate concentrations of DHPG required to elicit a reliable increase in ERK 1,2 phosphorylation under different conditions.

The results so far show that DHPG is able to increase ERK 1,2 phosphorylation in cerebrocortical nerve terminals. The next stage of this investigation looks to examine the pathway upstream of ERK 1,2 phosphorylation. Group I mGluRs are known to activate PKC and PKC is known, under some circumstances, to stimulate the ERK 1,2 signalling cascade (Roberson et al., 1999). PKC is also known to desensitise Group I mGluRs in a negative feedback loop (Gereau and Heinemann , 1998; Francesconi and Duvoisin, 2000), and so inhibition of PKC could result in increased activity downstream of the receptor. However, if the increase in ERK 1,2 phosphorylation is downstream of PKC activation then the increase should be blocked by a PKC inhibitor, whether the receptor is desensitised or not. The next series of experiments examines whether

A.

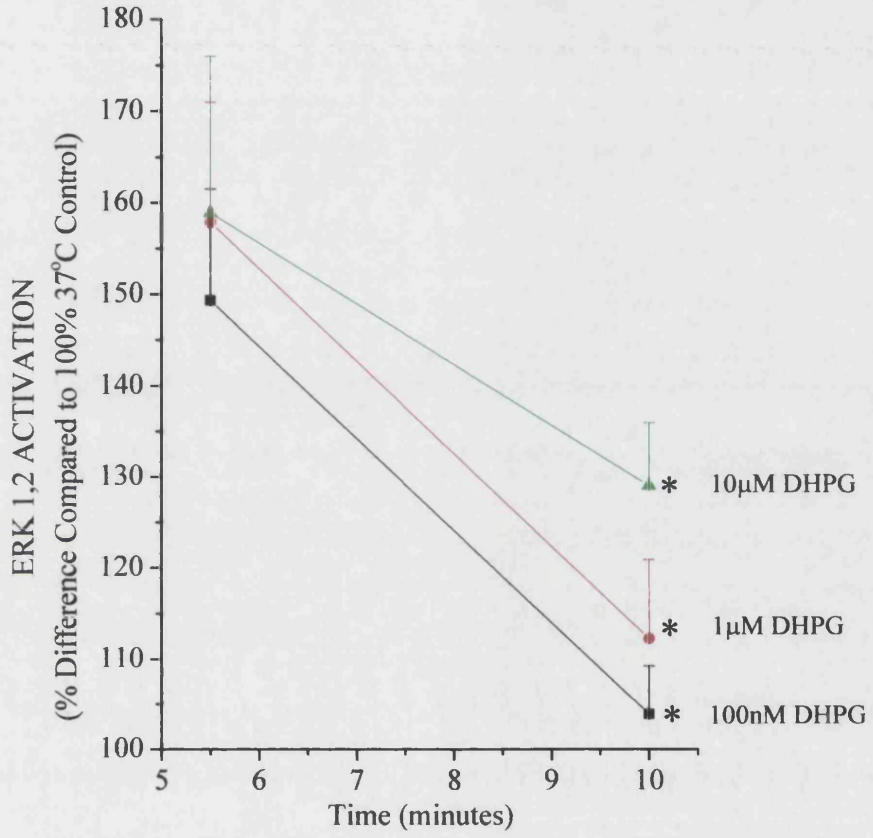


Figure 6.3 DHPG Stimulation of ERK 1,2 Activation Using Different Protocols. Synaptosomes were incubated at 37°C with 1mM $CaCl_2$. Immunoblots were labelled using phospho-MAPK antibody (NEB) and reported with ^{125}I-labelled protein A. Signals were quantified using phosphorimager spectroscopy and mean ± s.e.m. values were taken from several independent experiments and normalised to individual 100% 37°C controls. *P<0.05 (Students t-test) **A.** Quantification of ERK 1,2 phosphorylation levels where the total incubation time was 10 mins and the time stated is the time in the presence of DHPG (see section 6.2.1). Statistical analysis was of ERK 1,2 phosphorylation levels after 5 min 30 secs, compared to ERK 1,2 phosphorylation levels after 10 minutes, all in the presence of DHPG.

Group I mGluRs are acting through PKC to stimulate ERK 1,2 phosphorylation in cerebrocortical nerve terminals.

Figure 6.4 shows the effects of calphostin C, a PKC inhibitor, on the DHPG enhancement of ERK 1,2 phosphorylation levels (Kobayashi et al., 1989). A preincubation protocol was used (described in section 6.2.3), whereby synaptosomes were incubated with calphostin C for 20 minutes before being cooled back to 4°C. The synaptosomes were then reincubated at 37°C for 10 minutes, with DHPG being added 1 minute prior to termination of the reaction. This protocol was used because other studies using synaptosomes have utilised longer protocols to allow calphostin C to cross plasma membranes and enter the cell in sufficient quantity to inhibit PKC (Matveeva et al., 2001). The earlier data in this chapter also shows that the observed effect of DHPG on ERK 1,2 phosphorylation levels may desensitise in incubations lasting longer than 10 minutes, hence the cooling of synaptosomes back to 4°C between the preincubation and incubation steps was designed to limit this effect. The results obtained for the effects of calphostin C on ERK 1,2 phosphorylation levels using this protocol have already been shown in the previous chapter (Figure 5.4), where it was found that calphostin C increased ERK 1,2 phosphorylation even at 4°C levels. In order to account for the effects of calphostin C on basal phosphorylation levels, the samples containing DHPG were each normalised to their individual 37°C controls. Thus, the samples containing DHPG were normalised to 100% 37°C control values and the samples containing DHPG and calphostin C were normalised to 100% 37°C calphostin C values. DHPG was found to significantly increase ERK 1,2 phosphorylation levels in both the control sample and the sample containing calphostin C (mean ± s.e.m: control DHPG = 185 ± 24%, calphostin C DHPG = 172 ± 15%, $P<0.05$, ANOVA with Duncan's *post hoc* analysis). No significant difference was, however, found between the DHPG enhancement of ERK 1,2 phosphorylation levels in calphostin C and control samples.

These results suggest that, under the conditions used, the pathway between Group I mGluRs and ERK 1,2 is not dependent on the activation of PKC in as much as there is an apparent lack of sensitivity to calphostin C. However, the effect of calphostin C on basal ERK 1,2 phosphorylation levels could be confounding the observations in the presence of DHPG. It is also possible that the properties of the Group I mGluRs could

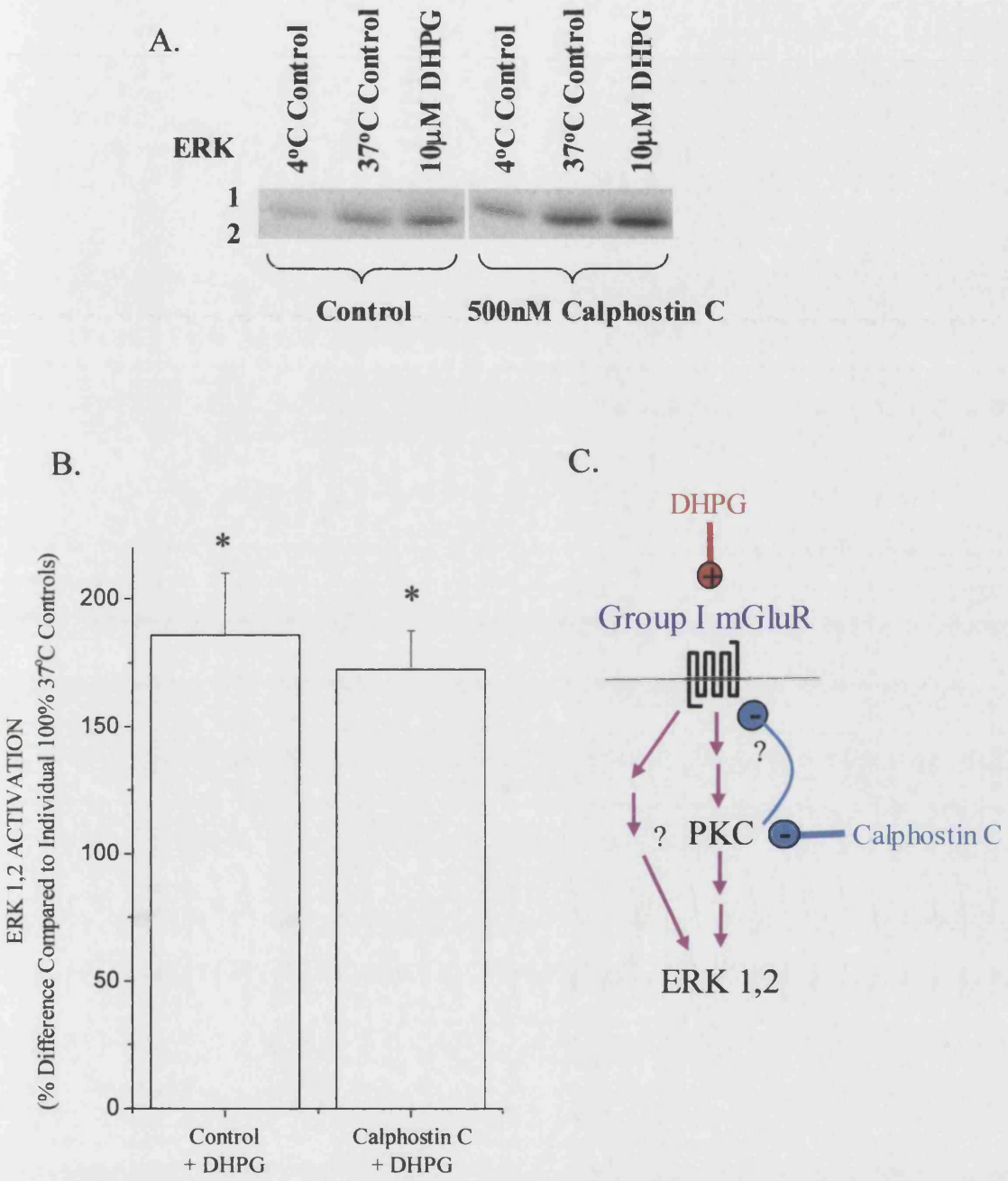


Figure 6.4 No Effect of Prolonged Calphostin C Exposure on DHPG-Stimulated ERK 1,2 Activation. Synaptosomes were preincubated as described in section 6.2.2 followed by a 10 min incubation with 1 min in the absence (control) or presence (+DHPG) of 10µM DHPG. 7.5% polyacrylamide gels were run. ERK 1,2 phosphorylation levels were detected using phospho-MAPK primary antibody (1:1000, NEB), reported with ^{125}I-Protein A, and measured using phosphorimager spectroscopy (Molecular Dynamics). Mean ± s.e.m. from 4 independent experiments, individually normalised to their own relevant 37°C control. *P<0.05 (ANOVA with Duncan's *post hoc* analysis, n=4). **A.** Phosphorimage of combined effects of calphostin C and DHPG. **B.** Quantification of combined effects. **C.** Proposed pathways involved in mediating these effects.

be changing with the long preincubation protocol necessary to administer the calphostin C. Considering these factors, the next experiment conducted looked at the effects of the PKC inhibitor, Ro-32-0432, on DHPG-enhancement of ERK 1,2 phosphorylation levels following a short incubation protocol (Kobayashi et al., 1989).

In the next series of experiments, the usual short incubation protocol was followed, whereby synaptosomes were incubated for 10 minutes in the presence of 1μM Ro-32-0432. DHPG (10μM) was used as this was the concentration shown to work best under the 10 minute incubation protocol. Results are shown in Figure 6.5 and, as expected, DHPG was found to significantly increase ERK 1,2 phosphorylation levels (mean ± s.e.m.: 146 ± 5% compared to 100% control, $P<0.05$, ANOVA followed by Duncan's *post hoc* analysis). In agreement with the results obtained in Chapter 5 (Figure 5.8), Ro-32-0432 was also found to significantly increase ERK 1,2 phosphorylation levels (mean ± s.e.m.: 154 ± 16% compared to 100% control, $P<0.05$, ANOVA followed by Duncan's *post hoc* analysis). When Ro-32-0432 and DHPG were added together, however, significant increases in ERK 1,2 phosphorylation levels were no longer observed (mean ± s.e.m.: 116 ± 18% compared to 100% control, $P>0.05$, ANOVA followed by Duncan's *post hoc* analysis). Although this in itself could suggest an inhibition of DHPG enhanced ERK 1,2 phosphorylation by Ro-32-0432, there was no statistically significant difference between the DHPG stimulated samples and the DHPG/Ro-32-0432 samples, possibly due to the low number of replicates (n=3). Notwithstanding, in every experiment performed (3/3), the levels of ERK 1,2 phosphorylation were found to be lower with DHPG/Ro-32-0432 compared to DHPG on its own.

The stimulatory effect of Ro-32-0432 on basal levels of ERK 1,2 phosphorylation appears to be peculiar to the ERK 1,2 pathway, as previous studies have demonstrated a clear inhibitory effect of the compound on PKC-dependent phosphorylation (Wilkinson et al., 1993). It remains to be seen, however, whether the stimulatory effect could be attributed to inhibition of phosphatase activation through inhibition of PKC or, to some other, non-specific effect due to interactions of Ro-32-0432 with enzymatic ATP binding sites, towards which the drug was originally targeted (Aiyar et al., 2000). Notwithstanding, this does invoke the possibility that the apparent inhibition of DHPG

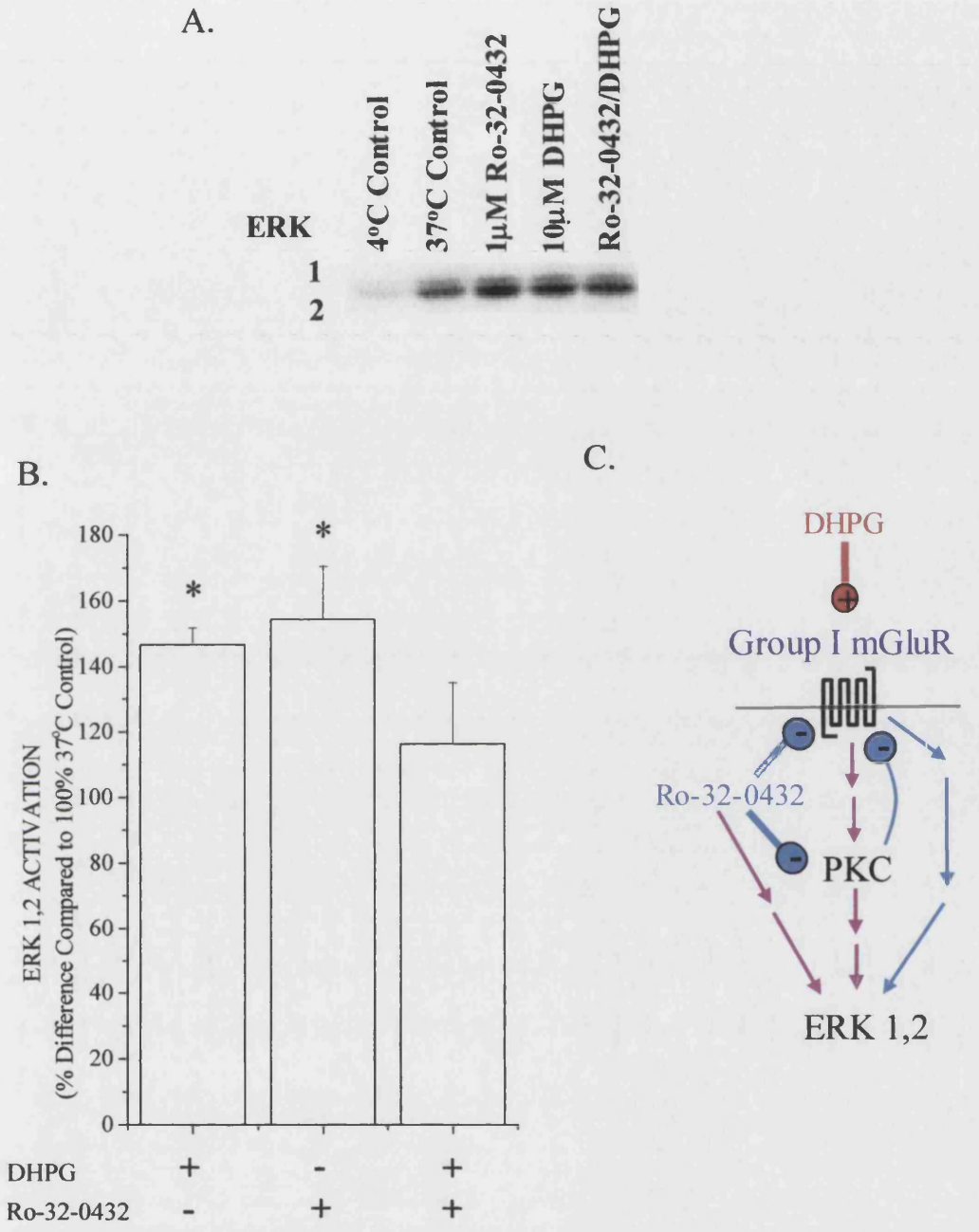


Figure 6.5 Ro-32-0432 Attenuates DHPG-Enhanced ERK 1,2 Activation. Synaptosomes were incubated for 10 mins with 1mM $CaCl_2$ either in the presence (+Ro-32-0432) or absence (control) of Ro-32-0432. DHPG was added to those samples labelled +DHPG, 1 min prior to the termination of the experiment. 7.5% polyacrylamide gels were used. Immunoblots were labelled with phospho-MAPK primary antibody (1:1000, NEB) and reported using ^{125}I-Protein A. ^{125}I-labelling was quantified using phosphorimager spectroscopy (Molecular Dynamics) with individual values normalised to 100% 37°C control samples. *P<0.05 compared to 37°C control (ANOVA with Duncan's *post hoc* analysis, n=3). **A.** Phosphorimage of DHPG and Ro-32-0432 effects. **B.** Quantification of DHPG and Ro-32-0432 effects. **C.** Possible pathways involved in mediating these effects.

activation of ERK 1,2 by Ro-32-0432 could also be due to a non-specific interaction of the drug rather than inhibition of PKC. The role of PKC in controlling MAPK phosphorylation levels seems to be complicated and remains unresolved with the PKC inhibitors used here. Given, however, that DHPG clearly affects ERK 1,2 phosphorylation, the next set of experiments looks at signalling upstream of PKC, at the level of the Group I mGluRs.

There are two receptors that constitute the Group I mGluRs, mGluR1 and mGluR5. The following experiments aimed to investigate which of the Group I mGluRs could be mediating the effect of DHPG on MAPK phosphorylation. In order to do this an mGluR5 specific non-competitive antagonist, MPEP, was added to the synaptosomal preparation one minute prior to the addition of DHPG (Gasparini et al., 1999). The results obtained from these experiments are illustrated in Figure 6.6. MPEP (10μM) was found to significantly increase basal levels of ERK 1,2 phosphorylation on its own (mean ± s.e.m. compared to 100% 37°C control samples: 181 ± 36%, $P<0.05$ ANOVA with Duncan's *post hoc* analysis). Addition of MPEP with DHPG (1μM) was found to have no significant effect on basal levels of ERK 1,2 phosphorylation and to significantly decrease the phosphorylation levels observed with MPEP alone (mean ± s.e.m.: 99 ± 5% compared to 100% basal levels). Despite DHPG values always being larger than control values (5/5 experiments) (mean ± s.e.m. for DHPG: 145 ± 15% compared to 100% basal levels), in this particular series of experiments, DHPG was not found to increase basal level of ERK 1,2 phosphorylation to statistical significance. Considering this protocol has yielded significant DHPG-induced increases in ERK 1,2 phosphorylation in previous experiments (see Figure 6.1), additional repetitions would be predicted to resolve the large variability of the current dataset with DHPG.

These results suggest that DHPG-induced enhanced ERK 1,2 phosphorylation is mediated through mGluR1 rather mGluR5. MPEP does appear to block DHPG–enhanced ERK 1,2 phosphorylation, but also has substantial positive effects on its own. This may suggest that the observed inhibition by MPEP is the result of it interacting with downstream pathways rather than as a direct effect of mGluR5 blockade. These results do imply that mGluR1 and mGluR5 occur in the same nerve terminals and that their downstream pathways can both regulate ERK 1,2 phosphorylation levels. Which

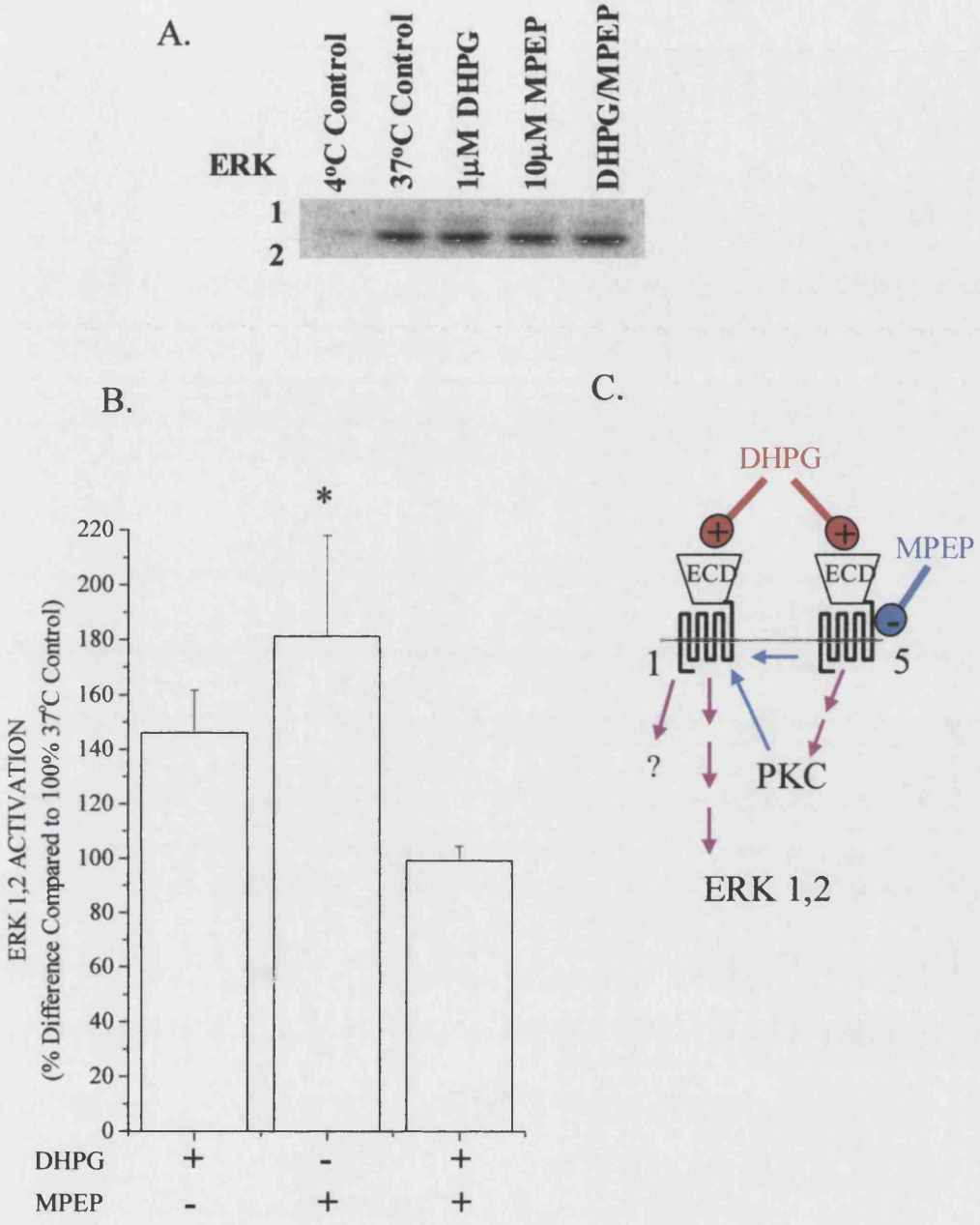


Figure 6.6 The Combined Effects of MPEP and DHPG on ERK 1,2 Activation
Synaptosomes were incubated at 37°C, for 10 mins, in the presence of 1mM $CaCl_2$. 10μM MPEP was added 2 mins prior, and 1μM DHPG 1 min prior to the termination of the reactions (labelled +MPEP, and +DHPG respectively). 7.5% polyacylamide gels were used. Western blots were performed, labelled with phosphospecific MAPK antibody (1:1000, NEB) and detected with (^{125}I)-labelled protein A. Signals from several independent experiments were quantified with phosphorimager spectroscopy and normalised to individual 100% 37°C controls. *P<0.05 compared to 37°C control, ANOVA with Duncan's *post hoc* analysis (n=5). **A.** Phosphorimage of MPEP and DHPG effects. **B.** Quantification of MPEP and DHPG effects. **C.** Possible pathways involved in mediating these effects. ECD = extracellular domain.

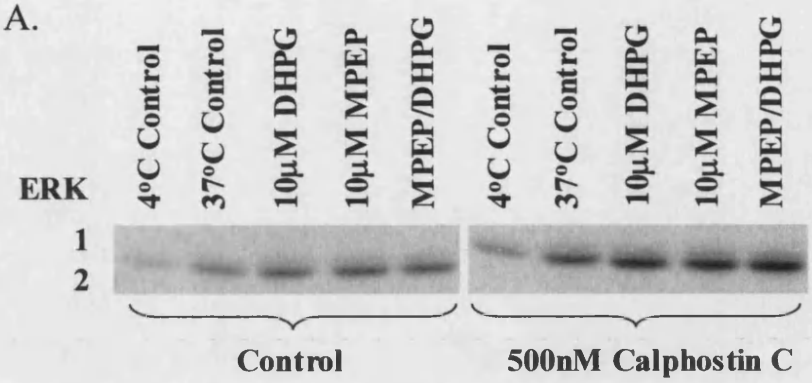
downstream pathways are involved still remains unclear.

It was interesting to observe that both Ro-32-0432, the PKC inhibitor, and MPEP, the mGluR5 inhibitor, gave the same pattern of results with regard to levels of ERK 1,2 phosphorylation in the presence and absence of DHPG. Recent evidence has suggested that the transmembrane domain of Group I mGluRs may be constitutively active (Pagano et al., 2000;Carroll et al., 2001). This could result in tonic inhibition of mGluR1 by mGluR5 through a PKC-dependent mechanism (Poisik et al., 2003). It may be that MPEP is removing a tonic inhibition of mGluR1 by mGluR5, leading to mGluR1-mediated stimulation of the ERK 1,2 signalling cascade. It is also possible that Ro-32-0432 is removing this tonic inhibition of mGluR1 by mGluR5 as well, by inhibiting the signalling pathway acting between mGluR5 and mGluR1. The Group I mGluRs have also been shown to be able to couple to different G proteins depending on their phosphorylation state, resulting in the activation of different signalling cascades (Francesconi and Duvoisin, 2000).

The next set of experiments conducted aimed to elucidate the pathways involved in regulating the crosstalk between mGluR1 and mGluR5 and the ERK 1,2 pathway in nerve terminals. The experiments have used the PKC inhibitor, calphostin C, along with MPEP and DHPG, to determine whether PKC was also involved in this case. The long preincubation protocol was used, as described before for Figure 6.4, with MPEP and DHPG being added 2 mins and 1 min prior to the termination of the reactions, respectively.

The blot shown in Figure 6.7 indicates that the treatment of synaptosomes with DHPG or MPEP still results in an increase in ERK 1,2 phosphorylation levels when the preincubation protocol is used. It is interesting to note that the results also suggest that the MPEP-mediated inhibition of DHPG-induced enhancement of ERK 1,2 phosphorylation is no longer present under these control conditions. This in itself implies that the inhibition seen with the short protocol is time-dependent and that when synaptosomes are preincubated, the signalling between the level of DHPG-simulation of Group I mGluRs and ERK 1,2 phosphorylation changes. It is possible that this change is mediated by a cross-talk between mGluR1 and mGluR5, and that it may no longer be

A.



B. Changes in ERK 1,2 Phosphorylation Levels.

	DHPG	MPEP	DHPG/MPEP
Short Incubation	↑	↑	—
Preincubation	↑	↑	↑
Preincubation + Calphostin C	↑	↑	↑↑

Figure 6.7 Combined Effects of Calphostin C, MPEP and DHPG on ERK 1,2 Activation. Synaptosomes were preincubated with 1mM $CaCl_2$ either in the presence (+ calphostin) or absence (control) of calphostin C as described in section 6.2.2. Samples were then incubated for a further 10 mins, with 1 min in the presence (+DHPG) or absence (-DHPG) of DHPG and/or 2 mins in the presence (+MPEP) or absence (-MPEP) of MPEP. Immunoblots were labelled with phospho-MAPK primary antibody and reported using 125I-labelled protein **A.** Phosphorimage of calphostin C, MPEP and DHPG effects. **B.** Table demonstrating the changes in ERK 1,2 phosphorylation/activation levels seen with different drug treatments and incubation protocols. (↑ = increase; — = no change).

present in preincubated synaptosomes. However, it is also possible that the lack of effect of MPEP on DHPG-induced ERK 1,2 phosphorylation is due to the higher concentration of DHPG used in this instance. Previous studies have found that inhibition of mGluR5 by MPEP can be overcome by treatment with higher concentrations of DHPG (Lorrain et al., 2002).

Figure 6.7 also indicates that the MPEP-induced increase in ERK 1,2 phosphorylation levels may not be PKC-dependent when the preincubation protocol is used. This is because ERK 1,2 phosphorylation levels still appear to be enhanced by MPEP despite treatment with calphostin C. The final point of note is that the ERK 1,2 phosphorylation levels induced by the combined treatment of DHPG and MPEP appear to be additive in the presence of calphostin C, compared to treatment with DHPG or MPEP alone. As this is only one blot, more experiments need to be conducted in order to confirm the results obtained here, but the indications are that the signalling between Group I mGluRs and ERK 1,2 is complex and that the specific aspects of the regulation revealed depends on the incubation protocol utilised.

Group I mGluRs can signal through PLC to evoke the hydrolysis of PI-4,5-P_2 into DAG and IP_3 (Sugiyama et al., 1987). IP_3 is known to stimulate the release of calcium from intracellular stores which, along with DAG, activates conventional forms of PKC (Kishimoto et al., 1980). The previous experiments in this chapter imply that PKC may be regulating ERK 1,2 phosphorylation following DHPG stimulation with the short incubation protocol, but not necessarily with the preincubation protocol. These experiments also suggest that if PKC is involved, its signalling may not necessarily be direct, though it has not been possible to clearly define this role using PKC inhibitors. Notwithstanding, Group I mGluRs could be signalling to ERK 1,2 in a PKC-independent manner, through direct DAG activation of a Ras-GEF (Ebinu et al., 1998; Kawasaki et al., 1998), or by a Ca^{2+}-dependent activation (Walker et al., 2003). To further examine the pathways involved downstream of mGluR stimulation leading up to ERK 1,2 phosphorylation, I examined the Ca^{2+}-dependency of the ERK 1,2 activation. If the enhancement in phosphorylation of ERK 1,2 with DHPG was found to be completely Ca^{2+}-dependent, then this would rule out a direct involvement of DAG signalling through a Ras-GEF, but include a Ca^{2+}-dependent activation of ERK 1,2 involving PKC and/or intracellular Ca^{2+} stores released by IP_3.

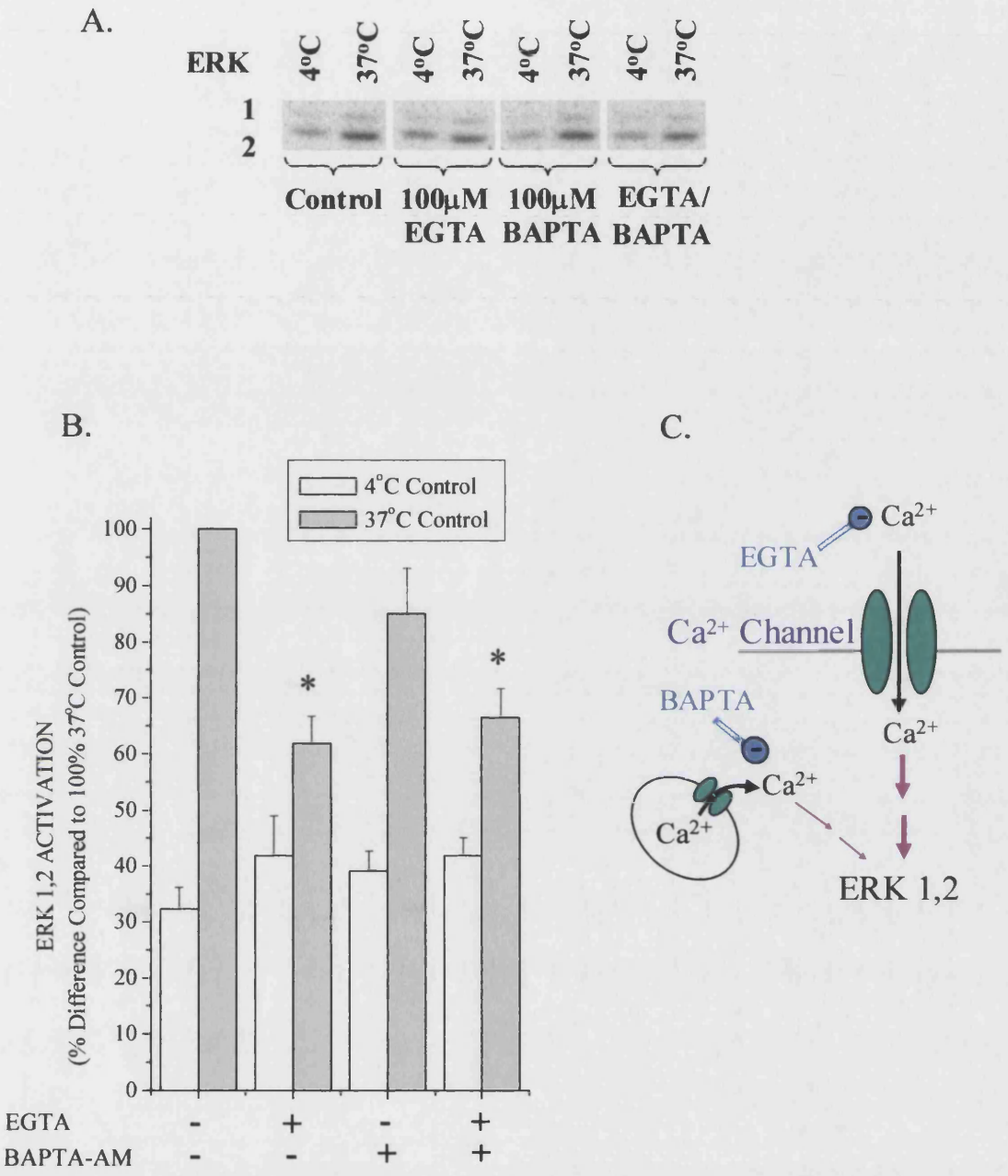


Figure 6.8 Ca^{2+}-Dependent Component to Basal ERK 1,2 Activation levels. Synaptosomes were preincubated with 1mM $CaCl_2$ either in the absence (-BAPTA) or presence (+BAPTA) of BAPTA-AM. The following incubation was then carried out in the presence (+EGTA) or absence (-EGTA) of EGTA, as described in section 6.2.2. 7.5% polyacrylamide gels were run. Immunoblots were labelled with phospho-MAPK primary antibody (1:1000, NEB) and reported using ^{125}I-Protein A. ^{125}I-labelling was quantified using phosphorimager spectroscopy (Molecular Dynamics) with individual values normalised to 100% 37°C control samples. 37°C samples were compared to 37°C control and 4°C samples were compared to 4°C control *P<0.05, ANOVA with Duncan's *post hoc* analysis (n=6). **A.** Phosphorimage of EGTA and BAPTA effects. **B.** Quantification of EGTA and BAPTA effects.**C.** Proposed pathways for these effects

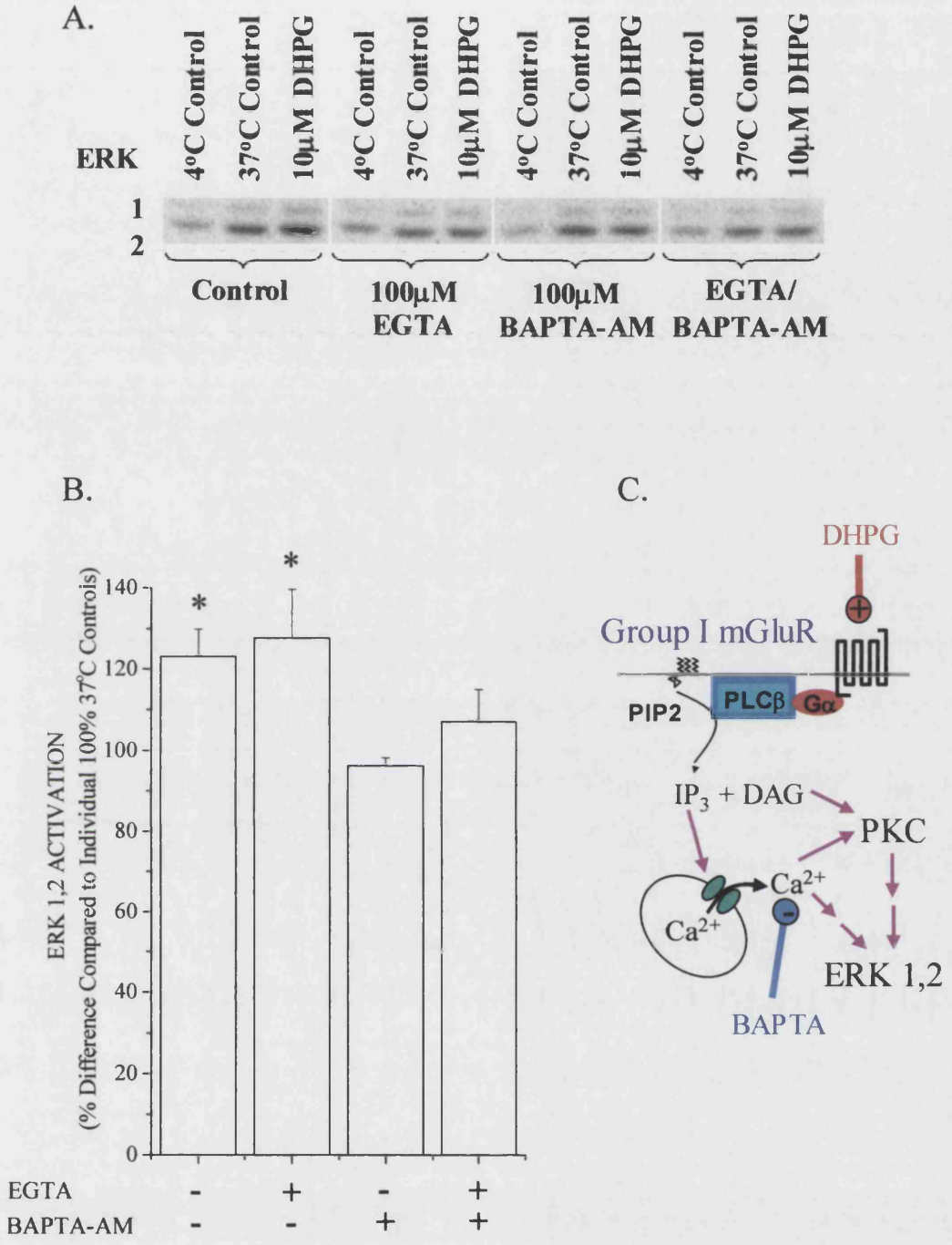


Figure 6.9 BAPTA Attenuates DHPG Stimulation of ERK 1,2 Activation. Synaptosomes were preincubated with 1mM $CaCl_2$ and either BAPTA-AM or DMSO. The following incubation was then carried out either in the presence of 1mM $CaCl_2$ (-EGTA) or EGTA (+EGTA). DHPG was added 1 min prior to the termination of the reactions. 7.5% polyacrylamide gels were used. Immunoblots were labelled with phospho-MAPK primary antibody (1:1000, NEB) and reported using ^{125}I-Protein A. ^{125}I-labelling was quantified using phosphorimager spectroscopy (Molecular Dynamics) with samples normalised to 100% individual 37°C controls. *P<0.05, ANOVA with Duncan's *post hoc* analysis (n=4). **A.** Phosphorimage of EGTA and BAPTA effects on DHPG-enhanced ERK 1,2 phosphorylation. **B.** Quantification of these effects. **C.** Proposed pathways involved.

BAPTA is a rapid chelator of Ca^{2+} which, with the addition of an acetoxymethyl ester group, is able to traverse cell membranes (Tsien, 1980; Lew et al., 1982). Upon entry to the cell, the acetoxymethyl ester group is removed, trapping the BAPTA within the cell, making it a valuable tool for studying the role of intracellular Ca^{2+}. EGTA is a slower chelator of Ca^{2+} which remains outside the cell (Tsien, 1980). The combined use of internal BAPTA (introduced as BAPTA-AM) and external EGTA makes it possible to examine the respective contributions of both intracellular and extracellular Ca^{2+}. Synaptosomes were first preincubated in the presence of 1mM $CaCl_2$ for 10 minutes to allow the filling of intracellular stores before the addition of either BAPTA-AM or DMSO for a further 20 minutes. EGTA was not added until the start of the incubation stage as described in section 6.2.2., with 1mM $CaCl_2$ being added to control samples. DHPG (10μM) was added 1 minute prior to the termination of the final incubation. It should be noted that buffered solutions were used to minimise the changes in pH that can occur following Ca^{2+} chelation by EGTA. No significant differences were found between the basal 4°C ERK 1,2 phosphorylation levels for each of the conditions (Figure 6.8) (mean ± s.e.m. for 6 independent experiments compared to 100% 37°C control: Control = 35 ± 4%, EGTA = 50 ± 10%, BAPTA = 45 ± 6%, EGTA/BAPTA = 45 ± 4%). However, the increase in ERK 1,2 phosphorylation levels observed when the synaptosomes are incubated at 37°C does appear to be partially dependent on extracellular Ca^{2+}, as phosphorylation levels were significantly decreased in the presence of EGTA (mean ± s.e.m. compared to 100% 37°C control: EGTA = 64 ± 4%, EGTA/BAPTA = 68 ±4%). BAPTA on its own did not significantly decrease the levels of ERK 1,2 phosphorylation found when synaptosomes were heated to 37°C (mean ± s.e.m. compared to 100% 37°C control: BAPTA = 89 ± 8%), suggesting that Ca^{2+} from intracellular stores is not responsible for the basal level of ERK 1,2 phosphorylation.

In contrast to the extracellular Ca^{2+}-dependency of basal ERK 1,2 phosphorylation levels, DHPG appears to require intracellular Ca^{2+} in order to increase the phosphorylation levels of ERK 1,2 (Figure 6.9). DHPG was found to significantly increase ERK 1,2 activation in control samples and in the presence of EGTA alone (mean ± s.e.m. of DHPG effect in % compared to individual 100% 37°C controls: DHPG = 123 ±6, EGTA = 127 ± 11). However, treatment with DHPG no longer caused

an enhancement of ERK 1,2 phosphorylation levels when BAPTA was also present (mean ± s.e.m. of DHPG effect in % compared to individual 100% 37°C controls: BAPTA = 96 ± 1, EGTA/BAPTA = 107 ± 8). This means that, when a preincubation protocol is followed, Group I mGluRs are likely to be signalling either through a conventional form of PKC, though a Ca^{2+}-dependent Ras-GEF, or another Ca^{2+}-dependent intermediary, to elicit the ERK 1,2 response observed in cerebrocortical nerve terminals.

This chapter, thus far, has only looked at signalling pathways between the Group I mGluRs and ERK 1,2, the next section looks at signalling downstream of ERK 1,2 and how it correlates with glutamate release. The first experiments looked at the phosphorylation of a protein, synapsin I, known to be a direct downstream substrate of ERK 1,2 in presynaptic nerve terminals (Jovanovic et al., 1996). Interestingly, neither 1μM or 10μM DHPG increased synapsin site 4/5 phosphorylation following the short incubation protocol (Figure 6.10) (mean ± sem in % compared to 100% 37°C control: 1μM DHPG = 106 ± 4, 10μM DHPG = 99 ± 2 (n=3)). This would appear to suggest that, if DHPG is able to increase glutamate release under these conditions, Group I mGluRs are not using the ERK/synapsin signalling cascade as part of this release regulation. Consistent with this, Figure 6.11 demonstrates, DHPG (1μM) was unable to enhance 4-AP elicited glutamate release from cerebrocortical synaptosomes (cumulative glutamate release (nmol/mg) following 5 min incubation with secretogogue: 4-AP = 10.1, 4-AP/DHPG = 10.5). In the same experimental instances, in contrast to the lack of effect of DHPG, I was able to demonstrate a strong facilitation of glutamate release with PDBu (Figure 6.11), both agents being able to increase levels of ERK 1,2 phosphorylation in positive control phosphorylation experiments carried out in parallel. The recalcitrance of glutamate modulation to DHPG is in agreement with previous studies conducted on cerebrocortical synaptosomes, where it was found that singular stimulation of Group I mGluRs alone was not able to enhance glutamate release, but rather required endogenous or exogenous arachidonic acid (AA) with DHPG, or the presence of a phosphatase 2B inhibitor (Coffey et al., 1994a; Sistiaga and Sanchez-Prieto, 2000a). In the experimental conditions used in this chapter, because synaptosomes are incubated in HBM containing BSA, the free fatty acid binding

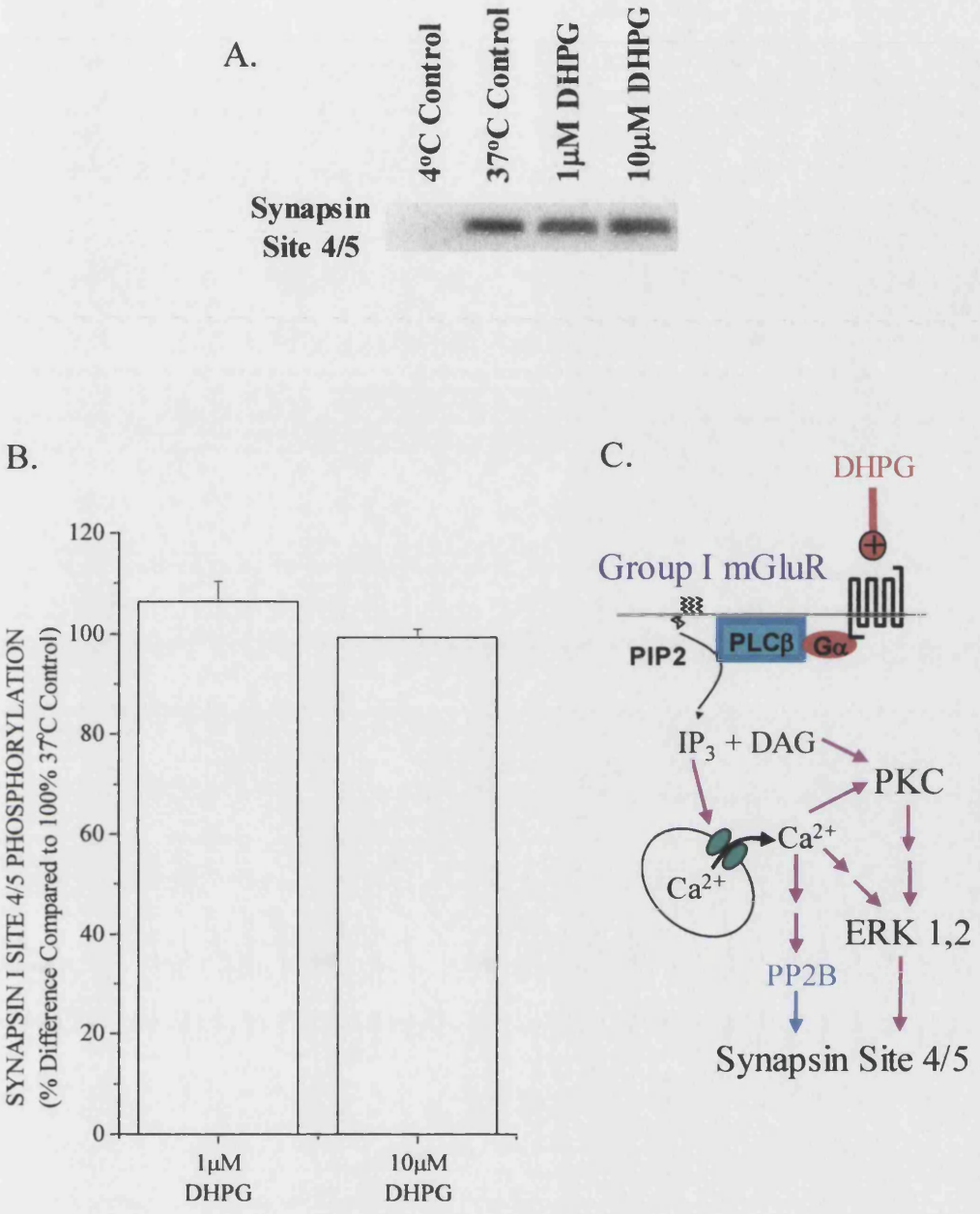


Figure 6.10 DHPG Does Not Enhance Total Levels of Synapsin Site 4/5 Phosphorylation. Synaptosomes were incubated for 10 mins with 1mM $CaCl_2$ either in the presence (+DHPG) or absence (control) of 1μM or 10μM DHPG for the final minute (detailed in section 6.2.1.). 7.5% polyacrylamide gels were run. Immunoblots were labelled with site 4/5 phospho-synapsin primary antibody (1:500, J.J.), with ^{125}I-Protein A used as the reporter. ^{125}I-levels were detected with phosphorimager spectroscopy. Data was obtained from 3 independent experiments and normalised to their individual 100% 37°C control values, no significant differences were found (Student's paired t-test, n=3). **A.** Phosphorimage of DHPG effect. **B.** Quantification of DHPG effect. **C.** Proposed pathway for DHPG effect.

A.

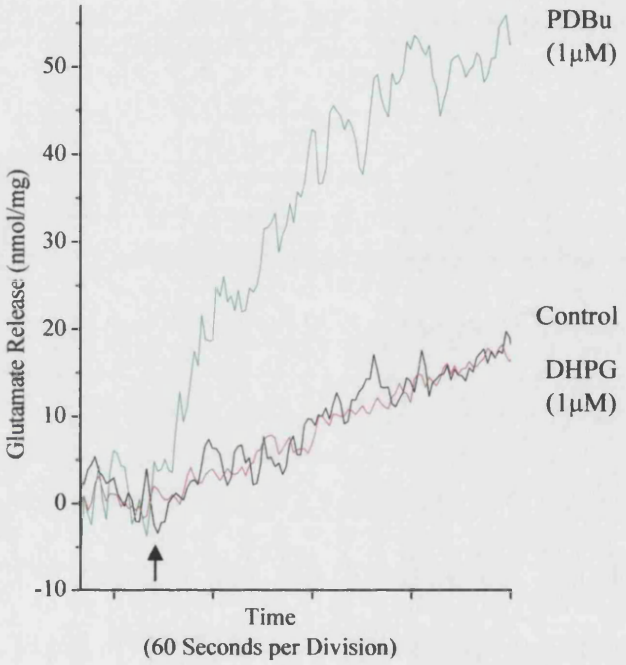


Figure 6.11 DHPG Does Not Appear to Enhance Glutamate Release on Its Own. Synaptosomes were incubated in the presence of 1mM $CaCl_2$ as indicated in section 5.2.1. **A.** Glutamate release was elicited with the addition of 4-AP (1mM, arrow) in the absence (control), or presence (+DHPG) of 1μM DHPG, or 1μM PDBu (+PDBu). DHPG was added 1 min prior, and PDBu added 30 secs prior to the secretogogue. Accumulative release was measured at 2 sec intervals. A representative experiment is shown.

capacity of the latter (including AA) ((Lombardi et al., 1996)), obviates any potential influence of endogenous AA. In the one specific instance where regulation by DHPG was reported to occur in the absence of AA, the regulation of [3H]-glutamate release was addressed, rather than endogenous neurotransmitter (Reid et al., 1999).

The results obtained so far have correlated a lack of increase in synapsin I site 4/5 phosphorylation with a lack in enhancement of glutamate release. The lack of increase in site 4/5 synapsin phosphorylation with DHPG, however, appears to be contrary to clear increases in phosphorylation observed in ERK 1,2, believed to be directly upstream of synapsin. The most facile explanation for this would be that ERK 1,2 is not the active kinase phosphorylating synapsin on site 4/5, despite a large body of data indicating to the contrary (Jovanovic et al., 1996; Yamagata et al., 2002). Alternatively, pathways acting in parallel to ERK 1,2 activation that are increasing the dephosphorylation of these sites on synapsin (Jovanovic et al., 2001), may play a role to reverse the effects of DHPG stimulated ERK 1,2 on the molecule. Group I mGluRs stimulate increases in intracellular Ca^{2+} levels through production of IP_3, as well as through PKC-dependent pathways. This increase, also alluded to in the foregoing mentioned series of experiments, could lead to the activation of Ca^{2+}-dependent phosphatases, such as calcineurin (PP2B) (Tonks and Cohen, 1983), known to dephosphorylate synapsin I on site 4/5 (Jovanovic et al., 2001). The earlier experiments described in this chapter looked at the roles of intracellular and extracellular Ca^{2+} on ERK 1,2 phosphorylation. It was, therefore, decided to return to use the same conditions to study the levels of synapsin site 4/5 phosphorylation, in conditions showing a Ca^{2+}-dependent activation of ERK 1,2.

Synaptosomes were preincubated with 1mM $CaCl_2$ for 10 mins before addition of BAPTA-AM. Preincubation continued for a further 20 mins before synaptosomes were harvested and washed. A standard 10 min incubation protocol was then followed in the presence of either 1mM $CaCl_2$ or EGTA, as described previously. The results obtained are shown in Figure 6.12 and agree with previous reports ((Jovanovic et al., 2001)), in that the levels of synapsin site 4/5 phosphorylation significantly increase with the removal of Ca^{2+} (mean ± s.e.m. of 37°C samples compared to 100% 37°C control: EGTA = 158 ± 13; BAPTA = 122 ± 7; EGTA/BAPTA = 163 ± 8). It is interesting to

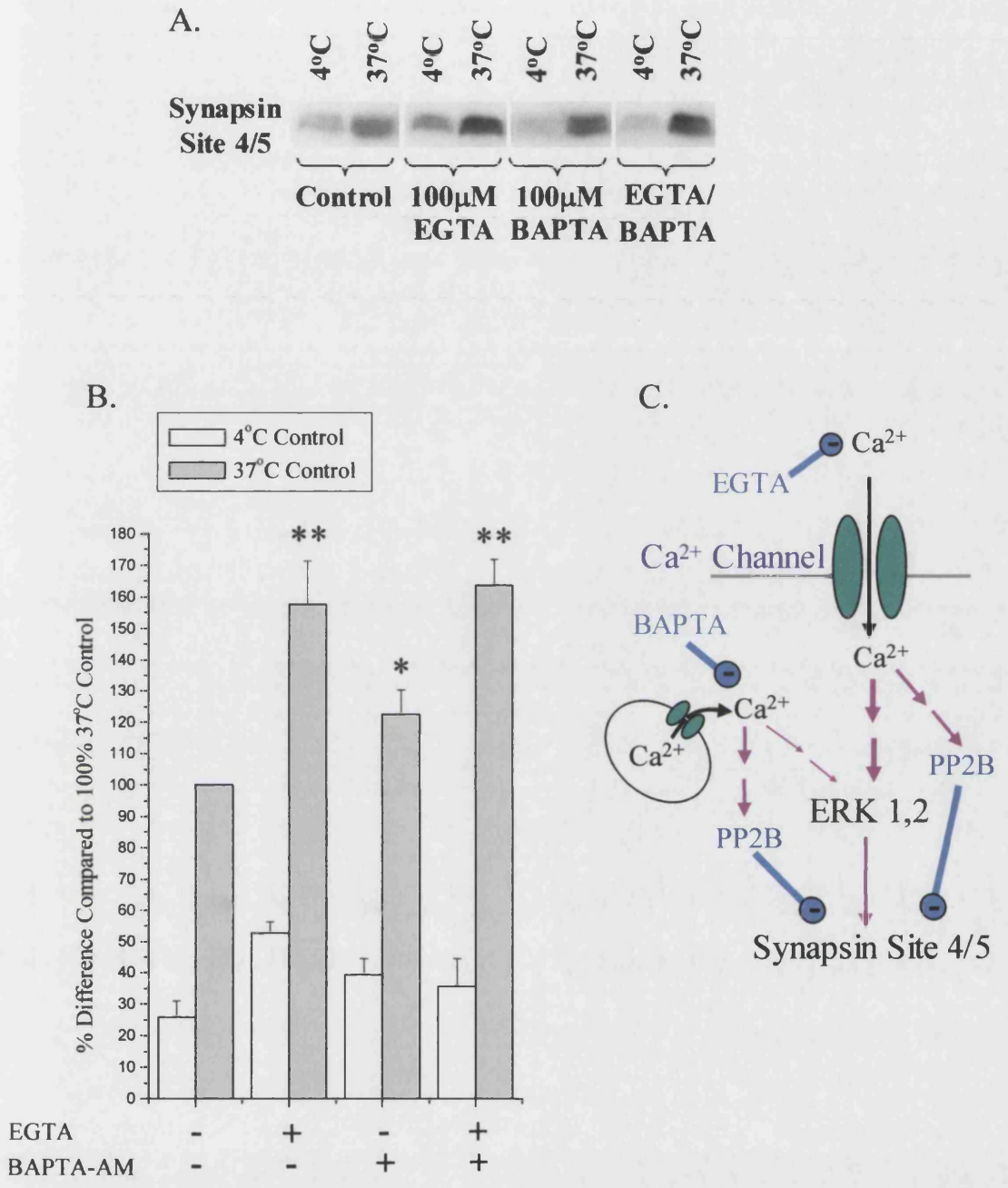


Figure 6.12 EGTA and BAPTA Stimulate Basal Levels of Synapsin Site 4/5 Phosphorylation. Synaptosomes were preincubated with 1mM $CaCl_2$ and either BAPTA-AM or DMSO. The following incubation was then carried out either in the presence of 1mM $CaCl_2$ (-EGTA) or EGTA (+EGTA) as described in 6.2.2. 7.5% polyacrylamide gels were used. Immunoblots were labelled with phospho-synapsin site 4/5 primary antibody (J.J, 1:500) and reported using ^{125}I-labelled Protein A. Signal levels were detected and analysed using phosphorimage spectroscopy (Molecular Dynamics). Samples were normalised to the 100% 37°C control value. **P<0.01, *P<0.05, ANOVA with Duncan's *post hoc* analysis (n=3) **A.** Phosphorimage of combined effects of BAPTA and EGTA. **B.** Quantification of these effects on synapsin site 4/5 phosphorylation. **C.** Possible pathways involved.

note that these increases occur despite the fact that, under the same conditions, ERK 1,2 phosphorylation levels were decreased compared to controls (Figure 6.9 and compared in Table 6.2). This suggests the presence of an alternative pathway involving a phosphatase, the activity of which is substantially suppressed with the removal of Ca^{2+}.

Table 6.2: Changing ERK 1,2 and Synapsin Site 4/5 Phosphorylation Levels.

	Control	Control			+ DHPG		
	DHPG	EGTA	BAPTA	BAPTA/ EGTA	EGTA	BAPTA	BAPTA/ EGTA
ERK 1,2	↑	↓	—	↓	↑	—	—
Synapsin 4/5	—	↑↑	↑	↑↑	—	↓	↑

The Ca^{2+}-dependency of the basal levels of ERK 1,2 and synapsin site 4/5 phosphorylation, combined with the opposing Ca^{2+}-dependency of DHPG-enhanced ERK 1,2 phosphorylation, has made it difficult to assess and interpret the levels of synapsin site 4/5 phosphorylation regulated by Ca^{2+}. For example, the blot shown in Figure 6.13, shows a slight DHPG-mediated increase in synapsin I site 4/5 phosphorylation in controls and EGTA/BAPTA containing samples. Apparently paradoxically, however, the BAPTA containing sample shows a slight DHPG-mediated decrease, with the synapsin I site 4/5 phosphorylation levels in the EGTA-containing case showing no response to the agonist. It should be noted that this was a representative blot and further experiments need to be conducted to confirm the validity of this study. However, these results do point towards the possibility for a Ca^{2+}-dependency of Group I mGluR-mediation of synapsin I site 4/5 phosphorylation levels. The effects of the different Ca^{2+} conditions in the presence and absence of DHPG on ERK 1,2 and synapsin site 4/5 phosphorylation are illustrated in Table 2, where it can be clearly seen that changes in ERK 1,2 activation are not necessarily conducive to the same changes in synapsin site 4/5 phosphorylation.

The final experiments described in this chapter elucidated on the proximity of the neurotrophin-mediated trkB/Ras/Raf/MEK/ERK 1,2/synapsin, and glutamate-mediated Group I mGluR/PKC or Ca^{2+}/ERK 1,2/synapsin signalling cascades. As both cascades

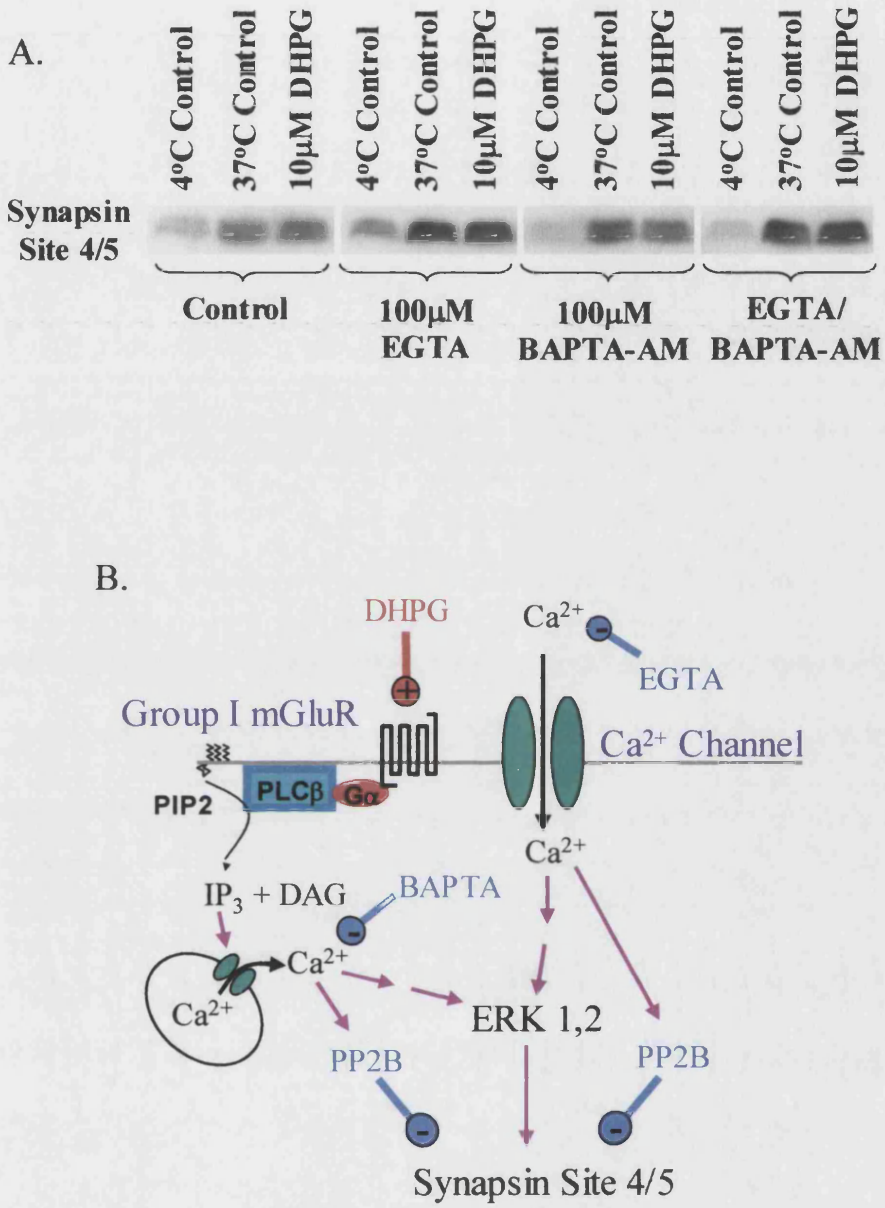


Figure 6.13 Combined Effects of Ca^{2+} and DHPG on Synapsin Site 4/5 Phosphorylation. Synaptosomes were preincubated with 1mM $CaCl_2$ and either BAPTA-AM or DMSO. The following incubation was then carried out either in the presence of 1mM $CaCl_2$ (-EGTA) or EGTA (+EGTA). DHPG was added 1 min prior to the termination of the reactions. 7.5% polyacrylamide gels were used. Immunoblots were labelled with synapsin site 4/5 primary antibody (1:500, J.J.) and reported using ^{125}I-Protein A. **A.** Phosphorimage of combined effects of EGTA, BAPTA and DHPG on synapsin site 4/5 phosphorylation. **B.** Schematic diagram showing the possible pathways involved in signalling to synapsin site 4/5 following this protocol.

are acting through increasing ERK 1,2 phosphorylation, it should be possible to examine their colocalisation by measuring levels of ERK 1,2 activation. Additive levels of ERK 1,2 phosphorylation could suggest the involvement of different populations of neurones, whereas lack of additivity or in fact, synergism, could be taken to indicate immediate intrasynaptosomal proximity or cross-talk of the pathways within neurones.

Cerebrocortical synaptosomes were incubated for a total time of 10 mins with 6mins in the presence of varying concentrations of BDNF, a trkB receptor agonist, and/or 5 mins 30 sec in the presence of varying concentrations of DHPG, a Group I mGluR agonist. The results obtained are shown in Figure 6.14 and Figure 6.15. Although 100nM DHPG appears to increase ERK 1,2 phosphorylation (Figure 6.14), the enhancement was not found to be statistically significant (mean ± s.e.m.: DHPG (100nM) = 148 ± 15). In contrast, both 50ng/ml and 100ng/ml BDNF were able to significantly increase ERK 1,2 activation (mean ± s.e.m.: BDNF (50ng/ml) = 174 ± 29; BDNF (100ng/ml) = 202 ± 34). It is interesting that the combined effects of these submaximal concentrations of DHPG and BDNF were neither synergistic nor additive, with increases in ERK 1,2 phosphorylation comparable with levels obtained with either drug alone (mean ± s.e.m.: DHPG/BDNF (100nM/50ng/ml) = 157 ± 18; DHPG/BDNF (100nM/100ng/ml) = 153 ± 15).

The results in Figure 6.15 led to similar conclusions as those in Figure 6.14. Both 100ng/ml and 200ng/ml BDNF significantly increased ERK 1,2 activation (mean ± s.e.m.: BDNF (100ng/ml) = 185 ± 29; BDNF (200ng/ml) = 233 ± 28), as did 1μM. DHPG (mean ± s.e.m.: DHPG A/C (1μM) = 171 ± 17; DHPG B/D (1μM) = 156 ± 13). The enhancement in ERK 1,2 activation levels upon combining 100ng/ml or 200ng/ml BDNF with 1μM DHPG was, however, comparable to that obtained with either neurotrophin or mGluR 1/5 agonist alone (mean = s.e.m.: DHPG/BDNF (1mM/100ng/ml) = 169 ± 9; DHPG/BDNF (1mM/200ng/ml) = 209 ± 32), indicating no additivity. The foregoing data suggest that the neurotrophin-mediated signalling through ERK 1,2 and the GPCR-mediated signalling through ERK 1,2 are likely occurring within the same population of nerve terminals

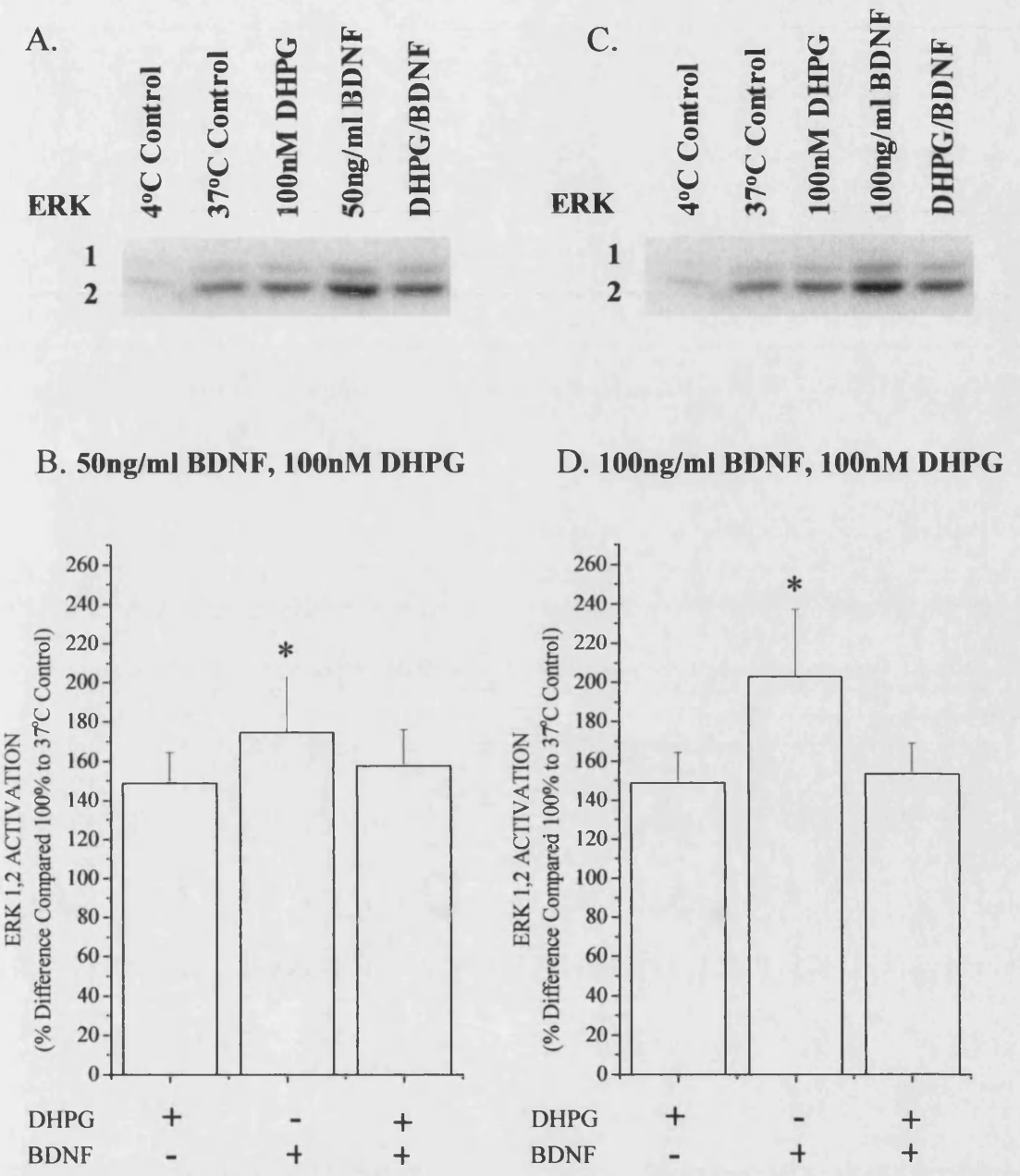


Figure 6.14 Combined Effects of DHPG and BDNF on ERK 1,2 Phosphorylation Levels. Synaptosomes were incubated at 37°C for 10 mins with 1mM $CaCl_2$. 50ng/ml or 100ng/ml BDNF (+BDNF) was added 6 mins prior to the end of the incubation. 100nM DHPG (+DHPG) was added 5 mins 30 sec prior to the termination of the incubation. 7.5% polyacrylamide gels were run. Immunoblots from several separate experiments were labelled with phospho-specific MAPK primary antibody (NEB, 1:1000) which was probed using ^{125}I-labelled protein A. Signal levels were quantified using phosphorimager spectroscopy. *P>0.05 (ANOVA with Duncan's *post hoc* analysis, n=3) **A and C**: Phosphorimages of BDNF and DHPG effects. **B and D**: Quantification of BDNF and DHPG effects.

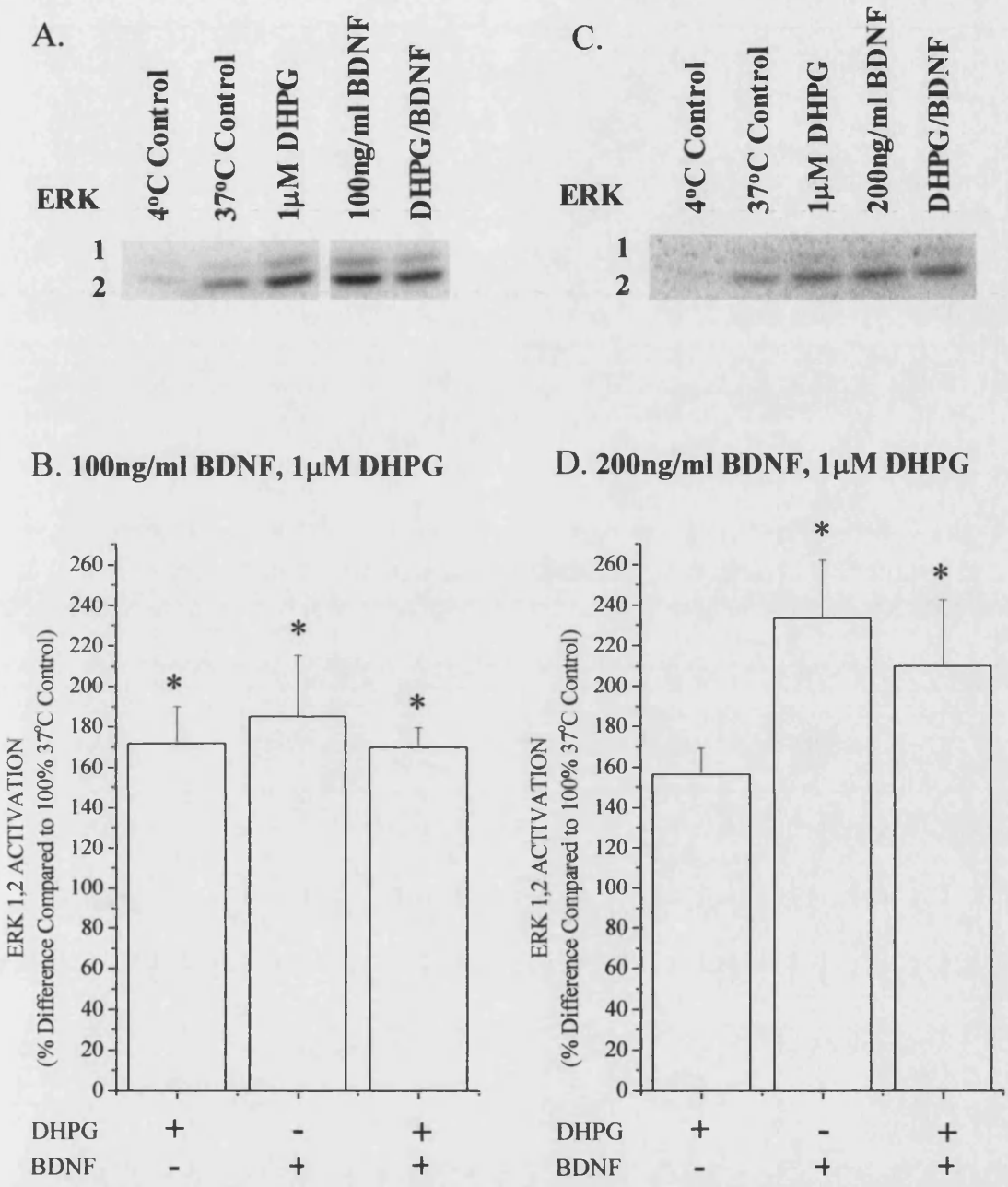


Figure 6.15 DHPG and BDNF-Mediated Increases in ERK 1,2 Activation are Not Additive. Synaptosomes were incubated with 1mM $CaCl_2$ for 10 mins in the presence of 100ng/ml or 200ng/ml BDNF (+BDNF) added 6 mins prior to the termination of the incubation, and/or 1μM DHPG (+DHPG) added 5 mins 30 sec prior to the end of the incubation. 7.5% polyacrylamide gels were run. Immunoblots were labelled with phospho-specific MAPK primary antibody and reported using ^{125}I-labelled protein A. ^{125}I-labelling was quantified using phosphorimager spectroscopy (Molecular Dynamics). *P>0.05 (ANOVA with Duncan's *post hoc* analysis, B: n=4; D: n=3). **A and C**: Phosphorimages of DHPG and BDNF effects. **B and D**: Quantification of DHPG and BDNF effects.

6.4 Summary of Results

- Group I mGluR agonist, DHPG, enhances p42 and p44 MAPK phosphorylation levels in presynaptic cerebrocortical nerve terminals.

- Group I mGluR-mediated enhancement of MAPK phosphorylation levels, although not desensitising as such, were dependent on agonist concentration and incubation protocol.

- mGluR1 is tonically inhibited under control, short incubation conditions.

- Removal of mGluR1 tonic inhibition leads to enhanced p42 and p44 MAPK phosphorylation levels in synaptosomes.

- Basal MAPK phosphorylation levels are dependent on extracellular Ca^{2+} in presynaptic cerebrocortical nerve terminals.

- Group I mGluR-mediated increase in MAPK phosphorylation is dependent on intracellular Ca^{2+}, but not PKC, when the preincubation protocol was followed.

- Synapsin I site 4/5 phosphorylation is highly dependent on intracellular and extracellular Ca^{2+} concentration, in a manner which does not bear relation to MAPK activation alone but additionally to the tone of Ca^{2+}-dependent dephosphorylation activity.

- Neurotrophin and GPCR signalling to MAPK occur in the same nerve terminals.

6.5 Discussion

The original basis for this work was to identify whether receptor linked pathways could be signalling through ERK 1,2 and synapsin as a common mechanism for regulating glutamate release from cerebrocortical nerve terminals. In support of this idea, this chapter has found that DHPG, a Group I mGluR agonist is able to increase levels of ERK 1 and ERK 2 phosphorylation in synaptosomes. Moreover, the evidence points to a Ca^{2+}-dependence of this activation appearing to be contingent on the release of intrasynaptosomal Ca^{2+}.

Characteristics of mGluR1/5 Activation: Desensitisation.

Previous studies have shown that the DHPG-mediated facilitation of glutamate release rapidly desensitises and is no longer present upon subsequent addition of the agonist (Herrero et al., 1998). The increase in levels of DAG produced by Group I mGluR stimulation was found to peak 5 seconds after treatment and be reduced back to basal following 5 minutes of incubation with DHPG. This effect was suggested to be due to phosphorylation of the C-terminal domain of the receptors by PKC, which is acting in a negative feedback loop (Herrero et al., 1998). During a desensitisation of a receptor-mediated response, it has traditionally been observed that increasing levels of agonist will increase the rate of desensitisation (Rodriguez-Moreno et al., 1998; Gereau and Heinemann , 1998). My investigations, looking at levels of ERK 1,2 phosphorylation following 10 min incubation with 1 min in the presence of varying concentrations of DHPG, indicate that the mean response does decrease with increasing concentrations of agonist, although at present, further experiments are warranted using the lowest concentrations of DHPG to obtain statistical support in this regard. Notwithstanding, it was interesting to note that although 100μM DHPG significantly increased ERK 1,2 phosphorylation levels following a 10 min incubation with the drug, the higher concentration (1mM DHPG) did not. While these data would in principle support a desensitisation argument, the validity of this result could be called into question on the basis of the extremely high concentration of agonist required. Especially considering the EC_{50} for DHPG stimulation of mGluR1 and mGluR5 demonstrated in oocytes was 6-10μM and 2μM respectively, notably some 100 fold lower than the concentration found here to 'desensitise' the receptor(s) (Ito et al., 1992).

In general, my investigations looking at the time course and dose response characteristics of the increase in ERK 1,2 phosphorylation levels with DHPG, found a pattern of response different to that observed with the desensitisation of the DAG response reported previously (Herrero et al., 1994). When synaptosomes were incubated for 10 minutes with 5mins 30sec in the presence of DHPG, the first signs of significant increase in the level of ERK 1,2 phosphorylation was obtained with 1nM DHPG. A similar level of increase in ERK 1,2 phosphorylation was still present with 100μM DHPG, despite having increased the drug concentration by 10^5 fold, suggesting that the ERK 1,2 response was certainly not diminishing with increasing concentration of agonist. When the incubation length with DHPG was increased to 10 mins, 100nM and 1μM DHPG were no longer able to significantly increase ERK 1,2 phosphorylation levels. This could be construed as a desensitisation effect (Rodriguez-Moreno et al., 1998; Gereau and Heinemann , 1998), but a higher concentration of 10μM DHPG was still able to significantly enhance levels of ERK 1,2 phosphorylation. These observations, suggesting that higher concentrations of DHPG are required to elicit increases in ERK 1,2 phosphorylation with increasing incubation times, are features contrary to conventional notions of desensitisation.

Underlying this unorthodox behaviour may be the presence of multiple convergent pathways with different characteristics. This lack of correlation between the time course of activation of DAG production by DHPG and of ERK 1,2 phosphorylation has, for example, also been reported in a study conducted in astrocytes (Peavy et al., 2002). In this study, mGluR5 activation was found to lead to increases in ERK 1,2 phosphorylation through a pathway dependent on Gαq, but completely independent of Gβγ regulation and PLCβ1 activation of PKC. Interestingly, this implied presence of two separate signalling pathways downstream of mGluR5 activation was also coupled with observations of unusual receptor desensitisation behaviour, in that the increase in ERK 1,2 activation in the continued presence of the Group I mGluR agonist, only diminished over periods extending to an hour. These types of observations suggest that experiments with lengthened incubation periods are warranted, to fully investigate any potential desensitisation of DHPG-induced increases in ERK 1,2 phosphorylation in synaptosomes.

Although the matter of desensitisation of the ERK 1,2 pathway downstream of Group I mGluR activation remains to be fully resolved, it is notable from the foregoing results and discussion that the levels of ERK 1,2 phosphorylation obtained in the presence of DHPG are evidently dependent on the incubation protocol and concentration of the drug used. Accordingly, my subsequent discussion of signalling pathways will be divided to assess separately effects found with a short 10 minute incubation, and those found following a preincubation protocol.

Group I mGluR Signalling to ERK 1,2: Role of PKC and Intrasynaptosomal Ca^{2+}.
Following on from the basic observations discussed above, my subsequent experiments related to investigating the nature of the pathway involved in signalling between the Group I mGluRs and ERK. Given that the facilitatory effects of Group I mGluRs in nerve terminals (Sistiaga and Sanchez-Prieto, 2000b), as well as the signalling to ERK 1,2 in other systems (Peavy and Conn, 1998), have been attributed to mGluR5 activation, I examined the involvement of this receptor subtype in the activation of ERK 1,2 in nerve terminals, using the non-competitive antagonist MPEP. Downstream of Group I mGluR activation, the stimulation of PKC has also been well documented (Pin and Duvoisin, 1995, review), as has the release of intracellular stores of Ca^{2+}. The relative contributions of these limbs of signalling, found downstream of PLCβ activation, to the Group I mGluR-mediated regulation of ERK 1,2 activation in nerve terminals was also assessed.

Group I metabotropic glutamate receptors contain a large N-terminal extracellular domain (ECD) consisting of two lobes which are able to exist in an open or closed conformation. The glutamate binding pocket is located in the hinge region in between the two lobes, and glutamate binding is believed to stabilise the ECD in the closed conformation and bring about activation of the receptor (Kunishima et al., 2000). Competitive antagonists are thought to bind at the same site as glutamate but not bring about the conformational changes and stabilisation of the receptor, resulting in the inhibition of activation. Non-competitive antagonists such as MPEP, however, are believed to act at the seven transmembrane domain (7TMD) of Group I mGluRs, to stabilise the conformation of this domain in an inactive state (Pagano et al., 2000; Malherbe et al., 2003). In the current study, MPEP effectively eliminated the agonist-

induced stimulation of ERK 1,2 activity, by presumably blocking transduction of signalling at the level of the 7TMD. However, additionally, the antagonist displayed agonist properties in causing ERK 1,2 activation by itself. Recent evidence has come to light which suggests that the 7TMD of mGluR1 and mGluR5 can be activated independently of agonist stimulation of the ECD (Ango et al., 2001; Goudet et al., 2004). Group I mGluRs have been found to be able to display a constitutive activity in heterologous cells which can only be attenuated by non-competitive antagonists, such as MPEP and BAY36-760, acting at the 7TMD, and not by competitive antagonists acting at the ECD. This has also led to the classification of MPEP as an inverse agonist (see (Gasparini et al., 2002, for review). However, with MPEP acting in this mode, if anything, the activity of ERK 1,2 might have been expected to have been diminished rather than enhanced in the manner observed here. Rationalization of this unexpected behaviour of MPEP may nonetheless be forthcoming with consideration of activation of multiple mGluRs in the system.

mGluR1 and mGluR5 have been found to coexist in many neuronal preparations, including striatal cholinergic interneurones (Pisani et al., 2001), type II globus pallidus neurones (Poisik et al., 2003), dorsal horn neurones (Karim et al., 2001), and CA1 pyramidal cells (Mannaioni et al., 2001; Ireland and Abraham, 2002). A recent study in type II globus pallidus neurones has proposed the existence of a modulating pathway between these two receptors, whereby, the constitutive activity of mGluR5 was suggested to be resulting in the tonic inhibition of mGluR1 through a PKC-mediated mechanism (Poisik et al., 2003). These findings could help explain the results I obtained showing a significant increase in ERK 1,2 phosphorylation levels in the presence of MPEP following the 10 minute incubation protocol. Thus, if MPEP, as an inverse agonist, attenuates the constitutive activity of the mGluR5 receptor, it would relieve inhibition of mGluR1 receptor activity and result in increased downstream ERK 1,2 activation. Such a mechanism is also consistent with the finding that treatment with MPEP alone is able to increase glutamate release in the medial vestibular nuclei of rat brainstem slices (Grassi et al., 2002).

The above discussion regarding the tonic inhibition of mGluR1 by mGluR5 mediated through a PKC-dependent pathway may also be relevant in synaptosomes, when considering the effects of PKC inhibitors. Of note in this regard was my observation of

the ability of the PKC inhibitor, Ro-32-0432, to increase ERK 1,2 phosphorylation levels on its own. Ro-32-0432 has, in fact, been shown to inhibit G-protein receptor kinase (GRK) activity of GRK5 (Moore and et al., 1998). In transfected HEK-293 cells, GRK5 has been shown to be able to mediate mGluR1a desensitisation and internalisation (Dale et al., 2000). The observations with Ro-32-0432 appear therefore to parallel those obtained with MPEP, in that it is a relief of the tonic inhibition of mGluR1 that is resulting in increased stimulation of ERK 1,2 phosphorylation through this receptor type in cerebrocortical synaptosomes.

Notwithstanding the aforementioned discussion, it is possible that the increases in MAPK phosphorylation found with treatment of synaptosomes with MPEP are due to non-specific actions of the drugs, rather than inhibition of the constitutive activation of mGluR5. Although, MPEP has been shown to inhibit the noradrenaline transporter in transfected cells, and to increase noradrenaline-mediated effects in vivo (Heidbreder et al., 2003), it does not, however, appear to have any effect on excitatory amino acid uptake (Fazal et al., 2003). At the receptor level, there are also suggestions that MPEP may be a positive allosteric modulator of mGluR4 in transfected cell lines (Mathiesen et al., 2003). This begs the use of newer non-competitive mGluR5 antagonists to authenticate or refute the finding here with MPEP. The effects of MPEP would also bear comparison with the effects of a selective competitive mGluR5 antagonist, LY34454, which should not regulate the constitutive activity of the receptor (Gasparini et al., 2002). Finally, it would be essential to try and elucidate on the relative contributions of mGluR1 and mGluR5 in DHPG-induced ERK 1,2 phosphorylation. CPCCOEt is a non-competitive mGluR1 antagonist which inhibits agonist-induced activation, but not constitutive activation, through inhibition of the interaction between the ECD and 7TMD (Pagano et al., 2000). This antagonist, together with another, BAY36-7620, which acts as a selective mGluR1 inverse agonist (Carroll et al., 2001), may be useful in investigating the role of the constitutive activity of mGluR1 in signalling to ERK 1,2.

Signalling Pathways Linking mGluR Stimulation with ERK 1,2 Activation.

Group I mGluRs have been shown to dynamically couple to different G-protein subunits and in doing so, regulate different downstream signalling pathways (De Blasi et al., 2001). It is possible that relief of inhibition of the 7TMD of the receptors,

combined with stimulation of the ECD by DHPG, switches the downstream coupling of the receptors to pathways not involving ERK 1,2. There is precedent for a switch in the control of glutamate release from nerve terminals following repeated exposure of Group I mGluRs to their agonist (Herrero et al., 1998). There is also evidence that Group I mGluR downstream signalling through PLC can be selectively desensitised by phosphorylation, whilst leaving signalling through cAMP intact (Francesconi and Duvoisin, 2000). Considering that coupling to Gq, Gi/o and Gs have all been shown to signal to ERK 1,2 in hippocampal neurones (Berkeley and Levey, 2003), the key question here is which particular signalling pathway(s) mediate the enhancement of ERK 1,2 phosphorylation in nerve terminal in response to DHPG. Given the known facilitatory effects of Group I mGluRs through a Gq mediated stimulation of PLCβ to produce the DAG and IP_3, the respective roles of the targets of these second messengers, i.e. PKC and Ca^{2+} stores, were examined.

Involvement of PKC.

Using the PKC inhibitors, calphostin C and Ro-32-0432, it has not been possible to absolutely confirm or refute PKC-mediated regulation of increases in ERK 1,2 phosphorylation downstream of Group I mGluR activation. Both calphostin and Ro-32-0432 per se actually produced ERK 1,2 activation themselves, although the latter did also inhibit the DHPG stimulation. As discussed in chapter 5, a major contributory factor to the complicated picture produced with inhibitor studies is likely the lack of complete specificity of the agents. Additionally, however, given the multiple roles of the enzyme in nerve, effects of inhibitors on the basal activity of the kinase would tend to confound interpretation.

In lieu of further studies to address the role of PKC in ERK 1,2 signalling, of note are several studies reporting a PKC-independence to the ERK 1,2 signalling downstream of Group I mGluRs, in a variety of systems, including, cortical astrocytes (Peavy et al., 2002), neostriatal slices (Tanaka and Nishizaki, 2003), and Chinese hamster ovary (CHO) cell lines (Thandi et al., 2002). Considering the diversity of messages involved within Group I mGluR signalling, other than DAG/PKC, a number of regulators including cAMP/PKA, PI3K and Ca^{2+}, could be involved (Gutkind, 2000, review). I

examined the last of these as a potential product of mGluR activation following PLC activation and the IP_3 generation.

Involvement of Intrasynaptosomal Ca^{2+}

Results presented here suggest that the while about 50% of the basal ERK 1,2 activity seen in nerve terminals seems to be supported by extracellular Ca^{2+} (external EGTA sensitive), the DHPG-mediated increase in ERK 1,2 phosphorylation, following a preincubation protocol, appears to be entirely dependent on intracellular Ca^{2+} (internal BAPTA sensitive). This suggests that, at least under these particular conditions, signalling between Group I mGluRs and ERK 1,2 is not occurring through DAG stimulation of a Ras-guanine nucleotide exchange factor (Ebinu et al., 1998). While it is possible that the observed enhancement of MAPK activation is occurring through a conventional PKC isoform, which requires Ca^{2+} for activation (Hug and Sarre, 1993), the lack of attenuation of the DHPG-mediated increase in ERK 1,2 phosphorylation by the PKC inhibitor, calphostin C, suggests that this may not be the case (although see caveats discussed above). The question remains then as to the mechanism by which Ca^{2+} mediates the stimulation of ERK 1,2. Potential crosstalk to the cAMP/PKA pathway is possible through the stimulation of Ca^{2+}/calmodulin sensitive isoforms of adenylate cyclase (Hurley, 1999). Linking into the Ras-cascade for ERK 1,2 activation is also possible through the activation of Ca^{2+} -sensitive non-receptor tyrosine kinases (nRTK) such as src. Given the recent co-localization of PI3K with synapsin I in nerve terminal (Cousin et al., 2003), PI3K may form another segway between Ca^{2+} and ERK 1,2 activation. Further experiments are clearly warranted to assess this potential involvement and contribution of cAMP, nRTKs or PI3K to the Ca^{2+}-mediated activation of MAPK in cerebrocortical nerve terminals reported herein.

Synapsin I: a Target for an mGluR/ERK 1,2 Signalling Cascade.

Having identified the presence of a MAPK signalling cascade downstream of Group I mGluR activation in cerebrocortical synaptosomes, in the subsequent experiments conducted I went on to investigate whether this cascade could be signalling through synapsin I in order to regulate glutamate release, as described with a neurotrophin-mediated pathway (Jovanovic et al., 2000).

Despite the clear effect of DHPG on ERK 1,2 activation, the agonist was unable to either enhance levels of 4-AP-elicited glutamate release under these conditions, or to increase synapsin site 4/5 phosphorylation. The lack of net increase in site 4/5 synapsin I phosphorylation levels is not necessarily a surprise as Group I mGluRs are known to increase production of IP_3, which in turn can increase internal Ca^{2+} concentrations through release from internal stores (Murphy and Miller, 1988; Dale et al., 2001). The increase in Ca^{2+} concentration can lead to the activation of the phosphatase calcineurin, which is known to dephosphorylate synapsin I at site 4/5, counteracting the increase in phosphorylation by ERK 1,2 (Jovanovic et al., 2001). This role of Ca^{2+} in determining the level of synapsin I phosphorylation at ERK 1,2 sites was evident from dramatic increases in synapsin I site 4/5 phosphorylation levels I obtained following the removal of Ca^{2+} from the experimental media using EGTA. It could therefore be postulated that, although Group I mGluRs are signalling through ERK 1,2, opposing or contradicting signals at the level of synapsin I prevent significant increases in phosphorylation of this protein, thus preventing increases in vesicle availability to support the facilitation of glutamate release.

Notwithstanding the postulated requirement for AA and for mGluR-mediated regulation of glutamate release, some studies have indeed found that DHPG is able to increase glutamate release without the need for exogenous AA (Reid et al., 1999). These differences in opinion could be consolidated in two ways: either that endogenous AA contributes in studies where no exogenous AA has been added (Thomas et al., 2000), or that modulation of release is not simply contingent on the presence of AA, but also determined by other factors including the Ca^{2+} concentrations of the preparation. With regard to the latter, as already discussed above, synapsin site 4/5 phosphorylation levels are highly regulated by Ca^{2+}, due to the activation of the Ca^{2+}-dependent phosphatase, calcineurin (Jovanovic et al., 2001). Thus, it could be that in preparations where no exogenous AA is required, basal Ca^{2+} levels are lower, and as such, affect lower levels of calcineurin-mediated synapsin I site 4/5 dephosphorylation and thereby promote facilitation of release. This hypothesis is in agreement with studies conducted in cerebrocortical synaptosomes which found that protein phosphatase 2B inhibitors (inhibitors of calcineurin) were able to mimic the action of AA on Group I mGluR-mediated increases in glutamate release (Sistiaga and Sanchez-Prieto, 2000b). Clearly, further experimentation is necessary to support this hypothesis. This would include

looking at ERK 1,2 and synapsin I phosphorylation levels in the presence of AA, as it is possible that AA is acting via several alternative mechanism to increase glutamate release, including increasing the activation of neuromodulin, a protein involved in the exocytotic machinery (Schaechter and Benowitz, 1993). With respect to the novel finding here that a Ca^{2+} dependent activation of ERK 1,2 can be affected by DHPG induced increases in intracellular Ca^{2+}, it is essential to establish that the lack of significant increase in synapsin I phosphorylation is indeed due to an opposing Ca^{2+} dependent dephosphorylation tone. This could be done by testing in detail the effects of FK506, a calcineurin inhibitor, on Group I mGluR-mediated phosphorylation of downstream signalling molecules and modulation of neurotransmitter release.

The identification of neurotrophin (BDNF)-mediated and GPCR-mediated (Group I mGluR) signalling operating on the same pool of ERK 1,2 in co-stimulation experiments, suggests these pathways coexist in the same presynaptic, glutamatergic nerve terminals It would be interesting to investigate the relative contributions of these pathways and whether signalling through one can influcence the nature of the signalling through the other.

This chapter has found that ERK 1,2 can be activated downstream of presynaptic Group I mGluR stimulation, but the pathways involved are complex and subject to regulation from multiple sources. It has not been possible, thus far, to confirm ERK 1,2 and synapsin I involvement in Group I mGluR-mediated increases in glutamate release from presynaptic nerve terminals although the indications are promising. The current work has invoked a number of further experiments which will be key to fully elucidating and detailing the mechanisms involved in regulating glutamate release downstream of presynaptic Group I mGluR activation.

7. Discussion

The protein kinases, ERK 1 and ERK 2, have been shown to be involved in many aspects of cell signalling, depending on cell type and situation (Gutkind, 2000). They were originally identified in mammalian cells as molecules involved in mediating the mitogenic effects of growth factors (Cooper and Hunter, 1983; Cooper and Hunter, 1981), and later in the decade as activities stimulated by insulin or various growth factors, that were able to phosphorylate the model substrates microtubule-associated protein 2 (MAP2) and myelin basic protein (MBP) (Ray and Sturgill, 1987; Hoshi et al., 1988; Hoshi et al., 1989). Numerous subsequent studies have suggested a central involvement of ERK1 and 2 in the regulation of cell proliferation through the transfer of signals from the cytosol to the nucleus, with constitutive activation of the pathway resulting in tumerogenesis (Pages et al., 1993; Mansour et al., 1994). Since then, work in mature neurones has also shown ERK1 and 2 to have major roles in conducting signals between the cytoplasm and nucleus in the regulation of synaptic plasticity, particularly at the level of the postsynaptic compartment (Sweatt, 2004; Thomas and Huganir, 2004). The majority of this latter work has focused on the hippocampus, an area of the brain where synaptic plasticity has been hypothesised to underlie the formation of memories (Sweatt, 2004; Malenka and Nicoll, 1999), but further roles for ERK 1,2 signalling are beginning to be uncovered in areas such as the dorsal root ganglia (Obata et al., 2003) and the striatum (Sanna et al., 2002). As research progresses into this postsynaptic ERK activation/phosphorylation, more and more upstream regulators are being identified, including PKA, PKC and Ca^{2+}, amongst others (Roberson et al., 1999; Thomas and Huganir, 2004). Considering these established notions concerning postsynaptic signalling, the work in this thesis investigated whether the cross-talk of signalling cascades with ERK 1,2 could also occur presynaptically, using the isolated presynaptic nerve terminal preparation.

The purified presynaptic nerve terminal (synaptosome) is obtained by using homogenisation to 'pinch off' presynaptic terminals from axons, followed by purification of the former using Percoll gradients (Sihra, 1997; Dunkley et al., 1986). Synaptosomes prepared in this manner have been shown to have intact membranes containing mitochondria and numerous small synaptic vesicles (Maycox et al., 1990).

They are also functionally competent, and able to undergo depolarisation-induced Ca^{2+}-dependent release of neurotransmitter (Sanchez-Prieto et al., 1987). The absence of postsynaptic domains, cell somas and nuclei from this preparation are advantageous for the examination of the functioning of the presynaptic nerve terminals in isolation, eliminating the complications arising from nuclear signalling pathways and postsynaptic events which could confound results (Maycox et al., 1990). A recent study, using this preparation, has identified a role for ERK 1,2 phosphorylation in enhancing glutamate release, downstream of TrkB receptor activation by BDNF (Jovanovic et al., 2000). A downstream effector for ERK 1,2 signalling, in this presynaptic preparation, was identified as synapsin I, a synaptic vesicle tethering protein involved in regulating synaptic vesicle availability (Hilfiker et al., 1999). Could it be that signalling molecules that were cross-talking with ERK 1,2 in the postsynaptic domain, could also be cross-talking in the presynaptic terminal, with a possible functional role in the regulation of glutamate release? If so, could the receptors upstream of these signalling cascades, also be identified? This study, has indeed, found that ERK 1,2 activation/phosphorylation is a highly regulated phenomenon in presynaptic cerebrocortical nerve terminals, integrating multiple signalling cascades involved in regulating glutamate release.

An apparent plethora of signals involved in regulating ERK 1,2 activation in cerebrocortical nerve terminals that has been unearthed in this thesis, has complicated the unambiguous dissection of interacting pathways. Nevertheless, I have found that PKA and Ca^{2+}, and possibly PKC, are all able to regulate ERK 1,2 phosphorylation levels in presynaptic nerve terminals, in a manner dependent on time and incubation protocol. In the subsequent sections these pathways are discussed depending on their temporal activity, with those affecting basal ERK 1,2 activation levels discussed first, followed by acute factors working on top of basal levels, and then pathways which are demonstrating tonic cross-talk activity.

Factors Regulating Basal ERK1,2 Phosphorylation

The first temporal delineation made was in examining the regulation of ERK 1,2 activation/phosphorylation with the exogenous application of receptor antagonists in the absence of exogenous agonists. The scientific rationale for this work was to elucidate on the involvement of constitutive receptor activity on the basal levels of ERK 1,2 activation. It was interesting to observe that both β-adrenoceptor inhibition and

mGluR5 inhibition resulted in the modulation of basal levels of ERK 1,2 activation, albeit in opposite directions. I found that basal levels of ERK 1,2 activation were reduced following treatment of synaptosomes with the β-adrenoceptor antagonist, propranolol, or with the PKA inhibitors, H-89 and KT-5720. This evidence, combined with the attenuation of the β-adrenoceptor agonist, isoproterenol, mediated stimulation of ERK 1,2 phosphorylation in the presence of PKA inhibitors or β-adrenoceptor antagonists, suggests that, in cerebrocortical synaptosomes at least, endogenous activation of β-adrenoceptors contributes to the basal levels of ERK 1,2 activation via PKA (Figure 7.1). These results are in agreement with other studies which have identified inverse agonist properties for propranolol, leading to the inhibition of cAMP production (Chidiac et al., 1994; Baker et al., 2003), and with those which have demonstrated that β-adrenoceptor stimulation can lead to a PKA-dependent increase in ERK 1,2 phosphorylation (Roberson et al., 1999; Schmitt and Stork, 2000). However, some of these studies have also suggested that propranolol can act as a dual efficacy ligand and stimulate ERK 1,2 activity through a mechanism independent of G-protein coupling (Azzi et al., 2003; Baker et al., 2003). It seems unlikely that propranolol is acting as a dual efficacy ligand in cerebrocortical synaptosomes as I have shown a decrease in ERK 1,2 activation with propranolol and not an increase.

There are several factors within my preparation that could be leading to the observed basal activity of β-adrenoceptors. The first is a constitutive activity of the 7-transmembrane spanning domain (7-TMD) in the absence of ligand binding to the extracellular domain (ECD), recently hypothesised to be a common property of GPCRs (Malherbe et al., 2003). The second is that endogenously released/leaked noradrenaline from the heterogenous population of synaptosomes is acting as an agonist at the β-adrenoceptors. Although the dilution of synaptosomes used in my preparation should be such that endogenous ligand cross-talk is kept to a minimum, this looks to be the likely explanation for the results I have obtained for ERK 1,2 activation downstream of β-adrenoceptors. The reason for this is that the effects downstream of constitutively active 7-TMDs are often found to differ from those obtained following agonist stimulation (Poisik et al., 2003), whereas I have found a consistent stimulation of ERK 1,2 activation, whether mediated downstream of endogenous activity of the β-adrenoceptor or through the exogenous application of an agonist. If this pathway is

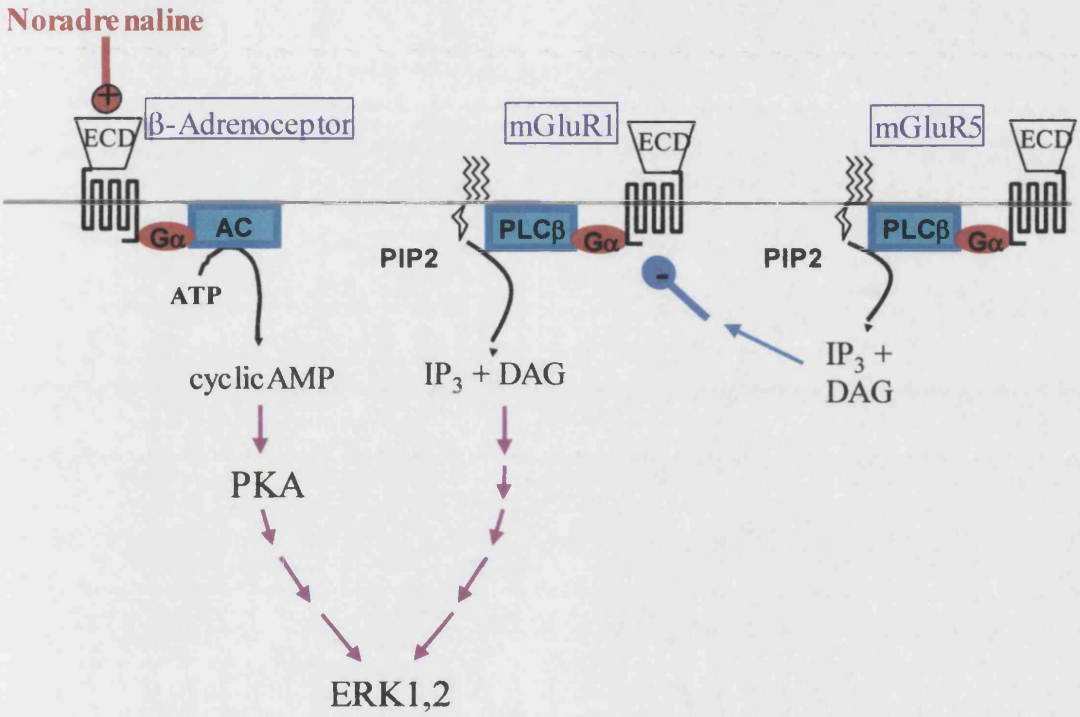


Figure 7.1 Schematic Diagram Illustrating the Possible Pathways, Identified in this Thesis, that May be Partly Involved in Controlling Basal Levels of ERK 1,2 Phosphorylation/Activation in Cerebrocortical Nerve Terminals. Endogenous activation of β-adrenoceptors appears to be partially responsible for basal ERK 1,2 stimulation, via a PKA-dependent pathway. Endogenous activation of mGluR5 appears to inhibit mGluR1-mediated signalling to ERK 1,2. However, the dependence of this signalling pathway on glutamate is yet to be determined. Abbreviations: AC = adenylate cyclase, ATP = adenosine triphosphate, cyclicAMP = cyclic adenosine monophosphate, PKA = cAMP-dependent protein kinase, ERK 1,2 = extracellular signal-regulated protein kinase 1,2, mGluR1 or 5 = metabotropic glutamate receptor 1 or 5, ECD = extracellular domain, PLC = phospholipase C, PIP2 = phosphatidylinositol-4,5-bisphosphate, DAG = diacylglycerol, IP3 = inositol-1,4,5-triphosphate. Pink/Red = stimulatory pathways, Blue = inhibitory pathways.

being activated by endogenously released/leaked noradrenaline, this increases its physiological relevance with regards to the endogenous regulation of ERK 1,2 activation. However, further experiments would be needed to be conducted to confirm this hypothesis, in particular, the addition of a different competitive β-adrenoceptor agonist, which should also inhibit downstream ERK 1,2 activation if it was mediated by agonist stimulaton of the receptor, but not if the ERK 1,2 activation was due to a basal constitutive activity of the 7TMD of the receptor.

Contrary to the β-adrenoceptor antagonist-mediated inhibition of basal levels ERK 1,2 activation, I found that application of the mGluR5 antagonist, MPEP, enhanced basal levels of ERK 1,2 activation. This was particularly intriguing considering that addition of the joint mGluR1 and mGluR5 agonist, DHPG, also stimulated ERK 1,2 activation, whereas application of the two drugs together resulted in no increase in ERK 1,2 activation in my system. Other studies have shown that MPEP can also act as a positive allosteric modulator at mGluR4 receptors which are negatively coupled, through Gi, to adenylate cyclase (Mathiesen et al., 2003). Although this MPEP-mediated stimulation of mGluR4 may be occurring in synaptosomes, this is unlikely to account for the observed enhancement in ERK 1,2 activation, especially considering that I have also shown that stimulation of adenylate cyclase enhances ERK 1,2 phosphorylation. As discussed in Chapter 6, a possible explanation for the stimulatory effect of MPEP on downstream ERK 1,2 activation is that mGluR1 is tonically inhibited by mGluR5, leading to the attenuation of mGluR1-mediated stimulation of ERK 1,2 phosphorylation (see Figure 7.1). This is supported by recent studies which have found that the 7TMD portion of the Group I mGluRs may be 'active' in the absence of external glutamate binding to the extracellular domain (Gasparini et al., 2002; Malherbe et al., 2003). It would be interesting to investigate whether the proposed inhibition of mGluR1 signalling to ERK 1,2 seen here, is as a result of extracellular glutamate binding to mGluR5, or of its constitutive activity. It may be possible to further examine this phenomenon by utilising the chemical components of the glutamate release assay. The addition of the enzyme GDH with NADP would metabolise any 'leaked' glutamate present in the preparation, therefore allowing the examination of this pathway in the absence of any endogenous glutamate-mediated activation of mGluR5. Basal effects of the PKC inhibitors were also observed on ERK 1,2 phosphorylation levels however, the

two inhibitors used, calphostin C and Ro-32-0432, are not entirely specific and had opposing effects on ERK 1,2 activation, meaning it was not possible to attribute a particular function to PKC (Davies et al., 2000).

The signalling pathways shown to regulate basal ERK 1,2 phosphorylation levels in cerebrocortical synaptosomes have been illustrated in Figure 7.1. It should be noted that the purified synaptosomes consist of a heterogenous population of presynaptic nerve terminals, with only global levels of ERK 1,2 phosphorylation being measured. This means that, although represented on the same diagram, these pathways may or may not be present within the same nerve terminal.

Factors Regulating Acute ERK1,2 Phosphorylation.

The next section in this discussion examines the signalling pathways involved in regulating ERK 1,2 activation/phosphorylation following the application of exogenous ligands (Figure 7.2). Again, due to the heterogenous nature of the synaptosome preparation, it is not yet known whether all these signalling pathways are occurring within the same nerve terminal. The results described in the signalling pathways labelled 1-3 in Figure 7.2 suggest that there may be more than one method of communicating between cAMP and ERK 1,2 in cerebrocortical nerve terminals. In the first pathway, I have shown a β-adrenoceptor-mediated enhancement of ERK 1,2 activation via a PKA-dependent pathway. This is in agreement with some of the studies conducted using different preparations, such as in HEK 293 cells and in slices from the hippocampal CA1 region (Roberson et al., 1999; Schmitt and Stork, 2000). However, other studies have also shown an ability for β-adrenoceptors to stimulate ERK 1,2 phosphorylation in a manner independent of PKA, through direct activation of a Rap1-GEF by cAMP (de Rooij et al., 1998).

Although it is possible that this could be happening in cerebrocortical nerve terminals, I believe this is unlikely to be the main pathway mediating ERK 1,2 activation downstream of β-adrenoceptor stimulation as I found that two different PKA inhibitors were able to attenuate this signalling between β-adrenoceptor and ERK 1,2. Having said this, my results also suggest that the majority of the signalling occurring following forskolin/IBMX-mediated stimulation of ERK 1,2 phosphorylation is PKA-independent

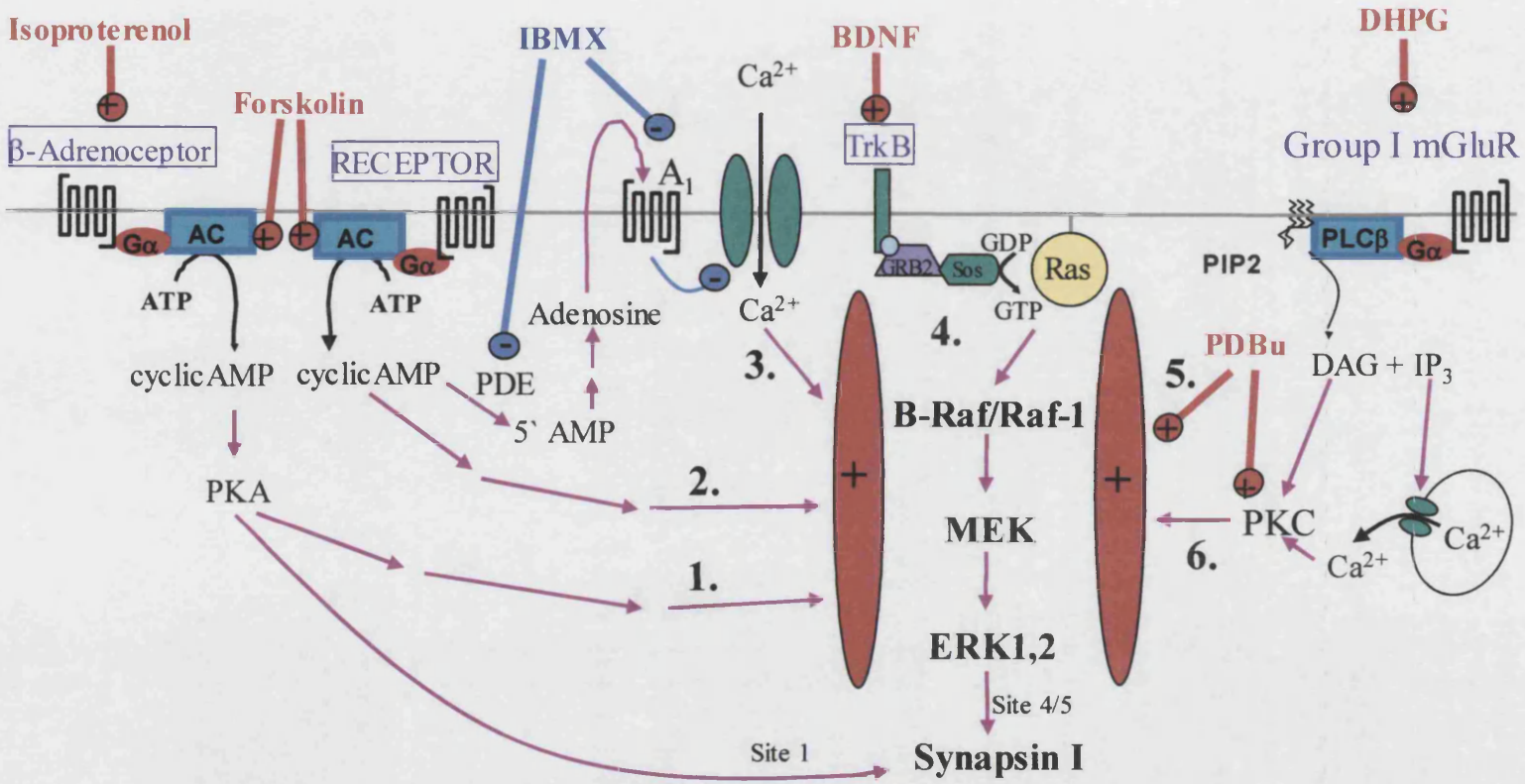


Figure 7.2 Schematic Diagram Illustrating the Possible Pathways Involved in Stimulating ERK 1,2 Activation/ Phosphorylation in Cerebrocortical Nerve Terminals. Abbreviations: AC = adenylate cyclase, cyclicAMP = cyclic adenosine monophosphate, PKA = cAMP-dependent protein kinase, PDE = phosphodiesterase, 5'AMP = 5'adenosine monophosphate, ERK = extracellular signal-regulated protein kinase, MEK = ERK kinase, PIP2 = phosphatidylinositol -4,5-bisphosphate, DAG = diacylglycerol, IP3 = inositol-1,4,5-triphosphate, PKC = protein kinase C, BDNF = brain-derived neurotrophic factor, PDBu = phorbol 12,13 dibutyrate , IBMX = 3-isobutyl-1-methylxanthine. Blue = inhibitory pathways, pink/red = stimulatory influences.

(Figure 7.2 pathways 2 and 3). This supports evidence from other laboratories that have found both PKA-dependent and –independent aspects to ERK 1,2 signalling downstream of AC activation (Ambrosini et al., 2000; Troadec et al., 2002; Baldassa et al., 2003; Lin et al., 2003). Though it is also possible that IBMX-mediated inhibition of adenosine A1 receptors is leading to disinhibition of Ca^{2+} channels, resulting in a Ca^{2+}-dependent increase in ERK 1,2 phosphorylation. Postsynaptic studies have demonstrated a clear dependency of ERK 1,2 signalling on extracellular Ca^{2+} (Rosen et al., 1994; Walker et al., 2003; Orban et al., 1999), and I have also shown a tonic regulation of ERK 1,2 by extracellular Ca^{2+}, suggesting the involvement of Ca^{2+} influx via Ca^{2+} channels. Further experiments are needed to delineate this PKA-independent component ERK 1,2 signalling, to identify the upstream receptors and to dissect the specific involvement of phosphodiesterase inhibition versus adenosine A1 receptor inhibition by IBMX. However, these results suggest that there are more, as yet unidentified, receptor signalling cascades involved in regulating ERK 1,2 phosphorylation levels in cerebrocortical nerve terminals. They also support the hypothesis that ERK 1,2 activation in cerebrocortical nerve terminals is a highly regulated process. It would also be interesting to investigate whether other receptor-linked PKA-dependent signalling pathways can also regulate ERK 1,2 phosphorylation in cerebrocortical nerve terminals, such as the Group II mGluRs (Huang et al., 1999).

The PKC signalling cascade has been shown to have many roles in the presynaptic regulation of glutamate release (Cousin et al., 1999; Berglund et al., 2002), as well as to be able to regulate signalling to ERK 1,2 in various systems (Roberson et al., 1999; Gao et al., 1999; Sundaresan et al., 1996). However, the studies conducted in this thesis were inconclusive with regards to PKC-dependency of ERK 1,2 phosphorylation in cerebrocortical nerve terminals. Nevertheless, both phorbol esters and Group I mGluR agonists, commonly found to stimulate PKC activation, were able to stimulate ERK 1,2 phosphorylation (Figure 7.2, pathways 5 and 6). Notwithstanding, it remains possible that the phorbol ester-mediated stimulation of ERK 1,2 is occurring through the activation of a DAG-dependent RasGEF (Lorenzo et al., 2000), and that Group I mGluR-mediated stimulation of ERK 1,2 is occurring through the activation of a Ca^{2+}-dependent RasGEF (Farnsworth et al., 1995), especially considering the results obtained using the preincubation protocol (Figure 7.3). A potential method for clarifying the role of PKC in these situations would be to use another PKC inhibitor (Davies et al., 2000).

Although the PKC inhibitors that have been used so far, and those that are currently available for use with short incubation protocols, are not necessarily completely specific, the use of a combination of inhibitors that function through different mechanisms could help to elucidate on the role of PKC in this system. For example, comparing the effects of the catalytic subunit inhibitors, Ro-32-0432 and calphostin C, with the effects of a regulatory subunit inhibitor, such as NPC 15437, could lead to the identification of common, and thus likely PKC-dependent, functions (Sullivan et al., 1992; Davies et al., 2000).

As mentioned above, purified cerebrocortical synaptosomes consist of a heterogenous population of nerve terminals. One possible method for identifying the proximity of signalling pathways in this system is through the use of additivity studies, in which signals utilising the same pool of ERK 1,2 will not have additive effects on ERK 1,2 phosphorylation when activated in parallel. The TrkB receptor-mediated signalling to ERK 1,2 (Figure 7.2, pathway 4) has already been identified as a pathway active in glutamatergic nerve terminals (Jovanovic et al., 2000), but was used here as a method of clarifying the location of β-adrenoceptor and Group I mGluR signalling to ERK 1,2. Additivity studies conducted in chapter 4 and in chapter 6 suggest that signalling from TrkB receptors and β-adrenoceptors regulates the same population of ERK 1,2, and that signalling from Group I mGluRs and TrkB receptors also regulates the same population of ERK 1,2. However, this cannot be extrapolated to say that β-adrenoceptors and Group I mGluRs signal to the same population of ERK 1,2, as the possibility that they may regulate different subsets of the TrkB regulated 'pool' has not been ruled out. It would be interesting to investigate whether these two receptors are able to cross-talk with the population of ERK 1,2 in cerebrocortical nerve terminals.

Forskolin/IBMX, isoproterenol, PDBu, and DHPG were all shown to enhance ERK 1,2 phosphorylation levels in cerebrocortical synaptosomes. The functional relevance of these signalling pathways was, in part, elucidated by examining a presynaptic downstream effector for ERK 1,2 signalling, synapsin I. Although, increases in ERK 1,2 phosphorylation did not always correlate with the enhancement of glutamate release from cerebrocortical nerve terminals, increases in the synapsin I phosphorylation sites regulated by ERK 1,2, did. It was interesting to observe that, although treatment with

DHPG enhanced ERK 1,2 phosphorylation levels, it did not enhance downstream synapsin I phosphorylation, which was correlated with a lack of effect of DHPG in the modulation of glutamate release. This was suggested from current results to be due to DHPG-induced increases in intracellular Ca^{2+} concentration leading to increases in Ca^{2+}-dependent phosphatase activity at the synapsin I phosphorylation site stimulated by ERK 1,2. The correlation of synapsin I phosphorylation levels at the site regulated by ERK 1,2, with the modulation of glutamate release from cerebrocortical nerve terminals, suggests that the functional importance of this signalling pathway may lie in regulation of the availability of glutamate-containing synaptic vesicle for release (Hilfiker et al., 1999). Although levels of ERK 1,2 phosphorylation seem to give a fairly good indication of a drugs ability to regulate glutamate release independently of Ca^{2+} channel regulation, they are not definitive, due to the opposing effects of phosphatases on site 4/5 synapsin I phosphorylation (Jovanovic et al., 2001). Further evidence for the involvement of ERK 1,2 in the regulation of glutamate release from nerve terminals has come from preliminary experiments that I have conducted. Results have shown that the cannabinoid receptor agonist, WIN 55,212-2, can not only decrease glutamate release from purified cerebrocortical nerve terminals, but can also decrease ERK 1,2 phosphorylation levels. This is interesting as it suggests that the proposed regulation of glutamate release by ERK 1,2 can be bidirectional, assuming a certain basal level of phosphorylation is present.

The working hypothesis that ERK 1,2 phosphorylation of site 4/5 on synapsin I, combined with the dephosphorylation regulated by calcineurin, contributes to the regulation of glutamate release through the tethering of synaptic vesicles, is supported by the data obtained in this thesis. However, levels of ERK 1,2 and synapsin I phosphorylation have only been correlated with glutamate release, and not directly implicated, in these studies. Recent work conducted in other laboratories has identified components of the exocytotic machinery that can be directly phosphorylated and regulated by PKA (Lonart et al., 2003; Nagy et al., 2004). This begs the question as to whether the Ca^{2+} channel independent regulation of glutamate release mediated by PKA and PKC is occurring through direct phosphorylation of the exocytotic machinery, rather than through regulation of the tethering of synaptic vesicles by synapsin I. Although phosphorylation of RIM1a by PKA has been shown to trigger LTP in the cerebellar parallel fibre synapse, the overexpression of a construct designed to mimic

constitutively active RIM1a it was not shown to regulate the EPSP amplitude (Lonart et al., 2003), questioning whether this phosphorylation really is sufficient for the induction of LTP in this system. PKA has also been shown to phosphorylate SNAP-25a to regulate vesicle priming and to possibly regulate the size of the readily releasable pool of synaptic vesicle in chromaffin cells (Nagy et al., 2004). However, the variability in the requirements for LTP induction and maintenance that has been shown to occur between synapses in different regions means that the relative importance of different signalling molecules in the modulation of presynaptic plasticity may also be subject to spatial regulation (Lonart and Sudhof, 1998). So, the apparent key requirement for PKA phosphorylation of RIM1a in cerebellar parallel fibre synapses, or the regulation of vesicle pool size by PKA phosphorylation of SNAP-25 in chromaffin cells, may not necessarily hold true for cerebrocortical synapses. Having said this, the possibility of direct regulation by PKA and PKC on the exocytotic machinery in cerebrocortical synaptosomes remains a tenable possibility and the relative contributions of these mechanisms, compared to the regulation of synaptic vesicle availability by synapsin I remains to be determined. Further experiments need to be conducted to confirm the roles of ERK 1,2 and synapsin I in the regulation of glutamate release downstream of β-adrenoceptor activation and phorbol ester treatment. These should perhaps be started by investigating the effects of the protein kinase A inhibitors on ionomycin-elicited glutamate release, to see if they correlate with the changes in ERK 1,2 phosphorylation levels.

The discussion so far has focused on synapsin I phosphorylation downstream of ERK 1,2 activation. However, it is also possible, and even likely, that ERK 1,2 is phosphorylating more than one downstream effector in nerve terminals. Studies conducted in other labs have suggested that ERK 1,2 can phosphorylate N-type Ca^{2+} channels and in doing so increase the Ca^{2+} influx into nerve terminals (Fitzgerald, 2002). It would be interesting to examine whether ERK 1,2 can also phosphorylate N-type Ca^{2+} channels in cerebrocortical nerve terminals, as well as to identify whether phosphorylation mediated by ERK 1,2 could also be involved in regulating other members of the synaptic vesicle exocytosis/endocytosis machinery.

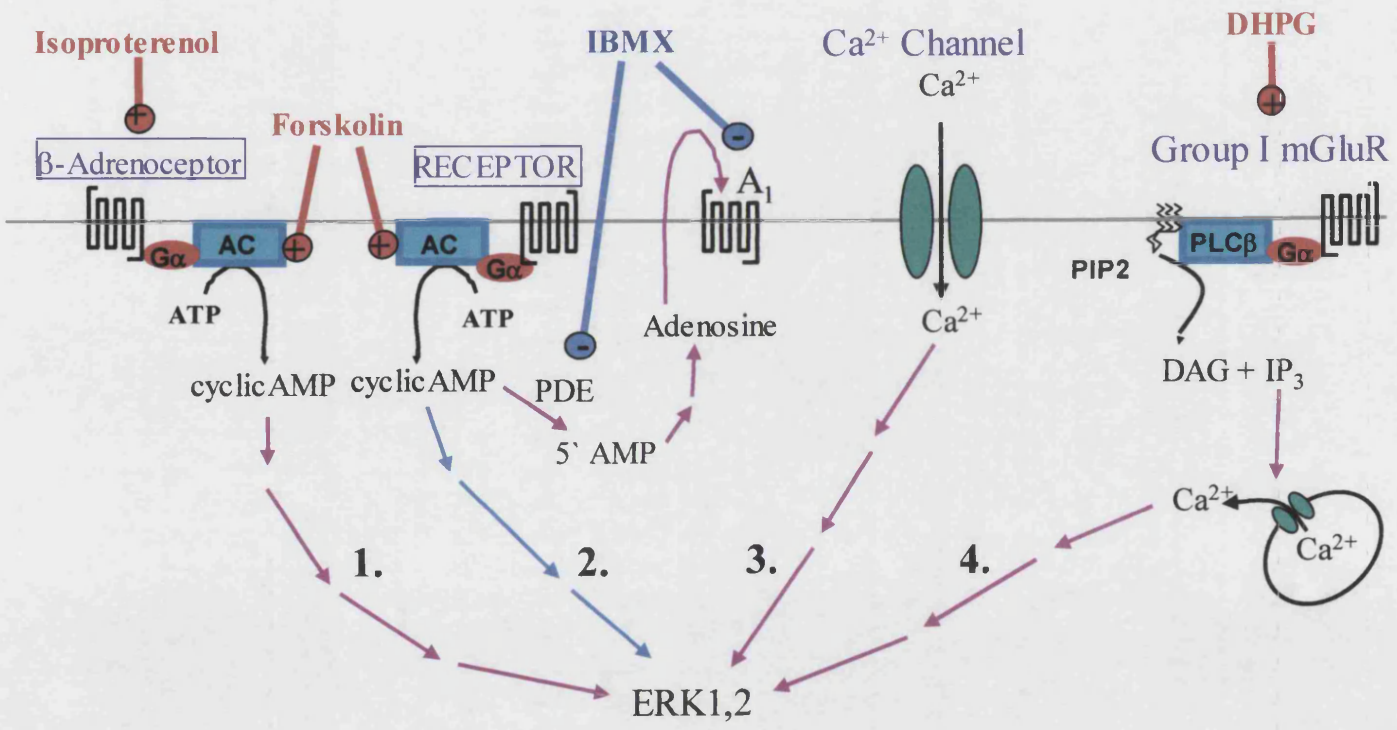


Figure 7.3 Schematic Diagram Illustrating the Pathways Identified, in this Thesis, that are Involved in Regulating Levels of ERK 1,2 Phosphorylation/Activation in Cerebrocortical Nerve Terminals Following Preincubation. Abbreviations: AC = adenylate cyclase, cyclicAMP = cyclic adenosine monophosphate, ATP = adenosine triphosphate, PDE = phosphodiesterase, 5'AMP = 5'adenosine monophosphate, ERK = extracellular signal-regulated protein kinase, PIP2 = phosphatidylinositol-4,5-bisphosphate, DAG = diacylglycerol, IP3 = inositol-1,4,5-triphosphate, Ca^{2+} = calcium, IBMX = 3-isobutyl-1-methylxanthine, DHPG = dihydroxyphenylglycine. Blue = inhibitory pathways, pink/red = stimulatory influences.

Factors Regulating Tonic ERK1,2 Phosphorylation

So far I have been examining the effects of acute cross-talk of signalling pathways. However, both β-adrenoceptors and Group I mGluRs have been shown to switch G-protein coupling following extended exposure to agonists. Daaka, et al., 1997 showed that the Gs-coupled β_2-adrenoceptor expressed in HEK 293 cells were phosphorylated by PKA in a feedback loop leading to switching of the receptor to Gi-coupling (Daaka et al., 1997). In contrast, the typically Gq-coupled Group I mGluRs can be phosphorylated by PKC, either in a feedback loop, or through cross-talk with each other, resulting in changes in the G-protein coupling (Gereau and Heinemann , 1998; Francesconi and Duvoisin, 2000; Poisik et al., 2003). As I have found that both the β-adrenoceptor and the Group I mGluRs are able to regulate basal levels of ERK 1,2 activation in purified cerebrocortical nerve terminals, it begged the question as to whether increased exposure to any endogenous ligands would change the nature of the exogenous agonist-mediated ERK 1,2 activation, perhaps through the modulation of G-protein coupling.

The tonic cross-talking properties of these pathways (as with the preincubation protocols) are illustrated in Figure 7.3. An interesting point, highlighted here, is that global enhancement of cAMP concentration results in an opposite effect on ERK 1,2 phosphorylation compared to the supposed localised enhancement following β-adrenoceptor stimulation (Figure 7.3, pathways 1 and 2). I found that stimulation of synaptosomes with forskolin/IBMX switches from having an acute stimulatory effect on ERK 1,2 phosphorylation levels to an inhibitory tonic effect, whereas the tonic β-adrenoceptor-mediated stimulation of ERK 1,2 activation remains. These results show that β-adrenoceptor signalling to ERK 1,2 in cerebrocortical synaptosomes is not subject to temporal regulation within the time points examined, but that the forskolin/IBMX-mediated signalling is. Studies conducted in other systems have shown that cAMP can regulate ERK 1,2 through more than one signalling pathway (Derkinderen et al., 2003; Roberson et al., 1999; Lin et al., 2003), and that PKA regulation of ERK 1,2 activation can at times be inhibitory (Konig et al., 2001; Mischak et al., 1996). The heterogenous nature of the synaptosome preparation means that these signalling pathways may not all be present in the same nerve terminal, and so could be dependent on terminal specificity, as has been demonstrated for cell type by Dhillon,

A.S. et al., (Dhillon et al., 2002). The specific requirements of this 'switching' of signalling pathways leading to ERK 1,2 regulation need to be examined further to determine their PKA- and adenosine-dependency.

The results obtained with the tonic regulation of ERK 1,2 by forskolin/IBMX and β-adrenoceptor agonists are in contrast to the phorbol ester and Group I mGluR-mediated pathways, which appear to share similar properties regarding their modulation of ERK 1,2 activity. Differences in the temporal regulation of ERK 1,2 activation by forskolin/IBMX, has highlighted difficulties with relating results between different incubation protocols. So, although the results obtained with Group I mGluR agonists and inhibitors of this pathway appear to be consistent between incubation protocols, it is perhaps wise to exercise caution in assuming that they really are the same.

Studies conducted using the preincubation protocol have identified roles for extracellular Ca^{2+}, in modulating basal levels of ERK 1,2 phosphorylation, and for intracellular Ca^{2+}, in regulating Group I mGluR signalling to ERK 1,2 (Figure 7.3, pathways 3 and 4). It would be interesting to further investigate the locus of the cross-talk of Ca^{2+} with the ERK 1,2 signalling cascade. One likely locus for the intracellular Ca^{2+} action is perhaps via a RasGEF, such as RasGRF, which has not only been localised to presynaptic nerve terminals, but has also been shown to be involved in regulating synaptic plasticity, albeit postsynaptically in this case (Walker et al., 2003). As RasGRF knockout mice have been developed (Brambilla et al., 1997), the simplest method for elucidating on the RasGRF-dependency of Group I mGluR signalling to ERK 1,2 via intracellular Ca^{2+} stores would be to examine this pathway in cerebrocortical nerve terminals from the knockout mice. The Ca^{2+}-dependency of ERK 1,2 activation downstream of Group I mGluR stimulation also provided a mechanism for the lack of net change in synapsin I site 4/5 phosphorylation downstream of ERK 1,2 activation, as these sites on synapsin I are dephosphorylated by the Ca^{2+}-dependent phosphatase, calcineurin. Experiments using the calcineurin inhibitor, FK506, would be needed to confirm the role of this pathway in regulating synapsin I phosphorylation downstream of Group I mGluR activation. It would also be interesting to discover at what point extracellular Ca^{2+} is regulating ERK 1,2 basal phosphorylation following a preincubation protocol. Considering the ability of β-adrenoceptors to mediate basal

ERK 1,2 phosphorylation following a standard incubation protocol via a PKA-dependent pathway, it is possible that extracellular Ca^{2+} could be involved in regulating the activation of a Ca^{2+}-dependent adenylate cyclase (Mons et al., 1998). Further experiments could use the preincubation protocol with BAPTA-AM and EGTA to examine the Ca^{2+}-dependency of β-adrenoceptor signalling to ERK 1,2 in cerebrocortical nerve terminals.

Although I have identified β-adrenoceptors and Group I mGluRs as being upstream of ERK 1,2 in cerebrocortical nerve terminals, I have not investigated the specific involvement or contribution of Ras and Raf in signalling between these two stages. The possible levels through which these signalling cascades could be cross-talking with the ERK 1,2 pathway have been discussed in each chapter and so, will not be repeated here. However, further elucidation on the levels of interaction could be obtained with future experiments using newly developed Raf-1 inhibitors (Lackey et al., 2000) to describe whether these receptor-linked signalling cascades require the activation of Raf-1 to mediate their stimulation of ERK 1,2 phosphorylation. Investigating the dependency of these signalling cascades on Ras activation would be a little more complicated, however, as inhibitors are not commonly available. Notwithstanding, it would be possible to measure Ras activation in nerve terminals using an immunoprecipitation assay, whereby glutathione sepharose beads are primed with Raf binding domains which will specifically bind to activated, GTP bound, Ras (de Rooij and Bos, 1997).

In conclusion, the evidence presented in this thesis has identified ERK 1,2 as a highly regulated presynaptic nerve terminal protein kinase, with possible roles in regulating synaptic transmission. It appears that cross-talk of presynaptic signalling cascades with the ERK 1,2 pathway are numerous and are coupled to, though not solely limited to, cascades found downstream of β-adrenoceptors or Group I mGluRs. These results show that ERK 1,2 is an important presynaptic signalling molecule which, with its downstream regulation of synapsin I phosphorylation, has the potential to regulate presynaptic plasticity.

Bibliography

Abe MK, Kahle KT, Saelzler MP, Orth K, Dixon JE, Rosner MR (2001) ERK7 Is an Autoactivated Member of the MAPK Family. J Biol Chem 276: 21272-21279.

Abe MK, Kuo WL, Hershenson MB, Rosner MR (1999) Extracellular Signal-Regulated Kinase 7á(ERK7), a Novel ERK with a C-Terminal Domain That Regulates Its Activity, Its Cellular Localization, and Cell Growth. Mol Cell Biol 19: 1301-1312.

Adams JA, McGlone ML, Gibson R, Taylor SS (1995) Phosphorylation modulates catalytic function and regulation in the cAMP-dependent protein kinase. Biochemistry (Mosc) 34: 2447-2454.

Adams JP, Roberson ED, English JD, Selcher JC, Sweatt JD (2000) MAPK regulation of gene expression in the central nervous system. Acta Neurobiol Exp 60: 377-394.

Aiyar N, Disa J, Dang K, Pronin AN, Benovic JL, Nambi P (2000) Involvement of G protein-coupled receptor kinase-6 in desensitization of CGRP receptors. Eur J Pharmacol 403: 1-7.

Alagarsamy S, Sorensen SD, Conn PJ (2001) Coordinate regulation of metabotropic glutamate receptors. Curr Opin Neurobiol 11: 357-362.

Alessi DR, Gomez N, Moorhead G, Lewis T, Keyse SM, Cohen P (1995) Inactivation of p42 MAP kinase by protein phosphatase 2A and a protein tyrosine phosphatase, but not CL100, in various cell lines. Curr Biol 5: 283-295.

Aley KO, Martin A, McMahon T, Mok J, Levine JD, Messing RO (2001) Nociceptor sensitization by extracellular signal-regulated kinases. J Neurosci 21: 6933-6939.

Allen PB, Ouimet CC, Greengard P (1997) Spinophilin, a novel protein phosphatase 1ábinding protein localized to dendriticáspines. PNAS 94: 9956-9961.

Allgaier C, Von Kugelgen O, Hertting G (1986) Enhancement of noradrenaline release by 12-O-tetradecanoyl phorbol-13-acetate, an activator of protein kinase C. Eur J Pharmacol 129: 389-392.

Allgaier C, Daschmann B, Huang HY, Hertting G (1988) Protein kinase C and presynaptic modulation of acetylcholine release in rabbit hippocampus. Br J Pharmacol 93: 525-534.

Ambrosini A, Tininini S, Barassi A, Racagni G, Sturani E, Zippel R (2000) cAMP cascade leads to Ras activation in cortical neurons. Brain Res Mol Brain Res 75: 54-60.

Anderson D, Koch CA, Grey L, Ellis C, Moran MF, Pawson T (1990) Binding of SH2 domains of phospholipase C gamma 1, GAP, and Src to activated growth factor receptors. Science 250: 979-982.

Anderson RA, Boronenkov IV, Doughman SD, Kunz J, Loijens JC (1999) Phosphatidylinositol Phosphate Kinases, a Multifaceted Family of Signaling Enzymes. J Biol Chem 274: 9907-9910.

Andjelkovic M, Jakubowicz T, Cron P, Ming XF, Han JW, Hemmings BA (1996) Activation and phosphorylation of a pleckstrin homology domain containing protein kinase (RAC-PK/PKB) promoted by serum and protein phosphatase inhibitors. PNAS 93: 5699-5704.

Ango F, Pin JP, Tu JC, Xiao B, Worley PF, Bockaert J, Fagni L (2000) Dendritic and axonal targeting of type 5 metabotropic glutamate receptor is regulated by homer1 proteins and neuronal excitation. J Neurosci 20: 8710-8716.

Ango F, Prezeau L, Muller T, Tu JC, Xiao B, Worley PF, Pin JP, Bockaert J, Fagni L (2001) Agonist-independent activation of metabotropic glutamate receptors by the intracellular protein Homer. Nature 411: 962-965.

Anthony FA, Winkler MA, Edwards HH, Cheung WY (1988) Quantitative subcellular localization of calmodulin-dependent phosphatase in chick forebrain. J Neurosci 8: 1245-1253.

Arcoleo JP, Weinstein IB (1985) Activation of protein kinase C by tumor promoting phorbol esters, teleocidin and aplysiatoxin in the absence of added calcium. Carcinogenesis 6: 213-217.

Augustine GJ (2001) How does calcium trigger neurotransmitter release? Curr Opin Neurobiol 11: 320-326.

Azzi M, Charest PG, Angers S, Rousseau G, Kohout T, Bouvier M, Pineyro G (2003) Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. Proc Natl Acad Sci U S A 100: 11406-11411.

Baker JG, Hall IP, Hill SJ (2003) Agonist and inverse agonist actions of beta-blockers at the human beta 2-adrenoceptor provide evidence for agonist-directed signaling. Mol Pharmacol 64: 1357-1369.

Baldassa S, Zippel R, Sturani E (2003) Depolarization-induced signaling to Ras, Rap1 and MAPKs in cortical neurons. Brain Res Mol Brain Res 119: 111-122.

Ballesteros JA, Jensen AD, Liapakis G, Rasmussen SG, Shi L, Gether U, Javitch JA (2001) Activation of the beta 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. J Biol Chem 276: 29171-29177.

Barclay JW, Craig TJ, Fisher RJ, Ciufo LF, Evans GJ, Morgan A, Burgoyne RD (2003) Phosphorylation of Munc18 by protein kinase C regulates the kinetics of exocytosis. J Biol Chem 278: 10538-10545.

Barrie AP, Nicholls DG, Sanchez-Prieto J, Sihra TS (1991) An ion channel locus for the protein kinase C potentiation of transmitter glutamate release from guinea pig cerebrocortical synaptosomes. J Neurochem 57: 1398-1404.

Bartmann P, Jackisch R, Hertting G, Allgaier C (1989) A role for protein kinase C in the electrically evoked release of [3H] gamma-aminobutyric acid in rabbit caudate nucleus. Naunyn Schmiedebergs Arch Pharmacol 339: 302-305.

Baude A, Nusser Z, Roberts JD, Mulvihill E, McIlhinney RA, Somogyi P (1993) The metabotropic glutamate receptor (mGluR1 alpha) is concentrated at perisynaptic membrane of neuronal subpopulations as detected by immunogold reaction. Neuron 11: 771-787.

Bear MF, Malenka RC (1994) Synaptic plasticity: LTP and LTD. Curr Opin Neurobiol 4: 389-399.

Beebe SJ (1994) The cAMP-dependent protein kinases and cAMP signal transduction. Semin Cancer Biol 5: 285-294.

Bell RM, Burns DJ (1991) Lipid activation of protein kinase C. J Biol Chem 266: 4661-4664.

Benedetti M, Levi A, Chao MV (1993) Differential expression of nerve growth factor receptors leads to altered binding affinity and neurotrophin responsiveness. Proc Natl Acad Sci U S A 90: 7859-7863.

Berglund K, Midorikawa M, Tachibana M (2002) Increase in the pool size of releasable synaptic vesicles by the activation of protein kinase C in goldfish retinal bipolar cells. J Neurosci 22: 4776-4785.

Berkeley JL, Levey AI (2003) Cell-specific extracellular signal-regulated kinase activation by multiple G protein-coupled receptor families in hippocampus. Mol Pharmacol 63: 128-135.

Berridge MJ (1993) Inositol trisphosphate and calcium signalling. Nature 361: 315-325.

Berruti G (2003) CAMP activates Rap1 in differentiating mouse male germ cells: a new signaling pathway mediated by the cAMP-activated exchange factor Epac? Cell Mol Biol 49: 381-388.

Berthet J, Rall TW, Sutherland EW (1957) The relationship of epinephrine and glucagon to liver phosphorylase. IV. Effect of epinephrine and glucagon on the reactivation of phosphorylase in liver homogenates. J Biol Chem 224: 463-475.

Betz A, Thakur P, Junge HJ, Ashery U, Rhee JS, Scheuss V, Rosenmund C, Rettig J, Brose N (2001) Functional interaction of the active zone proteins Munc13-1 and RIM1 in synaptic vesicle priming. Neuron 30: 183-196.

Bibel M, Hoppe E, Barde YA (1999) Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR. EMBO J 18: 616-622.

Birchall AM, Bishop J, Bradshaw D, Cline A, Coffey J, Elliott LH, Gibson VM, Greenham A, Hallam TJ, Harris W (1994) Ro 32-0432, a selective and orally active inhibitor of protein kinase C prevents T-cell activation. J Pharmacol Exp Ther 268: 922-929.

Bito H, Deisseroth K, Tsien RW (1996) CREB phosphorylation and dephosphorylation: a Ca(2+)- and stimulus duration-dependent switch for hippocampal gene expression. Cell 87: 1203-1214.

Blake RA, Broome MA, Liu X, Wu J, Gishizky M, Sun L, Courtneidge SA (2000) SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. Mol Cell Biol 20: 9018-9027.

Bloom O, Evergren E, Tomilin N, Kjaerulff O, Low P, Brodin L, Pieribone VA, Greengard P, Shupliakov O (2003) Colocalization of synapsin and actin during synaptic vesicle recycling. The Journal of Cell Biology 161: 737-747.

Bokemeyer D, Sorokin A, Yan M, Ahn NG, Templeton DJ, Dunn MJ (1996) Induction of Mitogen-activated Protein Kinase Phosphatase 1 by the Stress-activated Protein Kinase Signaling Pathway but Not by Extracellular Signal-regulated Kinase in Fibroblasts. J Biol Chem 271: 639-642.

Bolwell GP (1995) Cyclic AMP, the reluctant messenger in plants. Trends Biochem Sci 20: 492-495.

Bonanno G, Gemignani A, Fedele E, Fontana G, Raiteri M (1991) gamma-Aminobutyric acidB receptors mediate inhibition of somatostatin release from cerebrocortex nerve terminals. J Pharmacol Exp Ther 259: 1153-1157.

Bonanno G, Raiteri M (1992) Functional evidence for multiple gamma-aminobutyric acidB receptor subtypes in the rat cerebral cortex. J Pharmacol Exp Ther 262: 114-118.

Bossemeyer D, Engh RA, Kinzel V, Ponstingl H, Huber R (1993) Phosphotransferase and substrate binding mechanism of the cAMP-dependent protein kinase catalytic subunit from porcine heart as deduced from the 2.0 A structure of the complex with Mn2+ adenylyl imidodiphosphate and inhibitor peptide PKI(5-24). EMBO J 12: 849-859.

Botelho LH, Rothermel JD, Coombs RV, Jastorff B (1988) cAMP analog antagonists of cAMP action. Methods Enzymol 159: 159-172.

Botsford J, Harman JG (1992) Cyclic AMP in prokaryotes. Microbiol Rev 56: 100-122.

Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH, Yancopoulos GD (1991) ERKs: a family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 65: 663-675.

Bourne HR (1997) How receptors talk to trimeric G proteins. Curr Opin Cell Biol 9: 134-142.

Bowery NG (1989) GABA(B) receptors and their significance in mammalian pharmacology. Trends Pharmacol Sci 10: 401-406.

Bowery NG, Hill DR, Hudson AL, Doble A, Middlemiss DN, Shaw J, Turnbull M (1980) (-)Baclofen decreases neurotransmitter release in the mammalian CNS by an action at a novel GABA receptor. Nature 283: 92-94.

Bowie D, Lange GD (2002) Functional stoichiometry of glutamate receptor desensitization. J Neurosci 22: 3392-3403.

Bradford HF (1970a) Metabolic response of synaptosomes to electrical stimulation: release of amino acids. Brain Res 19: 239-247.

Bradford HF (1970b) Responses of synaptosomes to electrical stimulation. Biochem J 117: 36P.

Bradford HF, Bennett GW, Thomas AJ (1973) Depolarizing stimuli and the release of physiologically active amino acids from suspensions of mammalian synaptosomes. J Neurochem 21: 495-505.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248-254.

Bradley J, Li J, Davidson N, Lester HA, Zinn K (1994) Heteromeric olfactory cyclic nucleotide-gated channels: a subunit that confers increased sensitivity to cAMP. Proc Natl Acad Sci U S A 91: 8890-8894.

Bradley SR, Levey AI, Hersch SM, Conn PJ (1996) Immunocytochemical localization of group III metabotropic glutamate receptors in the hippocampus with subtype-specific antibodies. J Neurosci 16: 2044-2056.

Brambilla R, Gnesutta N, Minichiello L, White G, Roylance AJ, Herron CE, Ramsey M, Wolfer DP, Cestari V, Rossi-Arnaud C, Grant SG, Chapman PF, Lipp HP, Sturani E, Klein R (1997) A role for the Ras signalling pathway in synaptic transmission and long-term memory. Nature 390: 281-286.

Brautigan DL (1995) Flicking the switches: phosphorylation of serine/threonine protein phosphatases. Semin Cancer Biol 6: 211-217.

Brodie C, Bogi K, Acs P, Lazarovici P, Petrovics G, Anderson WB, Blumberg PM (1999) Protein kinase C-epsilon plays a role in neurite outgrowth in response to epidermal growth factor and nerve growth factor in PC12 cells. Cell Growth Differ 10: 183-191.

Brogley MA, Cruz M, Cheung HS (1999) Basic calcium phosphate crystal induction of collagenase 1 and stromelysin expression is dependent on a p42/44 mitogen-activated protein kinase signal transduction pathway. J Cell Physiol 180: 215-224.

Brondello JM, Brunet A, Pouyssegur J, McKenzie FR (1997) The Dual Specificity Mitogen-activated Protein Kinase Phosphatase-1 and -2Are Induced by the p42/p44MAPK Cascade. J Biol Chem 272: 1368-1376.

Brose N, Petrenko AG, Sudhof TC, Jahn R (1992) Synaptotagmin: a calcium sensor on the synaptic vesicle surface. Science 256: 1021-1025.

Brose N, Rosenmund C, Rettig J (2000) Regulation of transmitter release by Unc-13 and its homologues. Curr Opin Neurobiol 10: 303-311.

Brunger AT (2000) Structural insights into the molecular mechanism of Ca(2+)-dependent exocytosis. Curr Opin Neurobiol 10: 293-302.

Bruns RF, Miller FD, Merriman RL, Howbert JJ, Heath WF, Kobayashi E, Takahashi I, Tamaoki T, Nakano H (1991) Inhibition of protein kinase C by calphostin C is light-dependent. Biochem Biophys Res Commun 176: 288-293.

Burack WR, Sturgill TW (1997) The activating dual phosphorylation of MAPK by MEK is nonprocessive. Biochemistry 36: 5929-5933.

Burgering BM, de Vries-Smits AM, Medema RH, van Weeren PC, Tertoolen LG, Bos JL (1993) Epidermal growth factor induces phosphorylation of extracellular signal-regulated kinase 2 via multiple pathways. Mol Cell Biol 13: 7248-7256.

Burnette WN (1981) "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 112: 195-203.

Busca R, Abbe P, Mantoux F, Aberdam E, Peyssonnaux C, Eychene A, Ortonne JP, Ballotti R (2000) Ras mediates the cAMP-dependent activation of extracellular signal-regulated kinases (ERKs) in melanocytes. EMBO J 19: 2900-2910.

Camps M, Nichols A, Arkinstall S (2000) Dual specificity phosphatases: a gene family for control of MAP kinase function. FASEB J 14: 6-16.

Cao W, Luttrell LM, Medvedev AV, Pierce KL, Daniel KW, Dixon TM, Lefkowitz RJ, Collins S (2000) Direct binding of activated c-Src to the beta 3-adrenergic receptor is required for MAP kinase activation. J Biol Chem 275: 38131-38134.

Carroll FY, Stolle A, Beart PM, Voerste A, Brabet I, Mauler F, Joly C, Antonicek H, Bockaert J, Muller T, Pin JP, Prezeau L (2001) BAY36-7620: a potent non-competitive mGlu1 receptor antagonist with inverse agonist activity. Mol Pharmacol 59: 965-973.

Cartmell J, Schoepp DD (2000) Regulation of neurotransmitter release by metabotropic glutamate receptors. J Neurochem 75: 889-907.

Castagna M (1983) Transmembrane signalling systems. Biomed Pharmacother 37: 380-386.

Castillo PE, Schoch S, Schmitz F, Sudhof TC, Malenka RC (2002) RIM1alpha is required for presynaptic long-term potentiation. Nature 415: 327-330.

Castro-Alamancos MA, Calcagnotto ME (1999) Presynaptic long-term potentiation in corticothalamic synapses. J Neurosci 19: 9090-9097.

Catterall WA (2000) Structure and regulation of voltage-gated Ca2+ channels. Annu Rev Cell Dev Biol 16: 521-555.

Chang L, Karin M (2001) Mammalian MAP kinase signalling cascades. Nature 410: 37-40.

Chapman ER, Desai RC, Davis AF, Tornehl CK (1998) Delineation of the oligomerization, AP-2 binding, and synprint binding region of the C2B domain of synaptotagmin. J Biol Chem 273: 32966-32972.

Chapman ER, Hanson PI, An S, Jahn R (1995) Ca2+ regulates the interaction between synaptotagmin and syntaxin 1. J Biol Chem 270: 23667-23671.

Charles CH, Abler AS, Lau LF (1992) cDNA sequence of a growth factor-inducible immediate early gene and characterization of its encoded protein. Oncogene 7: 187-190.

Chavez-Noriega LE, Stevens CF (1994) Increased transmitter release at excitatory synapses produced by direct activation of adenylate cyclase in rat hippocampal slices. J Neurosci 14: 310-317.

Chen C, Regehr WG (1997) The mechanism of cAMP-mediated enhancement at a cerebellar synapse. J Neurosci 17: 8687-8694.

Chen CY, Gherzi R, Andersen JS, Gaietta G, Jurchott K, Royer HD, Mann M, Karin M (2000) Nucleolin and YB-1 are required for JNK-mediated interleukin-2 mRNA stabilization during T-cell activation. Genes Dev 14: 1236-1248.

Chen YA, Scales SJ, Patel SM, Doung YC, Scheller RH (1999) SNARE complex formation is triggered by Ca2+ and drives membrane fusion. Cell 97: 165-174.

Chen Y, Deng L, Maeno-Hikichi Y, Lai M, Chang S, Chen G, Zhang JF (2003) Formation of an endophilin-Ca2+ channel complex is critical for clathrin-mediated synaptic vesicle endocytosis. Cell 115: 37-48.

Chen Y, Harry A, Li J, Smit MJ, Bai X, Magnusson R, Pieroni JP, Weng G, Iyengar R (1997) Adenylyl cyclase 6 is selectively regulated by protein kinase A phosphorylation in a region involved in Galphas stimulation. Proc Natl Acad Sci U S A 94: 14100-14104.

Cheng M, Zhen E, Robinson MJ, Ebert D, Goldsmith E, Cobb MH (1996) Characterization of a Protein Kinase that Phosphorylates Serine 189 of the Mitogen-activated Protein Kinase Homolog ERK3. J Biol Chem 271: 12057-12062.

Cheng X, Ma Y, Moore M, Hemmings BA, Taylor SS (1998) Phosphorylation and activation of cAMP-dependent protein kinase by phosphoinositide-dependent protein kinase. Proc Natl Acad Sci U S A 95: 9849-9854.

Chheda MG, Ashery U, Thakur P, Rettig J, Sheng ZH (2001) Phosphorylation of Snapin by PKA modulates its interaction with the SNARE complex. Nat Cell Biol 3: 331-338.

Chi P, Greengard P, Ryan TA (2001) Synapsin dispersion and reclustering during synaptic activity. Nat Neurosci 4: 1187-1193.

Chi P, Greengard P, Ryan TA (2003) Synaptic vesicle mobilization is regulated by distinct synapsin I phosphorylation pathways at different frequencies. Neuron 38: 69-78.

Chidiac P, Hebert TE, Valiquette M, Dennis M, Bouvier M (1994) Inverse agonist activity of beta-adrenergic antagonists. Mol Pharmacol 45: 490-499.

Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, Hidaka H (1990) Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. J Biol Chem 265: 5267-5272.

Chittajallu R, Vignes M, Dev KK, Barnes JM, Collingridge GL, Henley JM (1996) Regulation of glutamate release by presynaptic kainate receptors in the hippocampus. Nature 379: 78-81.

Choe ES, Chung KT, Mao L, Wang JQ (2002) Amphetamine increases phosphorylation of extracellular signal-regulated kinase and transcription factors in the rat striatum via group I metabotropic glutamate receptors. Neuropsychopharmacology 27: 565-575.

Choe ES, Wang JQ (2001a) Group I metabotropic glutamate receptor activation increases phosphorylation of cAMP response element-binding protein, Elk-1, and extracellular signal-regulated kinases in rat dorsal striatum. Brain Research Molecular Brain Research 94: 75-84.

Choe ES, Wang JQ (2001b) Group I metabotropic glutamate receptors control phosphorylation of CREB, Elk-1 and ERK via a CaMKII-dependent pathway in rat striatum. Neurosci Lett 313: 129-132.

Choe ES, Wang JQ (2002) Regulation of transcription factor phosphorylation by metabotropic glutamate receptor-associated signaling pathways in rat striatal neurons. Neuroscience 114: 557-565.

Ciruela F, Soloviev MM, McIlhinney RA (1999) Co-expression of metabotropic glutamate receptor type 1alpha with homer-1a/Vesl-1S increases the cell surface expression of the receptor. Biochem J 341: 795-803.

Clapham DE, Neer EJ (1997) G protein beta gamma subunits. Annu Rev Pharmacol Toxicol 37: 167-203.

Cobb MH, Goldsmith EJ (1995) How MAP Kinases Are Regulated. J Biol Chem 270: 14843-14846.

Codina J, Hildebrandt J, Iyengar R, Birnbaumer L, Sekura RD, Manclark CR (1983) Pertussis toxin substrate, the putative Ni component of adenylyl cyclases, is an alpha beta heterodimer regulated by guanine nucleotide and magnesium. Proc Natl Acad Sci U S A 80: 4276-4280.

Coffey ET, Herrero I, Sihra TS, Sanchez-Prieto J, Nicholls DG (1994) Glutamate exocytosis and MARCKS phosphorylation are enhanced by a metabotropic glutamate receptor coupled to a protein kinase C synergistically activated by diacylglycerol and arachidonic acid. J Neurochem 63: 1303-1310.

Coffey ET, Sihra TS, Nicholls DG (1993) Protein kinase C and the regulation of glutamate exocytosis from cerebrocortical synaptosomes. J Biol Chem 268: 21060-21065.

Cohen PT (1997) Novel protein serine/threonine phosphatases: variety is the spice of life. Trends Biochem Sci 22: 245-251.

Cooper JA, Hunter T (1981) Similarities and differences between the effects of epidermal growth factor and Rous sarcoma virus. J Cell Biol 91: 878-883.

Cooper JA, Hunter T (1983) Identification and characterization of cellular targets for tyrosine protein kinases. J Biol Chem 258: 1108-1115.

Corbin JD, Francis SH (2002) Pharmacology of phosphodiesterase-5 inhibitors. Int J Clin Pract 56: 453-459.

Corbit KC, Trakul N, Eves EM, Diaz B, Marshall M, Rosner MR (2003) Activation of Raf-1 signaling by protein kinase C through a mechanism involving Raf kinase inhibitory protein. J Biol Chem 278: 13061-13068.

Correia-de-Sa P, Ribeiro JA (1994) Tonic adenosine A2A receptor activation modulates nicotinic autoreceptor function at the rat neuromuscular junction. Eur J Pharmacol 271: 349-355.

Corrodi H, Persson H, Carlsson A, Roberts J (1963) A new series of substances which block the adrenergic beta-receptors. J Med Chem 122: 751-755.

Corvera S, D'Arrigo A, Stenmark H (1999) Phosphoinositides in membrane traffic. Curr Opin Cell Biol 11: 460-465.

Cousin MA, Malladi CS, Tan TC, Raymond CR, Smillie KJ, Robinson PJ (2003) Synapsin I-associated phosphatidylinositol 3-kinase mediates synaptic vesicle delivery to the readily releasable pool. J Biol Chem 278: 29065-29071.

Cousin MA, McLaughlin M, Nicholls DG (1999) Protein kinase C modulates field-evoked transmitter release from cultured rat cerebellar granule cells via a dendrotoxin-sensitive K+ channel. Eur J Neurosci 11: 101-109.

Cousin MA, Robinson PJ (2000) Two mechanisms of synaptic vesicle recycling in rat brain nerve terminals. J Neurochem 75: 1645-1653.

Cousin MA, Robinson PJ (2001) The dephosphins: dephosphorylation by calcineurin triggers synaptic vesicle endocytosis. Trends Neurosci 24: 659-665.

Cousin MA, Tan TC, Robinson PJ (2001) Protein phosphorylation is required for endocytosis in nerve terminals: potential role for the dephosphins dynamin I and synaptojanin, but not AP180 or amphiphysin. J Neurochem 76: 105-116.

Coussens L, Parker PJ, Rhee L, Yang-Feng TL, Chen E, Waterfield MD, Francke U, Ullrich A (1986) Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. Science 233: 859-866.

Craxton A, Shu G, Graves JD, Saklatvala J, Krebs EG, Clark EA (1998) p38 MAPK Is Required for CD40-Induced Gene Expression and Proliferation in B Lymphocytes. J Immunol 161: 3225-3236.

Cremona O, De Camilli P (1997) Synaptic vesicle endocytosis. Curr Opin Neurobiol 7: 323-330.

Cremona O, Di Paolo G, Wenk MR, Luthi A, Kim WT, Takei K, Daniell L, Nemoto Y, Shears SB, Flavell RA, McCormick DA, De Camilli P (1999) Essential role of phosphoinositide metabolism in synaptic vesicle recycling. Cell 99: 179-188.

Crespo P, Xu N, Simonds WF, Gutkind JS (1994) Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. Nature 369: 418-420.

Cunha RA, Correia-de-Sa P, Sebastiao AM, Ribeiro JA (1996) Preferential activation of excitatory adenosine receptors at rat hippocampal and neuromuscular synapses by adenosine formed from released adenine nucleotides. Br J Pharmacol 119: 253-260.

Cunningham ME, Stephens RM, Kaplan DR, Greene LA (1997) Autophosphorylation of activation loop tyrosines regulates signaling by the TRK nerve growth factor receptor. J Biol Chem 272: 10957-10967.

Czernik AJ, Girault JA, Nairn AC, Chen J, Snyder G, Kebabian J, Greengard P (1991) Production of phosphorylation state-specific antibodies. Methods Enzymol 201: 264-283.

Czernik AJ, Pang DT, Greengard P (1987) Amino acid sequences surrounding the cAMP-dependent and calcium/calmodulin-dependent phosphorylation sites in rat and bovine synapsin I. Proc Natl Acad Sci U S A 84: 7518-7522.

Daaka Y, Luttrell LM, Ahn S, Della Rocca GJ, Ferguson SS, Caron MG, Lefkowitz RJ (1998) Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. J Biol Chem 273: 685-688.

Daaka Y, Luttrell LM, Lefkowitz RJ (1997) Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. Nature 390: 88-91.

Dai Y, Yu C, Singh V, Tang L, Wang Z, McInistry R, Dent P, Grant S (2001) Pharmacological inhibitors of the mitogen-activated protein kinase (MAPK) kinase/MAPK cascade interact synergistically with UCN-01 to induce mitochondrial dysfunction and apoptosis in human leukemia cells. Cancer Res 61: 5106-5115.

Dale L, Babwah AV, Bhattacharya M, Kelvin DJ, Ferguson SS (2001) Spatial-temporal patterning of metabotropic glutamate receptor-mediated inositol 1,4,5-triphosphate, calcium, and protein kinase C oscillations: protein kinase C-dependent receptor phosphorylation is not required. J Biol Chem 276: 35900-35908.

Dale L, Bhattacharya M, Anborgh PH, Murdoch B, Bhatia M, Nakanishi S, Ferguson SS (2000) G protein-coupled receptor kinase-mediated desensitization of metabotropic glutamate receptor 1A protects against cell death. J Biol Chem 275: 38213-38220.

Davies SP, Reddy H, Caivano M, Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351: 95-105.

Davis AF, Bai J, Fasshauer D, Wolowick MJ, Lewis JL, Chapman ER (1999) Kinetics of synaptotagmin responses to Ca2+ and assembly with the core SNARE complex onto membranes. Neuron 24: 363-376.

De Belleroche JS, Bradford HF (1977) On the site of origin of transmitter amino acids released by depolarization of nerve terminals in vitro. J Neurochem 29: 335-343.

De Blasi A, Conn PJ, Pin J, Nicoletti F (2001) Molecular determinants of metabotropic glutamate receptor signaling. Trends Pharmacol Sci 22: 114-120.

De Blassi A (1990) Beta-adrenergic receptors: structure, function and regulation. Drugs Exp Clin Res 16: 107-112.

De Lean A, Stadel JM, Lefkowitz RJ (1980) A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor. J Biol Chem 255: 7108-7117.

de Mendonca A, Ribeiro JA (1997) Influence of metabotropic glutamate receptor agonists on the inhibitory effects of adenosine A1 receptor activation in the rat hippocampus. Br J Pharmacol 121: 1541-1548.

de Novellis V, Marabese I, Palazzo E, Rossi F, Berrino L, Rodella L, Bianchi R, Rossi F, Maione S (2003) Group I metabotropic glutamate receptors modulate glutamate and gamma-aminobutyric acid release in the periaqueductal grey of rats. Eur J Pharmacol 462: 73-81.

de Rooij J, Bos JL (1997) Minimal Ras-binding domain of Raf1 can be used as an activation-specific probe for Ras. Oncogene 14: 623-625.

de Rooij J, Zwartkruis FJ, Verheijen MH, Cool RH, Nijman SM, Wittinghofer A, Bos JL (1998) Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature 396: 474-477.

de Souza NJ, Dohadwalla AN, Reden J (1983) Forskolin: a labdane diterpenoid with antihypertensive, positive inotropic, platelet aggregation inhibitory, and adenylate cyclase activating properties. Med Res Rev 3: 201-219.

Della Rocca GJ, Maudsley S, Daaka Y, Lefkowitz RJ, Luttrell LM (1999) Pleiotropic coupling of G protein-coupled receptors to the mitogen-activated protein kinase cascade. Role of focal adhesions and receptor tyrosine kinases. J Biol Chem 274: 13978-13984.

Dent P, Haser W, Haystead TA, Vincent LA, Roberts TM, Sturgill TW (1992) Activation of mitogen-activated protein kinase kinase by v-Raf in NIH 3T3 cells and in vitro. Science 257: 1404-1407.

Derkinderen P, Valjent E, Toutant M, Corvol JC, Enslen H, Ledent C, Trzaskos J, Caboche J, Girault JA (2003) Regulation of extracellular signal-regulated kinase by cannabinoids in hippocampus. J Neurosci 23: 2371-2382.

Dhillon AS, Pollock C, Steen H, Shaw PE, Mischak H, Kolch W (2002) Cyclic AMP-dependent kinase regulates Raf-1 kinase mainly by phosphorylation of serine 259. Mol Cell Biol 22: 3237-3246.

Dixon AK, Gubitz AK, Sirinathsinghji DJ, Richardson PJ, Freeman TC (1996) Tissue distribution of adenosine receptor mRNAs in the rat. Br J Pharmacol 118: 1461-1468.

Dohlman HG, Caron MG, Lefkowitz RJ (1987) A family of receptors coupled to guanine nucleotide regulatory proteins. Biochemistry 26: 2657-2664.

Dohovics R, Janaky R, Varga V, Hermann A, Saransaari P, Oja SS (2003a) Regulation of glutamatergic neurotransmission in the striatum by presynaptic adenylyl cyclase-dependent processes. Neurochem Int 42: 1-7.

Dohovics R, Janaky R, Varga V, Saransaari P, Oja SS (2003b) Cyclic AMP-mediated regulation of striatal glutamate release: interactions of presynaptic ligand- and voltage-gated ion channels and G-protein-coupled receptors. Neurochem Int 43: 425-430.

Dolphin AC (1995) The G.L. Brown Prize Lecture. Voltage-dependent calcium channels and their modulation by neurotransmitters and G proteins. Exp Physiol 80: 1-36.

Drummond GI (1973) Metabolism and functions of cyclic AMP in nerve. Prog Neurobiol 2: 119-176.

Dudley DT, Pang L, Decker SJ, Bridges AJ, Saltiel AR (1995) A synthetic inhibitor of the mitogen-activated protein kinase cascade. Proc Natl Acad Sci U S A 92: 7686-7689.

Dulubova I, Sugita S, Hill S, Hosaka M, Fernandez I, Sudhof TC, Rizo J (1999) A conformational switch in syntaxin during exocytosis: role of munc18. EMBO J 18: 4372-4382.

Dunkley PR, Heath JW, Harrison SM, Jarvie PE, Glenfield PJ, Rostas JA (1988) A rapid Percoll gradient procedure for isolation of synaptosomes directly from an S1 fraction: homogeneity and morphology of subcellular fractions. Brain Res 441: 59-71.

Dunkley PR, Jarvie PE, Heath JW, Kidd GJ, Rostas JA (1986) A rapid method for isolation of synaptosomes on Percoll gradients. Brain Res 372: 115-129.

Dunlap K, Luebke JI, Turner TJ (1995) Exocytotic Ca2+ channels in mammalian central neurons. Trends Neurosci 18: 89-98.

Dunwiddie TV, Hoffer BJ, Fredholm BB (1981) Alkylxanthines elevate hippocampal excitability. Evidence for a role of endogenous adenosine. Naunyn Schmiedebergs Arch Pharmacol 316: 326-330.

Dunwiddie TV, Taylor M, Heginbotham LR, Proctor WR (1992) Long-term increases in excitability in the CA1 region of rat hippocampus induced by beta-adrenergic stimulation: possible mediation by cAMP. J Neurosci 12: 506-517.

Earnest S, Khokhlatchev A, Albanesi JP, Barylko B (1996) Phosphorylation of dynamin by ERK2 inhibits the dynamin-microtubule interaction. FEBS Lett 396: 62-66.

Ebinu JO, Bottorff DA, Chan EY, Stang SL, Dunn RJ, Stone JC (1998) RasGRP, a Ras guanyl nucleotide- releasing protein with calcium- and diacylglycerol-binding motifs. Science 280: 1082-1086.

Eichholtz T, de Bont D, de Widt J, Liskamp R, Ploegh HL (1993) A myristolated pseudosubstrate peptide, a novel protein kinase C inhibitor. J Biol Chem 268: 1982-1986.

Engh RA, Bossemeyer D (2001) The protein kinase activity modulation sites: mechanisms for cellular regulation - targets for therapeutic intervention. Adv Enzyme Regul 41: 121-149.

English J, Pearson G, Wilsbacher J, Swantek J, Karandikar M, Xu S, Cobb MH (1999) New insights into the control of MAP kinase pathways. Exp Cell Res 253: 255-270.

English JM, Pearson G, Baer R, Cobb MH (1998) Identification of Substrates and Regulators of the Mitogen-activated Protein Kinase ERK5 Using Chimeric Protein Kinases. J Biol Chem 273: 3854-3860.

English JD, Sweatt JD (1997) A Requirement for the Mitogen-activated Protein Kinase Cascade in Hippocampal Long Term Potentiation. J Biol Chem 272: 19103-19106.

Enna SJ (2001) A GABAB Mystery: The Search for Pharmacologically Distinct GABAB Receptors. Mol Intervent 1: 208-218.

Erhardt P, Troppmair J, Rapp UR, Cooper GM (1995) Differential regulation of Raf-1 and B-Raf and Ras-dependent activation of mitogen-activated protein kinase by cyclic AMP in PC12 cells. Mol Cell Biol 15: 5524-5530.

Essen LO, Perisic O, Cheung R, Katan M, Williams RL (1996) Crystal structure of a mammalian phosphoinositide-specific phospholipase C delta. Nature 380: 595-602.

Evans DI, Jones RS, Woodhall G (2001a) Differential actions of PKA and PKC in the regulation of glutamate release by group III mGluRs in the entorhinal cortex. J Neurophysiol 85: 571-579.

Evans GJ, Wilkinson MC, Graham ME, Turner KM, Chamberlain LH, Burgoyne RD, Morgan A (2001b) Phosphorylation of cysteine string protein by protein kinase A. Implications for the modulation of exocytosis. J Biol Chem 276: 47877-47885.

Farago A, Nishizuka Y (1990) Protein kinase C in transmembrane signalling. FEBS Lett 268: 350-354.

Farnsworth CL, Freshney NW, Rosen LB, Ghosh A, Greenberg ME, Feig LA (1995) Calcium activation of Ras mediated by neuronal exchange factor Ras-GRF. Nature 376: 524-527.

Fasshauer D (2003) Structural insights into the SNARE mechanism. Biochim Biophys Acta 1641: 87-97.

Fasshauer D, Bruns D, Shen B, Jahn R, Brunger AT (1997a) A structural change occurs upon binding of syntaxin to SNAP-25. J Biol Chem 272: 4582-4590.

Fasshauer D, Otto H, Eliason WK, Jahn R, Brunger AT (1997b) Structural changes are associated with soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor complex formation. J Biol Chem 272: 28036-28041.

Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL, Scherle PA, Trzaskos JM (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem 273: 18623-18632.

Fazal A, Parker F, Palmer AM, Croucher MJ (2003) Characterisation of the actions of group I metabotropic glutamate receptor subtype selective ligands on excitatory amino acid release and sodium-dependent re-uptake in rat cerebrocortical minislices. J Neurochem 86: 1346-1358.

Feoktistov I, Biaggioni I (1997) Adenosine A2B receptors. Pharmacol Rev 49: 381-402.

Ferreira A, Paganoni S (2002) The formation of synapses in the central nervous system. Mol Neurobiol 26: 69-79.

Ferrell JEJ (1997) How responses get more switch-like as you move down a protein kinase cascade. Trends Biochem Sci 22: 288-289.

Ferrell J, Bhatt RR (1997) Mechanistic Studies of the Dual Phosphorylation of Mitogen-activated Protein Kinase. J Biol Chem 272: 19008-19016.

Feuerstein TJ, Allgaier C, Hertting G (1987) Possible involvement of protein kinase C (PKC) in the regulation of electrically evoked serotonin (5-HT) release from rabbit hippocampal slices. Eur J Pharmacol 139: 267-272.

Figurov A, Pozzo-Miller LD, Olafsson P, Wang T, Lu B (1996) Regulation of synaptic responses to high-frequency stimulation and LTP by neurotrophins in the hippocampus. Nature 381: 706-709.

Fischer von Mollard G, Mignery GA, Baumert M, Perin MS, Hanson TJ, Burger PM, Jahn R, Sudhof TC (1990) rab3 is a small GTP-binding protein exclusively localized to synaptic vesicles. Proc Natl Acad Sci U S A 87: 1988-1992.

Fitzgerald EM (2002) The presence of Ca2+ channel beta subunit is required for mitogen-activated protein kinase (MAPK)-dependent modulation of alpha1B Ca2+ channels in COS-7 cells. J Physiol 543: 425-437.

Folch J, Lebaron FN (1956) The chemistry of the phosphoinositides. Can J Med Sci 34: 305-319.

Foster AC, Fagg GE (1984) Acidic amino acid binding sites in mammalian neuronal membranes: their characteristics and relationship to synaptic receptors. Brain Res 319: 103-164.

Francesconi A, Duvoisin RM (2000) Opposing effects of protein kinase C and protein kinase A on metabotropic glutamate receptor signaling: selective desensitization of the inositol trisphosphate/Ca2+ pathway by phosphorylation of the receptor-G protein-coupling domain. Proc Natl Acad Sci U S A 97: 6185-6190.

Francis SH, Corbin JD (1994) Structure and function of cyclic nucleotide-dependent protein kinases. Annu Rev Physiol 56: 237-272.

Franklin CC, Kraft AS (1997) Conditional Expression of the Mitogen-activated Protein Kinase (MAPK) Phosphatase MKP-1 Preferentially Inhibits p38 MAPK and Stress-activated Protein Kinase in U937 Cells. J Biol Chem 272: 16917-16923.

Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 53: 527-552.

Friedman J, Babu B, Clark RB (2002) Beta(2)-adrenergic receptor lacking the cyclic AMP-dependent protein kinase consensus sites fully activates extracellular signal-regulated kinase 1/2 in human embryonic kidney 293 cells: lack of evidence for G(s)/G(i) switching. Mol Pharmacol 62: 1094-1102.

Fromm C, Coso OA, Montaner S, Xu N, Gutkind JS (1997) The small GTP-binding protein Rho links G protein-coupled receptors and Galpha12 to the serum response element and to cellular transformation. Proc Natl Acad Sci U S A 94: 10098-10103.

Fruman DA, Meyers RE, Cantley LC (1998) Phosphoinositide kinases. Annu Rev Biochem 67: 481-507.

Fujita T, Meguro T, Fukuyama R, Nakamuta H, Koida M (2002) New signaling pathway for parathyroid hormone and cyclic AMP action on extracellular-regulated kinase and cell proliferation in bone cells. Checkpoint of modulation by cyclic AMP. J Biol Chem 277: 22191-22200.

Fukuhara S, Murga C, Zohar M, Igishi T, Gutkind JS (1999) A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho. J Biol Chem 274: 5868-5879.

Gadbois DM, Crissman HA, Tobey RA, Bradbury EM (1992) Multiple kinase arrest points in the G1 phase of nontransformed mammalian cells are absent in transformed cells. Proc Natl Acad Sci U S A 89: 8626-8630.

Gao Z, Chen T, Weber MJ, Linden J (1999) A2B adenosine and P2Y2 receptors stimulate mitogen-activated protein kinase in human embryonic kidney-293 cells. cross-talk between cyclic AMP and protein kinase c pathways. J Biol Chem 274: 5972-5980.

Garner CC, Kindler S, Gundelfinger ED (2000) Molecular determinants of presynaptic active zones. Curr Opin Neurobiol 10: 321-327.

Garnier V, Zini R, Sapena R, Tillement JP (1997) A match between binding to beta-adrenoceptors and stimulation of adenylyl cyclase parameters of (-)isoproterenol and salbutamol on rat brain. Pharmacol Res 35: 303-312.

Garrington TP, Johnson GL (1999) Organization and regulation of mitogen-activated protein kinase signaling pathways. Curr Opin Cell Biol 11: 211-218.

Gasparini F, Kuhn R, Pin JP (2002) Allosteric modulators of group I metabotropic glutamate receptors: novel subtype-selective ligands and therapeutic perspectives. Curr Opin Pharmacol 2: 43-49.

Gasparini F, Lingenhohl K, Stoehr N, Flor PJ, Heinrich M, Vranesic I, Biollaz M, Allgeier H, Heckendorn R, Urwyler S, Varney MA, Johnson EC, Hess SD, Rao SP, Sacaan AI, Santori EM, Velicelebi G, Kuhn R (1999) 2-Methyl-6-(phenylethynyl)-pyridine (MPEP), a potent, selective and systemically active mGlu5 receptor antagonist. Neuropharmacology 38: 1493-1503.

Geppert M, Sudhof TC (1998) RAB3 and synaptotagmin: the yin and yang of synaptic membrane fusion. Annu Rev Neurosci 21: 75-95.

Gereau RW4, Conn PJ (1994) Potentiation of cAMP responses by metabotropic glutamate receptors depresses excitatory synaptic transmission by a kinase-independent mechanism. Neuron 12: 1121-1129.

Gereau RW4, Heinemann SF (1998) Role of protein kinase C phosphorylation in rapid desensitization of metabotropic glutamate receptor 5. Neuron 20: 143-151.

Giovannini MG, Blitzer RD, Wong T, Asoma K, Tsokas P, Morrison JH, Iyengar R, Landau EM (2001) Mitogen-activated protein kinase regulates early phosphorylation and delayed expression of Ca2+/calmodulin-dependent protein kinase II in long-term potentiation. J Neurosci 21: 7053-7062.

Girod A, Kinzel V, Bossemeyer D (1996) In vivo activation of recombinant cAPK catalytic subunit active site mutants by coexpression of the wild-type enzyme, evidence for intermolecular cotranslational phosphorylation. FEBS Lett 391: 121-125.

Gomeza J, Joly C, Kuhn R, Knopfel T, Bockaert J, Pin JP (1996) The second intracellular loop of metabotropic glutamate receptor 1 cooperates with the other intracellular domains to control coupling to G-proteins. J Biol Chem 271: 2199-2205.

Goncalves ML, Cunha RA, Ribeiro JA (1997) Adenosine A2A receptors facilitate 45Ca2+ uptake through class A calcium channels in rat hippocampal CA3 but not CA1 synaptosomes. Neurosci Lett 238: 73-77.

Goudet C, Gaven F, Kniazeff J, Vol C, Liu J, Cohen-Gonsaud M, Acher F, Prezeau L, Pin JP (2004) Heptahelical domain of metabotropic glutamate receptor 5 behaves like rhodopsin-like receptors. Proc Natl Acad Sci U S A 101: 378-383.

Graham ME, O'Callaghan DW, McMahon HT, Burgoyne RD (2002) Dynamin-dependent and dynamin-independent processes contribute to the regulation of single vesicle release kinetics and quantal size. Proc Natl Acad Sci U S A 99: 7124-7129.

Grassi S, Frondaroli A, Pettorossi VE (2002) Different metabotropic glutamate receptors play opposite roles in synaptic plasticity of the rat medial vestibular nuclei. J Physiol 543: 795-806.

Greengard P (2001) The neurobiology of slow synaptic transmission. Science 294: 1024-1030.

Grumont RJ, Rasko JE, Strasser A, Gerondakis S (1996) Activation of the mitogen-activated protein kinase pathway induces transcription of the PAC-1 phosphatase gene. Mol Cell Biol 16: 2913-2921.

Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 260: 3440-3450.

Gu JG, Foga IO, Parkinson FE, Geiger JD (1995) Involvement of bidirectional adenosine transporters in the release of L-[3H]adenosine from rat brain synaptosomal preparations. J Neurochem 64: 2105-2110.

Gubitz AK, Widdowson L, Kurokawa M, Kirkpatrick KA, Richardson PJ (1996) Dual signalling by the adenosine A2a receptor involves activation of both N- and P-type calcium channels by different G proteins and protein kinases in the same striatal nerve terminals. J Neurochem 67: 374-381.

Guo H, Damuni Z (1993) Autophosphorylation-Activated Protein Kinase Phosphorylates and Inactivates Protein Phosphatase 2A. PNAS 90: 2500-2504.

Gustafson EL, Girault JA, Hemmings HCJ, Nairn AC, Greengard P (1991) Immunocytochemical localization of phosphatase inhibitor-1 in rat brain. J Comp Neurol 310: 170-188.

Gustafsson J, Shupliakov O, Takei K, Low P, De Camilli P, Brodin L (1998) GTPγS induces an actin matrix associated with coated endocytic intermediates in presynaptic regions. Soc Neurosci Abstr 327: 19.

Gutkind JS (2000) Regulation of mitogen-activated protein kinase signaling networks by G protein-coupled receptors. Sci STKE 2000: RE1-RE13.

Hagemann C, Rapp UR (1999) Isotype-specific functions of Raf kinases. Exp Cell Res 253: 34-46.

Hamid J, Nelson D, Spaetgens R, Dubel SJ, Snutch TP, Zamponi GW (1999) Identification of an integration center for cross-talk between protein kinase C and G protein modulation of N-type calcium channels. J Biol Chem 274: 6195-6202.

Hamm HE (1998) The many faces of G protein signaling. J Biol Chem 273: 669-672.

Han J, Lee JD, Bibbs L, Ulevitch RJ (1994) A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 265: 808-811.

Hanks SK, Quinn AM (1991) Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. Methods Enzymol 200: 38-62.

Hanson PI, Heuser JE, Jahn R (1997) Neurotransmitter release - four years of SNARE complexes. Curr Opin Neurobiol 7: 310-315.

Harlow E, Lane DP (1988) Antibodies: A Laboratory Manual. New York: Cold Spring Harbor Laboratory.

Heidbreder CA, Bianchi M, Lacroix LP, Faedo S, Perdona E, Remelli R, Cavanni P, Crespi F (2003) Evidence that the metabotropic glutamate receptor 5 antagonist MPEP may act as an inhibitor of the norepinephrine transporter in vitro and in vivo. Synapse 50: 269-276.

Heidelberger R, Heinemann C, Neher E, Matthews G (1994) Calcium dependence of the rate of exocytosis in a synaptic terminal. Nature 371: 513-515.

Hepp R, Cabaniols JP, Roche PA (2002) Differential phosphorylation of SNAP-25 in vivo by protein kinase C and protein kinase A. FEBS Lett 532: 52-56.

Herb A, Burnashev N, Werner P, Sakmann B, Wisden W, Seeburg PH (1992) The KA-2 subunit of excitatory amino acid receptors shows widespread expression in brain and forms ion channels with distantly related subunits. Neuron 8: 775-785.

Herlitze S, Zhong H, Scheuer T, Catterall WA (2001) Allosteric modulation of Ca2+ channels by G proteins, voltage-dependent facilitation, protein kinase C, and Ca(v)beta subunits. Proc Natl Acad Sci U S A 98: 4699-4704.

Herrero I, Miras-Portugal MT, Sanchez-Prieto J (1994) Rapid desensitization of the metabotropic glutamate receptor that facilitates glutamate release in rat cerebrocortical nerve terminals. Eur J Neurosci 6: 115-120.

Herrero I, Miras-Portugal MT, Sanchez-Prieto J (1992) Positive feedback of glutamate exocytosis by metabotropic presynaptic receptor stimulation. Nature 360: 163-166.

Herrero I, Miras-Portugal MT, Sanchez-Prieto J (1998) Functional switch from facilitation to inhibition in the control of glutamate release by metabotropic glutamate receptors. J Biol Chem 273: 1951-1958.

Herrero I, Sanchez-Prieto J (1996) cAMP-dependent facilitation of glutamate release by beta-adrenergic receptors in cerebrocortical nerve terminals. J Biol Chem 271: 30554-30560.

Hibi M, Lin A, Smeal T, Minden A, Karin M (1993) Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. Genes Dev 7: 2135-2148.

Hilfiker S, Pieribone VA, Czernik AJ, Kao HT, Augustine GJ, Greengard P (1999) Synapsins as regulators of neurotransmitter release. Philos Trans R Soc Lond B Biol Sci 354: 269-279.

Hilfiker S, Schweizer FE, Kao HT, Czernik AJ, Greengard P, Augustine GJ (1998) Two sites of action for synapsin domain E in regulating neurotransmitter release. Nat Neurosci 1: 29-35.

Hill DR, Bowery NG (1981) 3H-baclofen and 3H-GABA bind to bicuculline-insensitive GABA B sites in rat brain. Nature 290: 149-152.

Holgado-Madruga M, Moscatello DK, Emlet DR, Dieterich R, Wong AJ (1997) Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor. Proc Natl Acad Sci U S A 94: 12419-12424.

Hollmann M, Heinemann S (1994) Cloned glutamate receptors. Annu Rev Neurosci 17: 31-108.

Holroyd P, Lang T, Wenzel D, De Camilli P, Jahn R (2002) Imaging direct, dynamin-dependent recapture of fusing secretory granules on plasma membrane lawns from PC12 cells. Proc Natl Acad Sci U S A 99: 16806-16811.

Hosaka M, Sudhof TC (1998) Synapsin III, a Novel Synapsin with an Unusual Regulation by Ca2+. J Biol Chem 273: 13371-13374.

Hoshi M, Nishida E, Sakai H (1989) Characterization of a mitogen-activated, Ca2+-sensitive microtubule-associated protein-2 kinase. Eur J Biochem 184: 477-486.

Hoshi M, Nishida E, Sakai H (1988) Activation of a Ca2+-inhibitable protein kinase that phosphorylates microtubule-associated protein 2 in vitro by growth factors, phorbol esters, and serum in quiescent cultured human fibroblasts. J Biol Chem 263: 5396-5401.

Houamed KM, Kuijper JL, Gilbert TL, Haldeman BA, O'Hara PJ, Mulvihill ER, Almers W, Hagen FS (1991) Cloning, expression, and gene structure of a G protein-coupled glutamate receptor from rat brain. Science 252: 1318-1321.

Huang CC, Hsu KS, Gean PW (1996) Isoproterenol potentiates synaptic transmission primarily by enhancing presynaptic calcium influx via P- and/or Q-type calcium channels in the rat amygdala. J Neurosci 16: 1026-1033.

Huang CC, Wang SJ, Gean PW (1998) Selective enhancement of P-type calcium currents by isoproterenol in the rat amygdala. J Neurosci 18: 2276-2282.

Huang L, Killbride J, Rowan MJ, Anwyl R (1999) Activation of mGluRII induces LTD via activation of protein kinase A and protein kinase C in the dentate gyrus of the hippocampus in vitro. Neuropharmacology 38: 73-83.

Huang YY, Kandel ER (1996) Modulation of both the early and the late phase of mossy fiber LTP by the activation of beta-adrenergic receptors. Neuron 16: 611-617.

Huang YY, Martin KC, Kandel ER (2000) Both protein kinase A and mitogen-activated protein kinase are required in the amygdala for the macromolecular synthesis-dependent late phase of long-term potentiation. J Neurosci 20: 6317-6325.

Hubert GW, Paquet M, Smith Y (2001) Differential Subcellular Localization of mGluR1a and mGluR5 in the Rat and Monkey Substantia Nigra. J Neurosci 21: 1838-1847.

Hug H, Sarre TF (1993) Protein kinase C isoenzymes: divergence in signal transduction? Biochem J 291: 329-343.

Hurley JH (1999) Structure, mechanism, and regulation of mammalian adenylyl cyclase. J Biol Chem 274: 7599-7602.

Hurley JH, Newton AC, Parker PJ, Blumberg PM, Nishizuka Y (1997) Taxonomy and function of C1 protein kinase C homology domains. Protein Sci 6: 477-480.

Huttner WB, DeGennaro LJ, Greengard P (1981) Differential phosphorylation of multiple sites in purified protein I by cyclic AMP-dependent and calcium-dependent protein kinases. J Biol Chem 256: 1482-1488.

Ikemoto A, Nakamura S, Akiguchi I, Hirano A (2002) Differential expression between synaptic vesicle proteins and presynaptic plasma membrane proteins in the anterior horn of amyotrophic lateral sclerosis. Acta Neuropathol (Berl) 103: 179-187.

Ireland DR, Abraham WC (2002) Group I mGluRs increase excitability of hippocampal CA1 pyramidal neurons by a PLC-independent mechanism. J Neurophysiol 88: 107-116.

Isaacson JS (1998) GABAB receptor-mediated modulation of presynaptic currents and excitatory transmission at a fast central synapse. J Neurophysiol 80: 1571-1576.

Ito I, Kohda A, Tanabe S, Hirose E, Hayashi M, Mitsunaga S, Sugiyama H (1992) 3,5-Dihydroxyphenyl-glycine: a potent agonist of metabotropic glutamate receptors. Neuroreport 3: 1013-1016.

Jiang H, Xiong F, Kong S, Ogawa T, Kobayashi M, Liu JO (1997) Distinct tissue and cellular distribution of two major isoforms of calcineurin. Mol Immunol 34: 663-669.

Johnson EM, Maeno H, Greengard P (1971) Phosphorylation of Endogenous Protein of Rat Brain by Cyclic Adenosine 3',5'-Monophosphate-dependent Protein Kinase. J Biol Chem 246: 7731-7739.

Jordan BA, Trapaidze N, Gomes I, Nivarthi R, Devi LA (2001) Oligomerization of opioid receptors with beta 2-adrenergic receptors: a role in trafficking and mitogen-activated protein kinase activation. Proc Natl Acad Sci U S A 98: 343-348.

Jovanovic JN, Czernik AJ, Fienberg AA, Greengard P, Sihra TS (2000) Synapsins as mediators of BDNF-enhanced neurotransmitter release. Nat Neurosci 3: 323-329.

Jovanovic JN, Sihra TS, Nairn AC, Hemmings HCJ, Greengard P, Czernik AJ (2001) Opposing changes in phosphorylation of specific sites in synapsin I during Ca2+-dependent glutamate release in isolated nerve terminals. J Neurosci 21: 7944-7953.

Jovanovic JN, Benfenati F, Siow YL, Sihra TS, Sanghera JS, Pelech SL, Greengard P, Czernik AJ (1996) Neurotrophins stimulate phosphorylation of synapsin I by MAP kinase and regulate synapsin I-actin interactions. PNAS 93: 3679-3683.

Kahn RA, Gilman AG (1984a) ADP-ribosylation of Gs promotes the dissociation of its alpha and beta subunits. J Biol Chem 259: 6235-6240.

Kahn RA, Gilman AG (1984b) Purification of a protein cofactor required for ADP-ribosylation of the stimulatory regulatory component of adenylate cyclase by cholera toxin. J Biol Chem 259: 6228-6234.

Kallunki T, Deng T, Hibi M, Karin M (1996) c-Jun can recruit JNK to phosphorylate dimerization partners via specific docking interactions. Cell 87: 929-939.

Kamiya H, Ozawa S (1998) Kainate receptor-mediated inhibition of presynaptic Ca2+ influx and EPSP in area CA1 of the rat hippocampus. J Physiol 509: 833-845.

Kamiya H, Yamamoto C (1997) Phorbol ester and forskolin suppress the presynaptic inhibitory action of group-II metabotropic glutamate receptor at rat hippocampal mossy fibre synapse. Neuroscience 80: 89-94.

Kandel ER, Schwartz JH, Jessell TM (1991) Principles of Neural Science. Conneticut: Appleton and Lange.

Kanterewicz BI, Urban NN, McMahon DB, Norman ED, Giffen LJ, Favata MF, Scherle PA, Trzskos JM, Barrionuevo G, Klann E (2000) The extracellular signal-regulated kinase cascade is required for NMDA receptor-independent LTP in area CA1 but not area CA3 of the hippocampus. J Neurosci 20: 3057-3066.

Kao HT, Porton B, Czernik AJ, Feng J, Yiu G, Haring M, Benfenati F, Greengard P (1998) A third member of the synapsin gene family. PNAS 95: 4667-4672.

Kaplan DR, Miller FD (2000) Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 10: 381-391.

Karim F, Wang CC, Gereau RW4 (2001) Metabotropic glutamate receptor subtypes 1 and 5 are activators of extracellular signal-regulated kinase signaling required for inflammatory pain in mice. J Neurosci 21: 3771-3779.

Kase H, Iwahashi K, Nakanishi S, Matsuda Y, Yamada K, Takahashi M, Murakata C, Sato A, Kaneko M (1987) K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. Biochem Biophys Res Commun 142: 436-440.

Katada T, Ui M (1982) Direct modification of the membrane adenylate cyclase system by islet-activating protein due to ADP-ribosylation of a membrane protein. Proc Natl Acad Sci U S A 79: 3129-3133.

Kato Y, Tapping RI, Huang S, Watson MH, Ulevitch RJ, Lee JD (1998) Bmk1/Erk5 is required for cell proliferation induced by epidermal growth factor. Nature 395: 713-716.

Katsuki H, Saito H, Satoh M (1992) The involvement of muscarinic, beta-adrenergic and metabotropic glutamate receptors in long-term potentiation in the fimbria-CA3 pathway of the hippocampus. Neurosci Lett 142: 249-252.

Kaupmann K, Huggel K, Heid J, Flor PJ, Bischoff S, Mickel SJ, McMaster G, Angst C, Bittiger H, Froestl W, Bettler B (1997) Expression cloning of GABA(B) receptors uncovers similarity to metabotropic glutamate receptors. Nature 386: 239-246.

Kawasaki H, Springett GM, Toki S, Canales JJ, Harlan P, Blumenstiel JP, Chen EJ, Bany IA, Mochizuki N, Ashbacher A, Matsuda M, Housman DE, Graybiel AM (1998) A Rap guanine nucleotide exchange factor enriched highly in the basal ganglia. Proc Natl Acad Sci U S A 95: 13278-13283.

Keinanen K, Wisden W, Sommer B, Werner P, Herb A, Verdoorn TA, Sakmann B, Seeburg PH (1990) A family of AMPA-selective glutamate receptors. Science 249: 556-560.

Kemp BE, Pearson RB (1990) Protein kinase recognition sequence motifs. Trends Biochem Sci 15: 342-346.

Kennedy MB, Greengard P (1981) Two calcium/calmodulin-dependent protein kinases, which are highly concentrated in brain, phosphorylate protein I at distinct sites. Proc Natl Acad Sci U S A 78: 1293-1297.

Keranen LM, Dutil EM, Newton AC (1995) Protein kinase C is regulated in vivo by three functionally distinct phosphorylations. Curr Biol 5: 1394-1403.

Kim J, Eckhart AD, Eguchi S, Koch WJ (2002) Beta-adrenergic receptor-mediated DNA synthesis in cardiac fibroblasts is dependent on transactivation of the epidermal growth factor receptor and subsequent activation of extracellular signal-regulated kinases. J Biol Chem 277: 32116-32123.

Kishimoto A, Takai Y, Mori T, Kikkawa U, Nishizuka Y (1980) Activation of calcium and phospholipid-dependent protein kinase by diacylglycerol, its possible relation to phosphatidylinositol turnover. J Biol Chem 255: 2273-2276.

Klagges BR, Heimbeck G, Godenschwege TA, Hofbauer A, Pflugfelder GO, Reifegerste R, Reisch D, Schaupp M, Buchner S, Buchner E (1996) Invertebrate Synapsins: A Single Gene Codes for Several Isoforms in Drosophila. J Neurosci 16: 3154-3165.

Klingauf J, Kavalali ET, Tsien RW (1998) Kinetics and regulation of fast endocytosis at hippocampal synapses. Nature 394: 581-585.

Klinger M, Freissmuth M, Nanoff C (2002) Adenosine receptors: G protein-mediated signalling and the role of accessory proteins. Cell Signal 14: 99-108.

Knighton DR, Zheng JH, Ten Eyck LF, Ashford VA, Xuong NH, Taylor SS, Sowadski JM (1991) Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. Science 253: 407-414.

Kobayashi E, Nakano H, Morimoto M, Tamaoki T (1989) Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Biochem Biophys Res Commun 159: 548-553.

Koch WJ, Hawes BE, Allen LF, Lefkowitz RJ (1994) Direct evidence that Gi-coupled receptor stimulation of mitogen-activated protein kinase is mediated by G beta gamma activation of p21ras. Proc Natl Acad Sci U S A 91: 12706-12710.

Koenig JH, Ikeda K (1996) Synaptic vesicles have two distinct recycling pathways. The Journal of Cell Biology 135: 797-808.

Kohara K, Ogura A, Akagawa K, Yamaguchi K (2001) Increase in number of functional release sites by cyclic AMP-dependent protein kinase in cultured neurons isolated from hippocampal dentate gyrus. Neurosci Res 41: 79-88.

Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marme D, Rapp UR (1993) Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature 364: 249-252.

Konig S, Guibert B, Morice C, Vernier P, Barnier JV (2001) Phosphorylation by PKA of a site unique to B-Raf kinase. C R Acad Sci III 324: 673-681.

Krueger BK, Forn J, Greengard P (1977) Depolarization-induced phosphorylation of specific proteins, mediated by calcium ion influx, in rat brain synaptosomes. J Biol Chem 252: 2764-2773.

Krupinski J, Coussen F, Bakalyar HA, Tang WJ, Feinstein PG, Orth K, Slaughter C, Reed RR, Gilman AG (1989) Adenylyl cyclase amino acid sequence: possible channel- or transporter-like structure. Science 244: 1558-1564.

Kuang Y, Wu Y, Smrcka A, Jiang H, Wu D (1996) Identification of a phospholipase C beta2 region that interacts with Gbeta-gamma. Proc Natl Acad Sci U S A 93: 2964-2968.

Kunishima N, Shimada Y, Tsuji Y, Sato T, Yamamoto M, Kumasaka T, Nakanishi S, Jingami H, Morikawa K (2000) Structural basis of glutamate recognition by a dimeric metabotropic glutamate receptor. Nature 407: 971-977.

Kwon KB, Kim JS, Chang BJ (2000) Translocational changes of localization of synapsin in axonal sprouts of regenerating rat sciatic nerves after ligation crush injury. J Vet Sci 1: 1-9.

Kyriakis JM, App H, Zhang XF, Banerjee P, Brautigan DL, Rapp UR, Avruch J (1992) Raf-1 activates MAP kinase-kinase. Nature 358: 417-421.

Kyriakis JM, Avruch J (1990) pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. J Biol Chem 265: 17355-17363.

Lackey K, Cory M, Davis R, Frye SV, Harris PA, Hunter RN, Jung DK, McDonald OB, McNutt RW, Peel MR, Rutkowske RD, Veal JM, Wood ER (2000) The discovery of potent cRaf1 kinase inhibitors. Bioorg Med Chem Lett 10: 223-226.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lafon-Cazal M, Viennois G, Kuhn R, Malitschek B, Pin JP, Shigemoto R, Bockaert J (1999) mGluR7-like receptor and GABA(B) receptor activation enhance neurotoxic effects of N-methyl-D-aspartate in cultured mouse striatal GABAergic neurones. Neuropharmacology 38: 1631-1640.

Lai HL, Yang TH, Messing RO, Ching YH, Lin SC, Chern Y (1997) Protein kinase C inhibits adenylyl cyclase type VI activity during desensitization of the A2a-adenosine receptor-mediated cAMP response. J Biol Chem 272: 4970-4977.

Lambright DG, Noel JP, Hamm HE, Sigler PB (1994) Structural determinants for activation of the alpha-subunit of a heterotrimeric G protein. Nature 369: 621-628.

Landis DM (1988) Membrane and cytoplasmic structure at synaptic junctions in the mammalian central nervous system. J Electron Microsc Tech 10: 129-151.

Lanthorn TH, Cotman CW (1981) Baclofen selectively inhibits excitatory synaptic transmission in the hippocampus. Brain Res 225: 171-178.

Laroche-Joubert N, Marsy S, Luriau S, Imbert-Teboul M, Doucet A (2003) Mechanism of activation of ERK and H-K-ATPase by isoproterenol in rat cortical collecting duct. Am J Physiol Renal Physiol 284: F948-F954.

Laroche-Joubert N, Marsy S, Michelet S, Imbert-Teboul M, Doucet A (2002) Protein kinase A-independent activation of ERK and H,K-ATPase by cAMP in native kidney cells: role of Epac I. J Biol Chem 277: 18598-18604.

Laurie DJ, Schoeffter P, Wiederhold KH, Sommer B (1997) Cloning, distribution and functional expression of the human mGlu6 metabotropic glutamate receptor. Neuropharmacology 36: 145-152.

Lebaron FN, Folch J (1957) Structure of brain tissue lipides. Physiol Rev 37: 539-561.

Lee FS, Chao MV (2001) Activation of Trk neurotrophin receptors in the absence of neurotrophins. Proc Natl Acad Sci U S A 98: 3555-3560.

Lee FS, Kim AH, Khursigara G, Chao MV (2001) The uniqueness of being a neurotrophin receptor. Curr Opin Neurobiol 11: 281-286.

Lee HK, Barbarosie M, Kameyama K, Bear MF, Huganir RL (2000) Regulation of distinct AMPA receptor phosphorylation sites during bidirectional synaptic plasticity. Nature 405: 955-959.

Lee JC, Laydon JT, McDonnell PC, Gallagher TF, Kumar S, Green D, McNulty D, Blumenthal MJ, Heys JR, Landvatter SW, et al. (1994) A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 372: 739-746.

Lee JD, Ulevitch RJ, Han J (1995) Primary structure of BMK1: a new mammalian map kinase. Biochem Biophys Res Commun 213: 715-724.

Lee SK, Qing WG, Mar W, Luyengi L, Mehta RG, Kawanishi K, Fong HH, Beecher CW, Kinghorn AD, Pezzuto JM (1998) Angoline and chelerythrine, benzophenanthridine alkaloids that do not inhibit protein kinase C. J Biol Chem 273: 19829-19833.

Lefkowitz RJ (1998) G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. J Biol Chem 273: 18677-18680.

Lerma J, Paternain AV, Rodriguez-Moreno A, Lopez-Garcia JC (2001) Molecular physiology of kainate receptors. Physiol Rev 81: 971-998.

Lerosey I, Pizon V, Tavitian A, de Gunzburg J (1991) The cAMP-dependent protein kinase phosphorylates the rap1 protein in vitro as well as in intact fibroblasts, but not the closely related rap2 protein. Biochem Biophys Res Commun 175: 430-436.

Leszkovszky G, Tardos L (1965) Some effects of propranolol on the central nervous system. J Pharm Pharmacol 17: 518-519.

Lew VL, Tsien RY, Miner C, Bookchin RM (1982) Physiological [Ca2+]i level and pump-leak turnover in intact red cells measured using an incorporated Ca chelator. Nature 298: 478-481.

Li L, Chin L, Shupliakov O, Brodin L, Sihra TS, Hvalby O, Jensen V, Zheng D, McNamara JO, Greengard P, Andersen P (1995) Impairment of Synaptic Vesicle Clustering and of Synaptic Transmission, and Increased Seizure Propensity, in Synapsin I-Deficient Mice. PNAS 92: 9235-9239.

Li N, Batzer A, Daly R, Yajnik V, Skolnik E, Chardin P, Bar-Sagi D, Margolis B, Schlessinger J (1993) Guanine-nucleotide-releasing factor hSos1 binds to Grb2 and links receptor tyrosine kinases to Ras signalling. Nature 363: 85-88.

Li WW, Luan S, Schreiber SL, Assmann SM (1994) CyclicAMP stimulates K+ channel activity in mesophyll cells of *Vicia faba* K. Plant Physiol 105: 957-961.

Lievens JC, Woodman B, Mahal A, Bates GP (2002) Abnormal phosphorylation of synapsin I predicts a neuronal transmission impairment in the R6/2 Huntington's disease transgenic mice. Mol Cell Neurosci 20: 638-648.

Lin SL, Johnson-Farley NN, Lubinsky DR, Cowen DS (2003) Coupling of neuronal 5-HT7 receptors to activation of extracellular-regulated kinase through a protein kinase A-independent pathway that can utilize Epac. J Neurochem 87: 1076-1085.

Linden DJ, Ahn S (1999) Activation of presynaptic cAMP-dependent protein kinase is required for induction of cerebellar long-term potentiation. J Neurosci 19: 10221-10227.

Linden J (2001) Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. Annu Rev Pharmacol Toxicol 41: 775-787.

Lombardi g, Leonardi P, Moroni F (1996) Metabotropic glutamate receptors, transmitter output and fatty acids: studies in rat brain slices. Br J Pharmacol 117: 189-195.

Lonart G, Janz R, Johnson KM, Sudhof TC (1998) Mechanism of action of rab3A in mossy fiber LTP. Neuron 21: 1141-1150.

Lonart G, Schoch S, Kaeser PS, Larkin CJ, Sudhof TC, Linden DJ (2003) Phosphorylation of RIM1alpha by PKA triggers presynaptic long-term potentiation at cerebellar parallel fiber synapses. Cell 115: 49-60.

Lonart G, Sudhof TC (1998) Region-specific phosphorylation of rabphilin in mossy fiber nerve terminals of the hippocampus. J Neurosci 18: 634-640.

Lonart G, Sudhof TC (2001) Characterization of rabphilin phosphorylation using phospho-specific antibodies. Neuropharmacology 41: 643-649.

Lopes LV, Cunha RA, Ribeiro JA (1999) Cross talk between A(1) and A(2A) adenosine receptors in the hippocampus and cortex of young adult and old rats. J Neurophysiol 82: 3196-3203.

Lopez-Ilasaca M, Crespo P, Pellici PG, Gutkind JS, Wetzker R (1997) Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase gamma. Science 275: 394-397.

Lorenzen A, Lang H, Schwabe U (1998) Activation of various subtypes of G-protein alpha subunits by partial agonists of the adenosine A1 receptor. Biochem Pharmacol 56: 1287-1293.

Lorenzo PS, Beheshti M, Pettit GR, Stone JC, Blumberg PM (2000) The guanine nucleotide exchange factor RasGRP is a high -affinity target for diacylglycerol and phorbol esters. Mol Pharmacol 57: 840-846.

Lorenzo PS, Kung JW, Bottorff DA, Garfield SH, Stone JC, Blumberg PM (2001) Phorbol esters modulate the Ras exchange factor RasGRP3. Cancer Res 61: 943-949.

Lorrain DS, Correa L, Anderson J, Varney M (2002) Activation of spinal group I metabotropic glutamate receptors in rats evokes local glutamate release and spontaneous nociceptive behaviors: effects of 2-methyl-6-(phenylethynyl)-pyridine pretreatment. Neurosci Lett 327: 198-202.

Lu KT, Gean PW (1999) Masking of forskolin-induced long-term potentiation by adenosine accumulation in area CA1 of the rat hippocampus. Neuroscience 88: 69-78.

Luttrell LM, Ferguson SS, Daaka Y, Miller WE, Maudsley S, Della Rocca GJ, Lin F, Kawakastu H, Owada K, Luttrell DK, Caron MG, Lefkowitz RJ (1999) Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes. Science 283: 655-661.

Luttrell LM, Hawes BE, van Biesen T, Luttrell DK, Lansing TJ, Lefkowitz RJ (1996) Role of c-Src tyrosine kinase in G protein-coupled receptor- and Gbetagamma subunit-mediated activation of mitogen-activated protein kinases. J Biol Chem 271: 19443-19450.

Lynch MA, Voss KL, Rodriguez J, Bliss TV (1994) Increase in synaptic vesicle proteins accompanies long-term potentiation in the dentate gyrus. Neuroscience 60: 1-5.

Ma L, Zablow L, Kandel ER, Siegelbaum SA (1999) Cyclic AMP induces functional presynaptic boutons in hippocampal CA3-CA1 neuronal cultures. Nat Neurosci 2: 24-30.

Macdonald SG, Crews CM, Wu L, Driller J, Clark R, Erikson RL, McCormick F (1993) Reconstitution of the Raf-1-MEK-ERK signal transduction pathway in vitro. Mol Cell Biol 13: 6615-6620.

Macek TA, Schaffhauser H, Conn PJ (1998) Protein kinase C and A3 adenosine receptor activation inhibit presynaptic metabotropic glutamate receptor (mGluR) function and uncouple mGluRs from GTP-binding proteins. J Neurosci 18: 6138-6146.

Majewski H, Iannazzo L (1998) Protein kinase C: a physiological mediator of enhanced transmitter output. Prog Neurobiol 55: 463-475.

Malenka RC, Nicoll RA (1999) Long-term potentiation--a decade of progress? Science 285: 1870-1874.

Malherbe P, Kratochwil N, Zenner MT, Piussi J, Diener C, Kratzeisen C, Fischer C, Porter RH (2003) Mutational analysis and molecular modeling of the binding pocket of the metabotropic glutamate 5 receptor negative modulator 2-methyl-6-(phenylethynyl)-pyridine. Mol Pharmacol 64: 823-832.

Malva JO, Ambrosio AF, Cunha RA, Ribeiro JA, Carvalho AP, Carvalho CM (1995) A functionally active presynaptic high-affinity kainate receptor in the rat hippocampal CA3 subregion. Neurosci Lett 185: 83-86.

Malva JO, Carvalho AP, Carvalho CM (1996) Domoic acid induces the release of glutamate in the rat hippocampal CA3 subregion. Neuroreport 7: 1330-1334.

Mannaioni G, Marino MJ, Valenti O, Traynelis SF, Conn PJ (2001) Metabotropic glutamate receptors 1 and 5 differentially regulate CA1 pyramidal cell function. J Neurosci 21: 5925-5934.

Mansour SJ, Matten WT, Hermann AS, Candia JM, Rong S, Fukasawa K, Vande Woude GF, Ahn NG (1994) Transformation of mammalian cells by constitutively active MAP kinase kinase. Science 265: 966-970.

Manzoni OJ, Finiels-Marlier F, Sassetti I, Blockaert J, le Peuch C, Sladeczek FA (1990) The glutamate receptor of the Qp-type activates protein kinase C and is regulated by protein kinase C. Neurosci Lett 109: 146-151.

Marais R, Light Y, Mason C, Paterson H, Olson MF, Marshall CJ (1998) Requirement of Ras-GTP-Raf complexes for activation of Raf-1 by protein kinase C. Science 280: 109-112.

Marks B, McMahon HT (1998) Calcium triggers calcineurin-dependent synaptic vesicle recycling in mammalian nerve terminals. Curr Biol 8: 740-749.

Marti M, Paganini F, Stocchi S, Bianchi C, Beani L, Morari M (2001) Presynaptic group I and II metabotropic glutamate receptors oppositely modulate striatal acetylcholine release. Eur J Neurosci 14: 1181-1184.

Martin TF (1998) Phosphoinositide lipids as signaling molecules: common themes for signal transduction, cytoskeletal regulation, and membrane trafficking. Annu Rev Cell Dev Biol 14: 231-264.

Martin TW (1988) Formation of diacylglycerol by a phospholipase D-phosphatidate phosphatase pathway specific for phosphatidylcholine in endothelial cells. Biochim Biophys Acta 962: 282-296.

Masu M, Tanabe Y, Tsuchida K, Shigemoto R, Nakanishi S (1991) Sequence and expression of a metabotropic glutamate receptor. Nature 349: 760-765.

Mathiesen JM, Svendsen N, Brauner-Osborne H, Thomsen C, Ramirez MT (2003) Positive allosteric modulation of the human metabotropic glutamate receptor 4 (hmGluR4) by SIB-1893 and MPEP. Br J Pharmacol 138: 1026-1030.

Matveeva EA, Whiteheart SW, Vanaman TC, Slevin JT (2001) Phosphorylation of the N-ethylmaleimide-sensitive factor is associated with depolarization-dependent neurotransmitter release from synaptosomes. J Biol Chem 276: 12174-12181.

Maudsley S, Pierce KL, Zamah AM, Miller WE, Ahn S, Daaka Y, Lefkowitz RJ, Luttrell LM (2000) The beta(2)-adrenergic receptor mediates extracellular signal-regulated kinase activation via assembly of a multi-receptor complex with the epidermal growth factor receptor. J Biol Chem 275: 9572-9580.

Maycox PR, Hell JW, Jahn R (1990) Amino acid neurotransmission: spotlight on synaptic vesicles. Trends Neurosci 13: 83-87.

McAvoy T, Allen PB, Obaishi H, Nakanishi H, Takai Y, Greengard P, Nairn AC, Hemmings HCJ (1999) Regulation of neurabin I interaction with protein phosphatase 1 by phosphorylation. Biochemistry 38: 12943-12949.

McGahon B, Lynch MA (1994) A study of the synergism between metabotropic glutamate receptor activation and arachidonic acid in the rat hippocampus. Neuroreport 5: 2353-2357.

McGahon B, Maguire C, Kelly A, Lynch MA (1999) Activation of p42 mitogen-activated protein kinase by arachidonic acid and trans-1-amino-cyclopentyl-1,3-dicarboxylate impacts on long-term potentiation in the dentate gyrus in the rat: analysis of age-related changes. Neuroscience 90: 1167-1175.

McGinty JF (1999) Regulation of neurotransmitter interactions in the ventral striatum. Ann N Y Acad Sci 877: 129-139.

McLennan H (1983) Receptors for the excitatory amino acids in the mammalian central nervous system. Prog Neurobiol 20: 251-271.

McMahon HT, Nicholls DG (1990) The relationship between cytoplasmic free Ca2+ and the release of glutamate from synaptosomes. Biochem Soc Trans 18: 375-377.

Meisenhelder J, Suh PG, Rhee SG, Hunter T (1989) Phospholipase C-gamma is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. Cell 57: 1109-1122.

Merrifield CJ, Moss SE, Ballestrem C, Imhof BA, Giese G, Wunderlich I, Almers W (1999) Endocytic vesicles move at the tips of actin tails in cultured mast cells. Nat Cell Biol 1: 72-74.

Millan C, Lujan R, Shigemoto R, Sanchez-Prieto J (2002) The inhibition of glutamate release by metabotropic glutamate receptor 7 affects both [Ca2+]c and cAMP: evidence for a strong reduction of Ca2+ entry in single nerve terminals. J Biol Chem 277: 14092-14101.

Min MY, Melyan Z, Kullmann D (1999) Synaptically released glutamate reduces gamma-aminobutyric acid (GABA)ergic inhibition in the hippocampus via kainate receptors. Proc Natl Acad Sci U S A 96: 9932-9937.

Minami N, Berglund K, Sakaba T, Kohmoto H, Tachibana M (1998) Potentiation of transmitter release by protein kinase C in goldfish retinal bipolar cells. J Physiol 512: 219-225.

Mineff E, Valtschanoff J (1999) Metabotropic glutamate receptors 2 and 3 expressed by astrocytes in rat ventrobasal thalamus. Neurosci Lett 270: 95-98.

Minneman KP, Hedberg A, Molinoff PB (1979) Comparison of beta adrenergic receptor subtypes in mammalian tissues. J Pharmacol Exp Ther 211: 502-508.

Mischak H, Seitz T, Janosch P, Eulitz M, Steen H, Schellerer M, Philipp A, Kolch W (1996) Negative regulation of Raf-1 by phosphorylation of serine 621. Mol Cell Biol 16: 5409-5418.

Misura KM, Scheller RH, Weis WI (2000) Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. Nature 404: 355-362.

Monaghan DT, Yao D, Cotman CW (1985) L-[3H]Glutamate binds to kainate-, NMDA- and AMPA-sensitive binding sites: an autoradiographic analysis. Brain Res 340: 378-383.

Mons N, Decorte L, Jaffard R, Cooper DM (1998) Ca2+-sensitive adenylyl cyclases, key integrators of cellular signalling. Life Sci 62: 1647-1652.

Moodie SA, Paris MJ, Kolch W, Wolfman A (1994) Association of MEK1 with p21ras.GMPPNP is dependent on B-Raf. Mol Cell Biol 14: 7153-7162.

Moodie SA, Willumsen BM, Weber MJ, Wolfman A (1993) Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase kinase. Science 260: 1659-1661.

Moore JB, et al. (1998) Inhibition of PTH desensitization by inhibition of the G protein-coupled receptor kinase-5 enzyme with Ro 32-0432. FASEB J 12: A741.

Morozov A, Muzzio IA, Bourtchouladze R, Van-Strien N, Lapidus K, Yin D, Winder DG, Adams JP, Sweatt JD, Kandel ER (2003) Rap1 couples cAMP signaling to a distinct pool of p42/44MAPK regulating excitability, synaptic plasticity, learning, and memory. Neuron 39: 309-325.

Morrison DK (2001) KSR: a MAPK scaffold of the Ras pathway? J Cell Sci 114: 1609-1612.

Moss J, Bruni P, Hsia JA, Tsai SC, Watkins PA, Halpern JL, Burns DL, Kanaho Y, Chang PP, Hewlett EL (1984) Pertussis toxin-catalyzed ADP-ribosylation: effects on the coupling of inhibitory receptors to the adenylate cyclase system. J Recept Res 4: 459-474.

Muda M, Boschert U, Dickinson R, Martinou JC, Martinou I, Camps M, Schlegel W, Arkinstall S (1996a) MKP-3, a Novel Cytosolic Protein-tyrosine Phosphatase That Exemplifies a New Class of Mitogen-activated Protein Kinase Phosphatase. J Biol Chem 271: 4319-4326.

Muda M, Boschert U, Smith A, Antonsson B, Gillieron C, Chabert C, Camps M, Martinou I, Ashworth A, Arkinstall S (1997) Molecular Cloning and Functional Characterization of a Novel Mitogen-activated Protein Kinase Phosphatase, MKP-4. J Biol Chem 272: 5141-5151.

Muda M, Theodosiou A, Rodrigues N, Boschert U, Camps M, Gillieron C, Davies K, Ashworth A, Arkinstall S (1996b) The Dual Specificity Phosphatases M3/6 and MKP-3 Are Highly Selective for Inactivation of Distinct Mitogen-activated Protein Kinases. J Biol Chem 271: 27205-27208.

Munno DW, Prince DJ, Syed NI (2003) Synapse number and synaptic efficacy are regulated by presynaptic cAMP and protein kinase A. J Neurosci 23: 4146-4155.

Munshi R, Pang IH, Sternweis PC, Linden J (1991) A1 adenosine receptors of bovine brain couple to guanine nucleotide-binding proteins Gi1, Gi2, and Go. J Biol Chem 266: 22285-22289.

Murphy SN, Miller RJ (1988) A glutamate receptor regulates Ca2+ mobilization in hippocampal neurons. Proc Natl Acad Sci U S A 85: 8737-8741.

Murthy VN, De Camilli P (2003) Cell biology of the presynaptic terminal. Annu Rev Neurosci 26: 701-728.

Nagy G, Reim K, Matti U, Brose N, Binz T, Rettig J, Neher E, Sorensen JB (2004) Regulation of releasable vesicle pool sizes by protein kinase A-dependent phosphorylation of SNAP-25. Neuron 41: 417-429.

Nakanishi S (1992) Molecular diversity of glutamate receptors and implications for brain function. Science 258: 597-603.

Neary JT, Kang Y, Bu Y, Yu E, Akong K, Peters CM (1999) Mitogenic Signaling by ATP/P2Y Purinergic Receptors in Astrocytes: Involvement of a Calcium-Independent Protein Kinase C, Extracellular Signal-Regulated Protein Kinase Pathway Distinct from the Phosphatidylinositol-Specific Phospholipase C/Calcium Pathway. J Neurosci 19: 4211-4220.

Newton AC (1995) Protein kinase C: structure, function, and regulation. J Biol Chem 270: 28495-28498.

Newton AC (1997) Regulation of protein kinase C. Curr Opin Cell Biol 9: 161-167.

Nguyen C, Bibb JA (2003) Cdk5 and the mystery of synaptic vesicle endocytosis. J Cell Biol 163: 679-699.

Nicholls DG (1989) Release of glutamate, aspartate, and gamma-aminobutyric acid from isolated nerve terminals. J Neurochem 52: 331-341.

Nicholls DG, Sihra TS (1986) Synaptosomes possess an exocytotic pool of glutamate. Nature 321: 772-773.

Nicholls DG, Sihra TS, Sanchez-Prieto J (1987) The role of the plasma membrane and intracellular organelles in synaptosomal calcium regulation. Soc Gen Physiol Ser 42: 31-43

Nichols RA, Haycock JW, Wang JK, Greengard P (1987) Phorbol ester enhancement of neurotransmitter release from rat brain synaptosomes. J Neurochem 48: 615-621.

Nicoletti F, Meek JL, Iadarola MJ, Chuang DM, Roth BL, Costa E (1986) Coupling of inositol phospholipid metabolism with excitatory amino acid recognition sites in rat hippocampus. J Neurochem 46: 40-46.

Nicoll RA (2003) Expression mechanisms underlying long-term potentiation: a postsynaptic view. Philos Trans R Soc Lond B Biol Sci 358: 721-726.

Nicoll RA, Malenka RC, Kauer JA (1990) Functional comparison of neurotransmitter receptor subtypes in mammalian central nervous system. Physiol Rev 70: 513-565.

Nishibe S, Wahl MI, Hernandez-Sotomayor SM, Tonks NK, Rhee SG, Carpenter G (1990) Increase of the catalytic activity of phospholipase C-gamma 1 by tyrosine phosphorylation. Science 250: 1253-1256.

Norman ED, Thiels E, Barrionuevo G, Klann E (2000) Long-term depression in the hippocampus in vivo is associated with protein phosphatase-dependent alterations in extracellular signal-regulated kinase. J Neurochem 74: 192-198.

Norum JH, Hart K, Levy FO (2003) Ras-dependent ERK activation by the human G(s)-coupled serotonin receptors 5-HT4(b) and 5-HT7(a). J Biol Chem 278: 3098-3104.

Obata K, Yamanaka H, Dai Y, Tachibana T, Fukuoka T, Tokunaga A, Yoshikawa H, Noguchi K (2003) Differential activation of extracellular signal-regulated protein kinase in primary afferent neurons regulates brain-derived neurotrophic factor expression after peripheral inflammation and nerve injury. J Neurosci 23: 4117-4126.

Obermeier A, Lammers R, Wiesmuller KH, Jung G, Schlessinger J, Ullrich A (1993) Identification of Trk binding sites for SHC and phosphatidylinositol 3'-kinase and formation of a multimeric signaling complex. J Biol Chem 268: 22963-22966.

Ohishi H, Ogawa-Meguro R, Shigemoto R, Kaneko T, Nakanishi S, Mizuno N (1994) Immunohistochemical localization of metabotropic glutamate receptors, mGluR2 and mGluR3, in rat cerebellar cortex. Neuron 13: 55-66.

Ohishi H, Shigemoto R, Nakanishi S, Mizuno N (1993) Distribution of the mRNA for a metabotropic glutamate receptor (mGluR3) in the rat brain: an in situ hybridization study. J Comp Neurol 335: 252-266.

Olsson AK, Vadhammar K, Nanberg E (2000) Activation and protein kinase C-dependent nuclear accumulation of ERK in differentiating human neuroblastoma cells. Exp Cell Res 256: 454-467.

Orban PC, Chapman PF, Brambilla R (1999) Is the Ras-MAPK signalling pathway necessary for long-term memory formation? Trends Neurosci 22: 38-44.

Orrego F, Villanueva S (1993) The chemical nature of the main central excitatory transmitter: a critical appraisal based upon release studies and synaptic vesicle localization. Neuroscience 56: 539-555.

Otto H, Hanson PI, Jahn R (1997) Assembly and disassembly of a ternary complex of synaptobrevin, syntaxin, and SNAP-25 in the membrane of synaptic vesicles. Proc Natl Acad Sci U S A 94: 6197-6201.

Pagano A, Ruegg D, Litschig S, Stoehr N, Stierlin C, Heinrich M, Floersheim P, Prezeau L, Carroll F, Pin JP, Cambria A, Vranesic I, Flor PJ, Gasparini F, Kuhn R (2000) The non-competitive antagonists 2-methyl-6-(phenylethynyl)pyridine and 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester interact with overlapping binding pockets in the transmembrane region of group I metabotropic glutamate receptors. J Biol Chem 275: 33750-33758.

Pages G, Lenormand P, L'Allemain G, Chambard J, Meloche S, Pouyssegur J (1993) Mitogen-Activated Protein Kinases p42mapk and p44mapk are Required for Fibroblast Proliferation. PNAS 90: 8319-8323.

Park DJ, Min HK, Rhee SG (1991a) IgE-induced tyrosine phosphorylation of phospholipase C-gamma 1 in rat basophilic leukemia cells. J Biol Chem 266: 24237-24240.

Park DJ, Rho HW, Rhee SG (1991b) CD3 stimulation causes phosphorylation of phospholipase C-gamma 1 on serine and tyrosine residues in a human T-cell line. Proc Natl Acad Sci U S A 88: 5453-5456.

Park KS, Jeong SW, Cha SK, Lee BS, Kong ID, Ikeda SR, Lee JW (2001) Modulation of N-type Ca2+ currents by A1-adenosine receptor activation in male rat pelvic ganglion neurons. J Pharmacol Exp Ther 299: 501-508.

Parker PJ, Coussens L, Totty N, Rhee L, Young S, Chen E, Stabel S, Waterfield MD, Ullrich A (1986) The complete primary structure of protein kinase C--the major phorbol ester receptor. Science 233: 853-859.

Parmentier ML, Prezeau L, Bockaert J, Pin JP (2002) A model for the functioning of family 3 GPCRs. Trends Pharmacol Sci 23: 268-274.

Pasantes-Morales H, Franco R, Ochoa L, Ordaz B (2002) Osmosensitive release of neurotransmitter amino acids: relevance and mechanisms. Neurochem Res 27: 59-65.

Pastorino L, Colciaghi F, Gardoni F, Albani-Torregrossa S, Pellegrini-Giampietro DE, Moroni F, De Graan PN, Cattabeni F, Di Luca M (2000) (+)-MCPG induces PKCepsilon translocation in cortical synaptosomes through a PLD-coupled mGluR. Eur J Neurosci 12: 1310-1318.

Patapoutian A, Reichardt LF (2001) Trk receptors: mediators of neurotrophin action. Curr Opin Neurobiol 11: 272-280.

Paternain AV, Rodriguez-Moreno A, Villarroel A, Lerma J (1998) Activation and desensitization properties of native and recombinant kainate receptors. Neuropharmacology 37: 1249-1259.

Payne DM, Rossomando AJ, Martino P, Erickson AK, Her JH, Shabanowitz J, Hunt DF, Weber MJ, Sturgill TW (1991) Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). EMBO J 10: 885-892.

Peavy RD, Conn PJ (1998) Phosphorylation of mitogen-activated protein kinase in cultured rat cortical glia by stimulation of metabotropic glutamate receptors. J Neurochem 71: 603-612.

Peavy RD, Sorensen SD, Conn PJ (2002) Differential regulation of metabotropic glutamate receptor 5-mediated phosphoinositide hydrolysis and extracellular signal-regulated kinase responses by protein kinase C in cultured astrocytes. J Neurochem 83: 110-118.

Pende M, Lanza M, Bonanno G, Raiteri M (1993) Release of endogenous glutamic and aspartic acids from cerebrocortex synaptosomes and its modulation through activation of a gamma-aminobutyric acidB (GABAB) receptor subtype. Brain Res 604: 325-330.

Pereira DB, Carvalho AP, Duarte CB (2002) Non-specific effects of the MEK inhibitors PD098,059 and U0126 on glutamate release from hippocampal synaptosomes. Neuropharmacology 42: 9-19.

Perkinton MS, Sihra TS (1998) Presynaptic GABA(B) receptor modulation of glutamate exocytosis from rat cerebrocortical nerve terminals: receptor decoupling by protein kinase C. J Neurochem 70: 1513-1522.

Perkinton MS, Sihra TS (1999) A high-affinity presynaptic kainate-type glutamate receptor facilitates glutamate exocytosis from cerebral cortex nerve terminals (synaptosomes). Neuroscience 90: 1281-1292.

Petralia RS, Wang YX, Singh S, Wu C, Shi L, Wei J, Wenthold RJ (1997) A monoclonal antibody shows discrete cellular and subcellular localizations of mGluR1 alpha metabotropic glutamate receptors. J Chem Neuroanat 13: 77-93.

Pevsner J, Hsu SC, Scheller RH (1994) n-Sec1: a neural-specific syntaxin-binding protein. Proc Natl Acad Sci U S A 91: 1445-1449.

Pierau FK, Zimmermann P (1973) Action of a GABA-derivative on postsynaptic potentials and membrane properties of cats' spinal motoneurones. Brain Res 54: 376-380.

Pierce KL, Maudsley S, Daaka Y, Luttrell LM, Lefkowitz RJ (2000) Role of endocytosis in the activation of the extracellular signal-regulated kinase cascade by sequestering and nonsequestering G protein-coupled receptors. Proc Natl Acad Sci U S A 97: 1489-1494.

Pieribone VA, Porton B, Rendon B, Feng J, Greengard P, Kao HT (2002) Expression of synapsin III in nerve terminals and neurogenic regions of the adult brain. J Comp Neurol 454: 105-114.

Pierret P, Mechawar N, Vallee A, Patel J, Priestley JV, Dunn RJ, Dower NA, Stone JC, Richardson PM (2002) Presence of Ras guanyl nucleotide-releasing protein in striosomes of the mature and developing rat. Neuroscience 111: 83-94.

Pierret P, Vallee A, Mechawar N, Dower NA, Stone JC, Richardson PM, Dunn RJ (2001) Cellular and subcellular localization of Ras guanyl nucleotide-releasing protein in the rat hippocampus. Neuroscience 108: 381-390.

Pin JP, Duvoisin R (1995) The metabotropic glutamate receptors: structure and functions. Neuropharmacology 34: 1-26.

Pin JP, Waeber C, Prezeau L, Bockaert J, Heinemann SF (1992) Alternative splicing generates metabotropic glutamate receptors inducing different patterns of calcium release in Xenopus oocytes. Proceedings of the National Academy of Sciences, USA 89: 10331-10335.

Pisani A, Bonsi P, Centonze D, Bernardi G, Calabresi P (2001) Functional coexpression of excitatory mGluR1 and mGluR5 on striatal cholinergic interneurons. Neuropharmacology 40: 460-463.

Pittaluga A, Raiteri M (1992) N-methyl-D-aspartic acid (NMDA) and non-NMDA receptors regulating hippocampal norepinephrine release. III. Changes in the NMDA receptor complex induced by their functional cooperation. J Pharmacol Exp Ther 263: 327-333.

Poirier MA, Xiao W, Macosko JC, Chan C, Shin YK, Bennett MK (1998) The synaptic SNARE complex is a parallel four-stranded helical bundle. Nat Struct Biol 5: 765-769.

Poisik OV, Mannaioni G, Traynelis S, Smith Y, Pin JP (2003) Distinct functional roles of the metabotropic glutamate receptors 1 and 5 in the rat globus pallidus. J Neurosci 23: 122-130.

Poo MM (2001) Neurotrophins as synaptic modulators. Nat Rev Neurosci 2: 24-32.

Porton B, Ferreira A, DeLisi LE, Kao HT (2004) A rare polymorphism affects a mitogen-activated protein kinase site in synapsin III: possible relationship to schizophrenia. Biol Psychiatry 55: 118-125.

Poskanzer KE, Marek KW, Sweeney ST, Davis GW (2003) Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis in vivo. Nature 426: 559-563.

Potier B, Dutar P (1993) Presynaptic inhibitory effect of baclofen on hippocampal inhibitory synaptic transmission involves a pertussis toxin-sensitive G-protein. Eur J Pharmacol 231: 427-433.

Powell KA, Valova VA, Malladi CS, Jensen ON, Larsen MR, Robinson PJ (2000) Phosphorylation of dynamin I on Ser-795 by protein kinase C blocks its association with phospholipids. J Biol Chem 275: 11610-11617.

Price NE, Mumby MC (1999) Brain protein serine/threonine phosphatases. Curr Opin Neurobiol 9: 336-342.

Prior IA, Hancock JF (2001) Compartmentalization of Ras proteins. J Cell Sci 114: 1603-1608.

Pujol MJ, Bosser R, Vendrell M, Serratosa J, Bachs O (1993) Nuclear calmodulin-binding proteins in rat neurons. J Neurochem 60: 1422-1428.

Qin S, Hu XY, Xu H, Zhou JN (2004) Regional alteration of synapsin I in the hippocampal formation of Alzheimer's disease patients. Acta Neuropathol 107: 209-215.

Rasenick MM, Watanabe M, Lazarevic MB, Hatta S, Hamm HE (1994) Synthetic peptides as probes for G protein function. Carboxyl-terminal G alpha s peptides mimic Gs and evoke high affinity agonist binding to beta-adrenergic receptors. J Biol Chem 269: 21519-21525.

Ray LB, Sturgill TW (1987) Rapid stimulation by insulin of a serine/threonine kinase in 3T3-L1 adipocytes that phosphorylates microtubule-associated protein 2 in vitro. Proc Natl Acad Sci U S A 84: 1502-1506.

Ray LB, Sturgill TW (1988) Insulin-stimulated microtubule-associated protein kinase is phosphorylated on tyrosine and threonine in vivo. Proc Natl Acad Sci U S A 85: 3753-3757.

Rebecchi MJ, Pentyala SN (2000) Structure, function, and control of phosphoinositide-specific phospholipase C. Physiol Rev 80: 1291-1335.

Reid ME, Toms NJ, Bedingfield JS, Roberts PJ (1999) Group I mGlu receptors potentiate synaptosomal [3H]glutamate release independently of exogenously applied arachidonic acid. Neuropharmacology 38: 477-485.

Reppert SM, Weaver DR, Stehle JH, Rivkees SA (1991) Molecular cloning and characterization of a rat A1-adenosine receptor that is widely expressed in brain and spinal cord. Mol Endocrinol 5: 1037-1048.

Rhee SG, Bae YS (1997) Regulation of phosphoinositide-specific phospholipase C isozymes. J Biol Chem 272: 15045-15048.

Ribeiro JA, Sebastiao AM (1987) On the role, inactivation and origin of endogenous adenosine at the frog neuromuscular junction. J Physiol (Lond) 384: 571-585.

Ribeiro JA, Sebastiao AM, de Mendonca A (2003) Adenosine receptors in the nervous system: pathophysiological implications. Prog Neurobiol 68: 377-392.

Rice LM, Brunger AT (1999) Crystal structure of the vesicular transport protein Sec17: implications for SNAP function in SNARE complex disassembly. Mol Cell 4: 85-95.

Ringstad N, Gad H, Low P, Di Paolo G, Brodin L, Shupliakov O, De Camilli P (1999) Endophilin/SH3p4 is required for the transition from early to late stages in clathrin-mediated synaptic vesicle endocytosis. Neuron 24: 143-154.

Risinger C, Bennett MK (1999) Differential phosphorylation of syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25) isoforms. J Neurochem 72: 614-624.

Robbe D, Alonso G, Chaumont S, Bockaert J, Manzoni OJ (2002) Role of p/q-Ca2+ channels in metabotropic glutamate receptor 2/3-dependent presynaptic long-term depression at nucleus accumbens synapses. J Neurosci 22: 4346-4356.

Roberson ED, English JD, Adams JP, Selcher JC, Kondratick C, Sweatt JD (1999) The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. J Neurosci 19: 4337-4348.

Robinson MJ, Xu B, Stippec S, Cobb MH (2002) Different Domains of the Mitogen-activated Protein Kinases ERK3 and ERK2 Direct Subcellular Localization and Upstream Specificity in Vivo. J Biol Chem 277: 5094-5100.

Roche KW, Tingley WG, Huganir RL (1994) Glutamate receptor phosphorylation and synaptic plasticity. Curr Opin Neurobiol 4: 383-388.

Rodriguez-Moreno A, Herreras O, Lerma J (1997) Kainate receptors presynaptically downregulate GABAergic inhibition in the rat hippocampus. Neuron 19: 893-901.

Rodriguez-Moreno A, Sihra TS (2004) Presynaptic kainate receptor facilitation of glutamate release involves protein kinase A in the rat hippocampus. J Physiol 557: 733-745.

Rodriguez-Moreno A, Sistiaga A, Lerma J, Sanchez-Prieto J (1998) Switch from facilitation to inhibition of excitatory synaptic transmission by group I mGluR desensitization. Neuron 21: 1477-1486.

Rodriguez-Viciana P, Warne PH, Dhand R, Vanhaesebroeck B, Gout I, Fry MJ, Waterfield MD, Downward J (1994) Phosphatidylinositol-3-OH kinase as a direct target of Ras.. Nature 370: 527-532.

Rosen LB, Ginty DD, Weber MJ, Greenberg ME (1994) Membrane depolarization and calcium influx stimulate MEK and MAP kinase via activation of Ras. Neuron 12: 1207-1221.

Rosenmund C, Stern-Bach Y, Stevens CF (1998) The tetrameric structure of a glutamate receptor channel. Science 280: 1596-1599.

Runden E, Seglen PO, Haug FM, Ottersen OP, Wieloch T, Shamloo M, Laake JH (1998) Regional Selective Neuronal Degeneration after Protein Phosphatase Inhibition in Hippocampal Slice Cultures: Evidence for a MAP Kinase-Dependent Mechanism. J Neurosci 18: 7296-7305.

Rusanescu G, Gotoh T, Tian X, Feig LA (2001) Regulation of Ras signaling specificity by protein kinase C. Mol Cell Biol 21: 2650-2658.

Rusnak F, Mertz P (2000) Calcineurin: Form and Function. Physiol Rev 80: 1483-1521.

Saint DA, Thomas T, Gage PW (1990) GABAB agonists modulate a transient potassium current in cultured mammalian hippocampal neurons. Neurosci Lett 118: 9-13.

Sakurada K, Kato H, Nagumo H, Hiraoka H, Furuya K, Ikuhara T, Yamakita Y, Fukunaga K, Miyamoto E, Matsumura F, Matsuo YI, Naito Y, Sasaki Y (2002) Synapsin I is phosphorylated at Ser603 by p21-activated kinases (PAKs) in vitro and in PC12 cells stimulated with bradykinin. J Biol Chem 277: 45473-45479.

Salin PA, Malenka RC, Nicoll RA (1996) Cyclic AMP mediates a presynaptic form of LTP at cerebellar parallel fiber synapses. Neuron 16: 797-803.

Sanchez-Prieto J, Budd DC, Herrero I, Vazquez E, Nicholls DG (1996) Presynaptic receptors and the control of glutamate exocytosis. Trends Neurosci 19: 235-239.

Sanchez-Prieto J, Sihra TS, Nicholls DG (1987) Characterization of the exocytotic release of glutamate from guinea-pig cerebral cortical synaptosomes. J Neurochem 49: 58-64.

Sando JJ, Chertihin OI (1996) Activation of protein kinase C by lysophosphatidic acid: dependence on composition of phospholipid vesicles. Biochem J 317: 583-588.

Sanna PP, Simpson C, Lutjens R, Koob G (2002) ERK regulation in chronic ethanol exposure and withdrawal. Brain Res 948: 186-191.

Sassa T, Harada S, Ogawa H, Rand JB, Maruyama IN, Hosono R (1999) Regulation of the UNC-18-Caenorhabditis elegans syntaxin complex by UNC-13. J Neurosci 19: 4772-4777.

Scanziani M, Gahwiler BH, Charpak S (1998) Target cell-specific modulation of transmitter release at terminals from a single axon. Proc Natl Acad Sci U S A 95: 12004-12009.

Scanziani M, Salin PA, Vogt KE, Malenka RC, Nicoll RA (1997) Use-dependent increases in glutamate concentration activate presynaptic metabotropic glutamate receptors. Nature 385: 630-634.

Scarpace PJ, Tumer N, Mader SL (1991) Beta-adrenergic function in aging. Basic mechanisms and clinical implications. Drugs Aging 1: 116-129.

Schaechter JD, Benowitz LI (1993) Activation of protein kinase C by arachidonic acid selectively enhances the phosphorylation of GAP-43 in nerve terminal membranes. J Neurosci 13: 4361-4371.

Schaeffer HJ, Catling AD, Eblen ST, Collier LS, Krauss A, Weber MJ (1998) MP1: a MEK binding partner that enhances enzymatic activation of the MAP kinase cascade. Science 281: 1668-1671.

Schaffhauser H, Cai Z, Hubalek F, Macek TA, Pohl J, Murphy TJ, Conn PJ (2000) cAMP-Dependent Protein Kinase Inhibits mGluR2 Coupling to G-Proteins by Direct Receptor Phosphorylation. J Neurosci 20: 5663-5670.

Schaffhauser H, Knoflach F, Pink JR, Bleuel Z, Cartmell J, Goepfert F, Kemp JA, Richards JG, Adam G, Mutel V (1998) Multiple pathways for regulation of the KCl-induced [3H]-GABA release by

metabotropic glutamate receptors, in primary rat cortical cultures. Brain Res 782: 91-104.

Schiavo G, Stenbeck G, Rothman JE, Sollner TH (1997) Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin-treated synapses. Proc Natl Acad Sci U S A 94: 997-1001.

Schikorski T, Stevens CF (2001) Morphological correlates of functionally defined synaptic vesicle populations. Nat Neurosci 4: 391-395.

Schmitt JM, Stork PJ (2000) beta 2-adrenergic receptor activates extracellular signal-regulated kinases (ERKs) via the small G protein rap1 and the serine/threonine kinase B-Raf. J Biol Chem 275: 25342-25350.

Schmitt JM, Stork PJ (2002) Galpha and Gbeta gamma require distinct Src-dependent pathways to activate Rap1 and Ras. J Biol Chem 277: 43024-43032.

Schmitz D, Frerking M, Nicoll RA (2000) Synaptic activation of presynaptic kainate receptors on hippocampal mossy fiber synapses. Neuron 27: 327-338.

Schoepp D, Bockaert J, Sladeczek F (1990) Pharmacological and functional characteristics of metabotropic excitatory amino acid receptors. Trends Pharmacol Sci 11: 508-515.

Schuler V, Luscher C, Blanchet C, Klix N, Sansig G, Klebs K, Schmutz M, Heid J, Gentry C, Urban L, Fox A, Spooren W, Jaton AL, Vigouret J, Pozza M, Kelly PH, Mosbacher J, Froestl W, Kaslin E, Korn R, Bischoff S, Kaupmann K, van der Putten H, Bettler B (2001) Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic GABA(B) responses in mice lacking GABA(B(1)). Neuron 31: 47-58.

Schulte G, Fredholm BB (2000) Human adenosine A(1), A(2A), A(2B), and A(3) receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2. Mol Pharmacol 58: 477-482.

Schulte G, Fredholm BB (2002) Signaling pathway from the human adenosine A(3) receptor expressed in Chinese hamster ovary cells to the extracellular signal-regulated kinase 1/2. Mol Pharmacol 62: 1137-1146.

Schulte G, Fredholm BB (2003) Signalling from adenosine receptors to mitogen-activated protein kinases. Cell Signal 15: 813-827.

Schutz W, Freissmuth M (1992) Reverse intrinsic activity of antagonists on G protein-coupled receptors. Trends Pharmacol Sci 13: 376-380.

Sciancalepore M, Stratta F, Fisher ND, Cherubini E (1995) Activation of metabotropic glutamate receptors increase the frequency of spontaneous GABAergic currents through protein kinase A in neonatal rat hippocampal neurons. J Neurophysiol 74: 1118-1122.

Scott JD (1991) Cyclic nucleotide-dependent protein kinases. Pharmacol Ther 50: 123-145.

Seamon KB, Daly JW (1981) Forskolin: a unique diterpene activator of cyclic AMP-generating systems. J Cyclic Nucleotide Res 7: 201-224.

Sebastiao AM, Ribeiro JA (1996) Adenosine A2 receptor-mediated excitatory actions on the nervous system. Prog Neurobiol 48: 167-189.

Seeburg PH (1993) The TINS/TiPS Lecture. The molecular biology of mammalian glutamate receptor channels. Trends Neurosci 16: 359-365.

Seger R, Ahn NG, Posada J, Munar ES, Jensen AM, Cooper JA, Cobb MH, Krebs EG (1992) Purification and characterization of mitogen-activated protein kinase activator(s) from epidermal growth factor-stimulated A431 cells. J Biol Chem 267: 14373-14381.

Seger R, Krebs EG (1995) The MAPK signaling cascade. FASEB J 9: 726-735.

Shearman MS, Sekiguchi K, Nishizuka Y (1989) Modulation of ion channel activity: a key function of the protein kinase C enzyme family. Pharmacol Rev 41: 211-237.

Sheng M, Kim MJ (2002) Postsynaptic signaling and plasticity mechanisms. Science 298: 776-780.

Sherr CJ (1996) Cancer cell cycles. Science 274: 1672-1677.

Shields JM, Pruitt K, McFall A, Shaub A, Der CJ (2000) Understanding Ras: 'it ain't over 'til it's over'. Trends Cell Biol 10: 147-154.

Shigemoto R, Kinoshita A, Wada E, Nomura S, Ohishi H, Takada M, Flor PJ, Neki A, Abe T, Nakanishi S, Mizuno N (1997) Differential presynaptic localization of metabotropic glutamate receptor subtypes in the rat hippocampus. J Neurosci 17: 7503-7522.

Shigemoto R, Kulik A, Roberts JD, Ohishi H, Nusser Z, Kaneko T, Somogyi P (1996) Target-cell-specific concentration of a metabotropic glutamate receptor in the presynaptic active zone. Nature 381: 523-525.

Shigemoto R, Nomura S, Ohishi H, Sugihara H, Nakanishi S, Mizuno N (1993) Immunohistochemical localization of a metabotropic glutamate receptor, mGluR5, in the rat brain. Neurosci Lett 163: 53-57.

Siddhanti SR, Hartle JE2, Quarles LD (1995) Forskolin inhibits protein kinase C-induced mitogen activated protein kinase activity in MC3T3-E1 osteoblasts. Endocrinology 136: 4834-4841.

Sidovar MF, Kozlowski P, Lee JW, Collins MA, He Y, Graves LM (2000) Phosphorylation of serine 43 is not required for inhibition of c-Raf kinase by the cAMP-dependent protein kinase. J Biol Chem 275: 28688-28694.

Sihra TS (1997) Protein phosphorylation and dephosphorylation in isolated nerve terminals (synaptosomes). In: Regulatory protein modification: techniques and protocols (Hemmings HCJ, ed), pp 67-119. Humana Press Inc.

Sihra TS, Bogonez E, Nicholls DG (1992) Localized Ca2+ entry preferentially effects protein dephosphorylation, phosphorylation, and glutamate release. J Biol Chem 267: 1983-1989.

Sihra TS, Piomelli D, Nichols RA (1993) Barium evokes glutamate release from rat brain synaptosomes by membrane depolarization: involvement of K+, Na+, and Ca2+ channels. J Neurochem 61: 1220-1230.

Simonds WF (1999) G protein regulation of adenylate cyclase. Trends Pharmacol Sci 20: 66-73.

Singer WD, Brown HA, Sternweis PC (1997) Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D. Annu Rev Biochem 66: 475-509.

Sistiaga A, Sanchez-Prieto J (2000a) Protein phosphatase 1 and 2A inhibitors prolong the switch in the control of glutamate release by group I metabotropic glutamate receptors: characterization of the inhibitory pathway. J Neurochem 75: 1566-1574.

Sistiaga A, Sanchez-Prieto J (2000b) Protein phosphatase 2B inhibitors mimic the action of arachidonic acid and prolong the facilitation of glutamate release by group I mGlu receptors. Neuropharmacology 39: 1544-1553.

Smellie FW, Davis CW, Daly JW, Wells JN (1979) Alkylxanthines: inhibition of adenosine-elicited accumulation of cyclic AMP in brain slices and of brain phosphodiesterase activity. Life Sci 24: 2475-2482.

Smith FD, Oxford GS, Milgram SL (1999) Association of the D2 Dopamine Receptor Third Cytoplasmic Loop with Spinophilin, a Protein Phosphatase-1-interacting Protein. J Biol Chem 274: 19894-19900.

Smrcka AV, Hepler JR, Brown KO, Sternweis PC (1991) Regulation of polyphosphoinositide-specific phospholipase C activity by purified Gq. Science 251: 804-807.

Soderling TR (1993) Protein kinases and phosphatases: regulation by autoinhibitory domains. Biotechnol Appl Biochem 18: 185-200.

Sommer B, Burnashev N, Verdoorn TA, Keinanen K, Sakmann B, Seeburg PH (1992) A glutamate receptor channel with high affinity for domoate and kainate. EMBO J 11: 1651-1656.

Song W, Zinsmaier KE (2003) Endophilin and synaptojanin hook up to promote synaptic vesicle endocytosis. Neuron 40: 665-667.

Sontag E (2001) Protein phosphatase 2A: the Trojan Horse of cellular signaling. Cell Signal 13: 7-16.

Sontag E, Fedorov S, Kamibayashi C, Robbins D, Cobb M, Mumby M (1993) The interaction of SV40 small tumor antigen with protein phosphatase 2A stimulates the map kinase pathway and induces cell proliferation. Cell 75: 887-897.

Söllner TH (1995) SNAREs and targeted membrane fusion. FEBS Lett 369: 80-83.

Stahl B, Chou JH, Li C, Sudhof TC, Jahn R (1996) Rab3 reversibly recruits rabphilin to synaptic vesicles by a mechanism analogous to raf recruitment by ras. EMBO J 15: 1799-1809.

Stanton PK, Sarvey JM (1985a) Blockade of norepinephrine-induced long-lasting potentiation in the hippocampal dentate gyrus by an inhibitor of protein synthesis. Brain Res 361: 276-283.

Stanton PK, Sarvey JM (1985b) Depletion of norepinephrine, but not serotonin, reduces long-term potentiation in the dentate gyrus of rat hippocampal slices. J Neurosci 5: 2169-2176.

Stawowy P, Goetze S, Margeta C, Fleck E, Graf K (2003) LPS regulate ERK1/2-dependent signaling in cardiac fibroblasts via PKC-mediated MKP-1 induction. Biochem Biophys Res Commun 303: 74-80.

Stella SLJ, Bryson EJ, Thoreson WB (2002) A2 adenosine receptors inhibit calcium influx through L-type calcium channels in rod photoreceptors of the salamander retina. J Neurophysiol 87: 351-360.

Stephenson FA (1988) Understanding the GABAA receptor: a chemically gated ion channel. Biochem J 249: 21-32.

Stork PJ, Schmitt JM (2002) Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol 12: 258-266.

Storm SM, Khawaja XZ (1999) Probing for drug-induced multiplex signal transduction pathways using high resolution two-dimensional gel electrophoresis: application to beta-adrenoceptor stimulation in the rat C6 glioma cell. Brain Res Mol Brain Res 71: 50-60.

Strack S, Barban MA, Wadzinski BE, Colbran RJ (1997) Differential inactivation of postsynaptic density-associated and soluble Ca2+/calmodulin-dependent protein kinase II by protein phosphatases 1 and 2A. J Neurochem 68: 2119-2128.

Strack S, Kini S, Ebner FF, Wadzinski BE, Colbran RJ (1999) Differential cellular and subcellular localization of protein phosphatase 1 isoforms in brain. J Comp Neurol 413: 373-384.

Strader CD, Fong TM, Tota MR, Underwood D, Dixon RA (1994) Structure and function of G protein-coupled receptors. Annu Rev Biochem 63: 101-132.

Sudhof TC (2000) The synaptic vesicle cycle revisited. Neuron 28: 317-320.

Sudhof TC, Czernik AJ, Kao HT, Takei K, Johnston PA, Horiuchi A, Kanazir SD, Wagner MA, Perin MS, De Camilli P, et al. (1989) Synapsins: mosaics of shared and individual domains in a family of synaptic vesicle phosphoproteins. Science 245: 1474-1480.

Sugiyama H, Ito I, Hirono C (1987) A new type of glutamate receptor linked to inositol phospholipid metabolism. Nature 325: 531-533.

Sullivan JP, Connor JR, Shearer BG, Burch RM (1992) 2,6-Diamino-N-([1-(1-oxotridecyl)-2-piperidinyl] methyl)hexanamide (NPC 15437): a novel inhibitor of protein kinase C interacting at the regulatory domain. Mol Pharmacol 41: 38-44.

Sumida M, Hong RM, Tagaya M (1994) Role of two nucleotide-binding regions in an N-ethylmaleimide-sensitive factor involved in vesicle-mediated protein transport. J Biol Chem 269: 20636-20641.

Summers ST, Cronin MJ (1986) Phorbol esters enhance basal and stimulated adenylate cyclase activity in a pituitary cell line. Biochem Biophys Res Commun 135: 276-281.

Sun H, Charles CH, Lau LF, Tonks NK (1993) MKP-1 (3CH134), an immediate early gene product, is a dual specificity phosphatase that dephosphorylates MAP kinase in vivo. Cell 75: 487-493.

Sunahara RK, Dessauer CW, Gilman AG (1996) Complexity and diversity of mammalian adenylyl cyclases. Annu Rev Pharmacol Toxicol 36: 461-480.

Sundaresan S, Colin IM, Pestell RG, Jameson JL (1996) Stimulation of mitogen-activated protein kinase by gonadotropin-releasing hormone: evidence for the involvement of protein kinase C. Endocrinology 137: 304-311.

Surchev L (1987) Freeze-etching study of synaptosomes from rat cerebral cortex. Acta Anat (Basel) 128: 210-213.

Sutton RB, Ernst JA, Brunger AT (1999) Crystal structure of the cytosolic C2A-C2B domains of synaptotagmin III. Implications for Ca(+2)-independent snare complex interaction. J Cell Biol 147: 589-598.

Sutton RB, Fasshauer D, Jahn R, Brunger AT (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature 395: 347-353.

Sweatt JD (2001) The neuronal MAP kinase cascade: a biochemical signal integration system subserving synaptic plasticity and memory. J Neurochem 76: 1-10.

Sweatt JD (2004) Mitogen-activated protein kinases in synaptic plasticity and memory. Curr Opin Neurobiol 14: 311-317.

Takahashi T, Kajikawa Y, Tsujimoto T (1998) G-Protein-coupled modulation of presynaptic calcium currents and transmitter release by a GABAB receptor. J Neurosci 18: 3138-3146.

Takai K, Ushiro H, Noda Y, Narumiya S, Tokuyama T (1977) Crystalline hemoprotein from Pseudomonas that catalyzes oxidation of side chain of tryptophan and other indole derivatives. J Biol Chem 252: 2648-2656.

Takao-Rikitsu E, Mochida S, Inoue E, Deguchi-Tawarada M, Inoue M, Ohtsuka T, Takai Y (2004) Physical and functional interaction of the active zone proteins, CAST, RIM1, and Bassoon, in neurotransmitter release. J Cell Biol 164: 301-311.

Takei K, Mundigl O, Daniell L, De Camilli P (1996) The synaptic vesicle cycle: a single vesicle budding step involving clathrin and dynamin. The Journal of Cell Biology 133: 1237-1250.

Tanaka A, Nishizaki T (2003) The newly synthesized linoleic acid derivative FR236924 induces a long-lasting facilitation of hippocampal neurotransmission by targeting nicotinic acetylcholine receptors. Bioorg Med Chem Lett 13: 1037-1040.

Tang WJ, Gilman AG (1995) Construction of a soluble adenylyl cyclase activated by Gs alpha and forskolin. Science 268: 1769-1772.

Tang WJ, Hurley JH (1998) Catalytic mechanism and regulation of mammalian adenylyl cyclases. Mol Pharmacol 54: 231-240.

Tanoue T, Adachi M, Moriguchi T, Nishida E (2000) A conserved docking motif in MAP kinases common to substrates, activators and regulators. Nat Cell Biol 2: 110-116.

Tasken K, Skalhegg BS, Tasken KA, Solberg R, Knutsen HK, Levy FO, Sandberg M, Orstavik S, Larsen T, Johansen AK, Vang T, Schrader HP, Reinton NT, Torgersen KM, Hansson V, Jahnsen T (1997) Structure, function, and regulation of human cAMP-dependent protein kinases. Adv Second Messenger Phosphoprotein Res 31: 191-204.

Taylor DL, Diemel LT, Pocock JM (2003) Activation of microglial group III metabotropic glutamate receptors protects neurons against microglial neurotoxicity. J Neurosci 23: 2150-2160.

Taylor SS, Zheng J, Radzio-Andzelm E, Knighton DR, Ten Eyck LF, Sowadski JM, Herberg FW, Yonemoto WM (1993) cAMP-dependent protein kinase defines a family of enzymes. Philos Trans R Soc Lond B Biol Sci 340: 315-324.

Thandi S, Blank JL, Challiss RA (2002) Group-I metabotropic glutamate receptors, mGlu1a and mGlu5a, couple to extracellular signal-regulated kinase (ERK) activation via distinct, but overlapping, signalling pathways. J Neurochem 83: 1139-1153.

Thomas GM, Huganir RL (2004) MAPK cascade signalling and synaptic plasticity. Nat Rev Neurosci 5: 173-183.

Thomas KL, Hunt SP (1993) The regional distribution of extracellularly regulated kinase-1 and -2 messenger RNA in the adult rat central nervous system. Neuroscience 56: 741-757.

Thomas LS, Jane DE, Harris JR, Croucher MJ (2000) Metabotropic glutamate autoreceptors of the mGlu(5) subtype positively modulate neuronal glutamate release in the rat forebrain in vitro. Neuropharmacology 39: 1554-1566.

Thorner J (1982) An essential role for cyclic AMP in growth control: the case for yeast. Cell 30: 5-6.

Tibbs GR, Barrie AP, Van Mieghem FJ, McMahon HT, Nicholls DG (1989) Repetitive action potentials in isolated nerve terminals in the presence of 4-aminopyridine: effects on cytosolic free Ca2+ and glutamate release. J Neurochem 53: 1693-1699.

Toker A, Meyer M, Reddy KK, Falck JR, Aneja R, Aneja S, Parra A, Burns DJ, Ballas LM, Cantley LC (1994) Activation of protein kinase C family members by the novel polyphosphoinositides PtdIns-3,4-P2 and PtdIns-3,4,5-P3. J Biol Chem 269: 32358-32367.

Tonks NK, Cohen P (1983) Calcineurin is a calcium ion-dependent, calmodulin-stimulated protein phosphatase. Biochim Biophys Acta 747: 191-193.

Topham MK, Prescott SM (1999) Mammalian Diacylglycerol Kinases, a Family of Lipid Kinases with Signaling Functions. J Biol Chem 274: 11447-11450.

Troadec JD, Marien M, Mourlevat S, Debeir T, Ruberg M, Colpaert F, Michel PP (2002) Activation of the mitogen-activated protein kinase (ERK(1/2)) signaling pathway by cyclic AMP potentiates the neuroprotective effect of the neurotransmitter noradrenaline on dopaminergic neurons. Mol Pharmacol 62: 1043-1052.

Trudeau LE, Emery DG, Haydon PG (~~1996~~) Direct modulation of the secretory machinery underlies PKA-dependent synaptic facilitation in hippocampal neurons. Neuron 17: 789-797.

Tsien RY (1980) New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. Biochemistry 19: 2396-2404.

Ueda Y, Iwakabe H, Masu M, Suzuki M, Nakanishi S (1997) The mGluR6 5' upstream transgene sequence directs a cell-specific and developmentally regulated expression in retinal rod and ON-type cone bipolar cells. J Neurosci 17: 3014-3023.

Usui H, Inoue R, Tanabe O, Nishito Y, Shimizu M, Hayashi H, Kagamiyama H, Takeda M (1998) Activation of protein phosphatase 2A by cAMP-dependent protein kinase-catalyzed phosphorylation of the 74-kDa B" (delta) regulatory subunit in vitro and identification of the phosphorylation sites. FEBS Lett 430: 312-316.

Vanhoose AM, Emery M, Jimenez L, Winder DG (2002) ERK activation by G-protein-coupled receptors in mouse brain is receptor identity-specific. J Biol Chem 277: 9049-9053.

Varma DR, Shen H, Deng XF, Peri KG, Chemtob S, Mulay S (1999) Inverse agonist activities of beta-adrenoceptor antagonists in rat myocardium. Br J Pharmacol 127: 895-902.

Vawter MP, Thatcher L, Usen N, Hyde TM, Kleinman JE, Freed WJ (2002) Reduction of synapsin in the hippocampus of patients with bipolar disorder and schizophrenia. Mol Psychiatry 7: 571-578.

Vazquez E, Herrero I, Miras-Portugal MT, Sanchez-Prieto J (1994) Role of arachidonic acid in the facilitation of glutamate release from rat cerebrocortical synaptosomes independent of metabotropic glutamate receptor responses. Neurosci Lett 174: 9-13.

Vazquez E, Sanchez-Prieto J (1997) Presynaptic modulation of glutamate release targets different calcium channels in rat cerebrocortical nerve terminals. Eur J Neurosci 9: 2009-2018.

Verhage M, McMahon HT, Ghijsen WE, Boomsma F, Scholten G, Wiegant VM, Nicholls DG (1991) Differential release of amino acids, neuropeptides, and catecholamines from isolated nerve terminals. Neuron 6: 517-524.

Verkhratsky AJ, Petersen OH (1998) Neuronal calcium stores. Cell Calcium 24: 333-343.

Vignes M, Clarke VR, Parry MJ, Bleakman D, Lodge D, Ornstein PL, Collingridge GL (1998) The GluR5 subtype of kainate receptor regulates excitatory synaptic transmission in areas CA1 and CA3 of the rat hippocampus. Neuropharmacology 37: 1269-1277.

Vossler MR, Yao H, York RD, Pan MG, Rim CS, Stork PJ (1997) cAMP activates MAP kinase and Elk-1 through a B-Raf- and Rap1-dependent pathway. Cell 89: 73-82.

Walaas SI (1999) Regulation of calcium-dependent [3H]noradrenaline release from rat cerebrocortical synaptosomes by protein kinase C and modulation of the actin cytoskeleton. Neurochem Int 34: 221-233.

Walaas SI, Aswad DW, Greengard P (1983) A dopamine- and cyclic AMP-regulated phosphoprotein enriched in dopamine-innervated brain regions. Nature 301: 69-71.

Walker SA, Cullen PJ, Taylor JA, Lockyer PJ (2003) Control of Ras cycling by Ca2+. FEBS Lett 546: 6-10.

Waltereit R, Weller M (2003) Signaling from cAMP/PKA to MAPK and synaptic plasticity. Mol Neurobiol 27: 99-106.

Wang JK (1991) Presynaptic glutamate receptors modulate dopamine release from striatal synaptosomes. J Neurochem 57: 819-822.

Wang JK, Walaas SI, Greengard P (1988) Protein phosphorylation in nerve terminals: comparison of calcium/calmodulin-dependent and calcium/diacylglycerol-dependent systems. J Neurosci 8: 281-288.

Wang JH, Desai R (1977) Modulator binding protein. Bovine brain protein exhibiting the Ca2+-dependent association with the protein modulator of cyclic nucleotide phosphodiesterase. J Biol Chem 252: 4175-4184.

Wang SJ, Coutinho V, Sihra TS (2002) Presynaptic cross-talk of beta-adrenoreceptor and 5-hydroxytryptamine receptor signalling in the modulation of glutamate release from cerebrocortical nerve terminals. Br J Pharmacol 137: 1371-1379.

Wang SJ, Sihra TS (2003) Opposing facilitatory and inhibitory modulation of glutamate release elicited by cAMP production in cerebrocortical nerve terminals (synaptosomes). Neuropharmacology 44: 686-697.

Wang Y, Okamoto M, Schmitz F, Hofmann K, Sudhof TC (1997) Rim is a putative Rab3 effector in regulating synaptic-vesicle fusion. Nature 388: 593-598.

Watabe AM, Zaki PA, O'Dell TJ (2000) Coactivation of beta-adrenergic and cholinergic receptors enhances the induction of long-term potentiation and synergistically activates

mitogen-activated protein kinase in the hippocampal CA1 region. J Neurosci 20: 5924-5931.

Watanabe S, Hoffman DA, Migliore M, Johnston D (2002) Dendritic K+ channels contribute to spike-timing dependent long-term potentiation in hippocampal pyramidal neurons. Proc Natl Acad Sci U S A 99: 8366-8371.

Watanabe Y, Ikegaya Y, Saito H, Abe K (1996) Opposite regulation by the beta-adrenoceptor-cyclic AMP system of synaptic plasticity in the medial and lateral amygdala in vitro. Neuroscience 71: 1031-1035.

Way KJ, Chou E, King GL (2000) Identification of PKC-isoform-specific biological actions using pharmacological approaches. Trends Pharmacol Sci 21: 181-187.

Weisskopf MG, Castillo PE, Zalutsky RA, Nicoll RA (1994) Mediation of hippocampal mossy fiber long-term potentiation by cyclic AMP. Science 265: 1878-1882.

Wess J (1997) G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. FASEB J 11: 346-354.

Wheeler DB, Randall A, Tsien RW (1994) Roles of N-type and Q-type Ca2+ channels in supporting hippocampal synaptic transmission. Science 264: 107-111.

White AM, Kylanpaa RA, Christie LA, McIntosh SJ, Irving AJ, Platt B (2003) Presynaptic group I metabotropic glutamate receptors modulate synaptic transmission in the rat superior colliculus via 4-AP sensitive K(+) channels. Br J Pharmacol 140: 1421-1433.

Whiteheart SW, Rossnagel K, Buhrow SA, Brunner M, Jaenicke R, Rothman JE (1994) N-ethylmaleimide-sensitive fusion protein: a trimeric ATPase whose hydrolysis of ATP is required for membrane fusion. The Journal of Cell Biology 126: 945-954.

Wilkie TM, Gilbert DJ, Olsen AS, Chen XN, Amatruda TT, Korenberg JR, Trask BJ, de Jong P, Reed RR, Simon MI, et al. (1992) Evolution of the mammalian G protein alpha subunit multigene family. Nat Genet 1: 85-91.

Wilkinson SE, Parker PJ, Nixon JS (1993) Isoenzyme specificity of bisindolylmaleimides, selective inhibitors of protein kinase C. Biochem J 294: 335-337.

Winder DG, Martin KC, Muzzio IA, Rohrer D, Chruscinski A, Kobilka B, Kandel ER (1999) ERK plays a regulatory role in induction of LTP by theta frequency stimulation and its modulation by beta-adrenergic receptors. Neuron 24: 715-726.

Winder DG, Sweatt JD (2001) Roles of serine/threonine phosphatases in hippocampal synaptic plasticity. Nat Rev Neurosci 2: 461-474.

Wu J, Dent P, Jelinek T, Wolfman A, Weber MJ, Sturgill TW (1993) Inhibition of the EGF-activated MAP kinase signaling pathway by adenosine 3',5'-monophosphate. Science 262: 1065-1069.

Wu LG, Borst JG, Sakmann B (1998) R-type Ca2+ currents evoke transmitter release at a rat central synapse. Proc Natl Acad Sci U S A 95: 4720-4725.

Wu LG, Westenbroek RE, Borst JG, Catterall WA, Sakmann B (1999) Calcium channel types with distinct presynaptic localization couple differentially to transmitter release in single calyx-type synapses. J Neurosci 19: 726-736.

Xia Y, Makris C, Su B, Li E, Yang J, Nemerow GR, Karin M (2000) MEK kinase 1 is critically required for c-Jun N-terminal kinase activation by proinflammatory stimuli and growth factor-induced cell migration. PNAS 97: 5243-5248.

Xia Y, Wu Z, Su B, Murray B, Karin M (1998) JNKK1 organizes a MAP kinase module through specific and sequential interactions with upstream and downstream components mediated by its amino-terminal extension. Genes Dev 12: 3369-3381.

Yamada K, Yu B, Gallagher JP (1999) Different subtypes of GABAB receptors are present at pre- and postsynaptic sites within the rat dorsolateral septal nucleus. J Neurophysiol 81: 2875-2883.

Yamagata Y, Jovanovic JN, Czernik AJ, Greengard P, Obata K (2002) Bidirectional changes in synapsin I phosphorylation at MAP kinase-dependent sites by acute neuronal excitation in vivo. J Neurochem 80: 835-842.

Yamaguchi T, Nagao S, Wallace DP, Belibi FA, Cowley BD, Pelling JC, Grantham JJ (2003) Cyclic AMP activates B-Raf and ERK in cyst epithelial cells from autosomal-dominant polycystic kidneys. Kidney Int 63: 1983-1994.

Yan SZ, Huang ZH, Shaw RS, Tang WJ (1997) The conserved asparagine and arginine are essential for catalysis of mammalian adenylyl cyclase. J Biol Chem 272: 12342-12349.

York RD, Yao H, Dhillon T, Ellig CL, Eckert SP, McCleskey EW, Stork PJ (1998) Rap1 mediates sustained MAP kinase activation induced by nerve growth factor. Nature 392: 622-626.

Yu R, Mandlekar S, Tan TH, Kong AN (2000) Activation of p38 and c-Jun N-terminal kinase pathways and induction of apoptosis by chelerythrine do not require inhibition of protein kinase C. J Biol Chem 275: 9612-9619.

Yu TP, McKinney S, Lester HA, Davidson N (2001) Gamma-aminobutyric acid type A receptors modulate cAMP-mediated long-term potentiation and long-term depression at monosynaptic CA3-CA1 synapses. Proc Natl Acad Sci U S A 98: 5264-5269.

Yujiri T, Sather S, Fanger GR, Johnson GL (1998) Role of MEKK1 in cell survival and activation of JNK and ERK pathways defined by targeted gene disruption. Science 282: 1911-1914.

Zamah AM, Delahunty M, Luttrell LM, Lefkowitz RJ (2002) Protein kinase A-mediated phosphorylation of the beta 2-adrenergic receptor regulates its coupling to Gs and Gi. Demonstration in a reconstituted system. J Biol Chem 277: 31249-31256.

Zhang B, Koh YH, Beckstead RB, Budnik V, Ganetzky B, Bellen HJ (1998) Synaptic vesicle size and number are regulated by a clathrin adaptor protein required for endocytosis. Neuron 21: 1465-1475.

Zhang F, Strand A, Robbins D, Cobb MH, Goldsmith EJ (1994) Atomic structure of the MAP kinase ERK2 at 2.3 A resolution. Nature 367: 704-711.

Zhang ZY, Zhou B, Xie L (2002) Modulation of protein kinase signaling by protein phosphatases and inhibitors. Pharmacol Ther 93: 307-317.

Zhao L, Brinton RD (2003) Vasopressin-induced cytoplasmic and nuclear calcium signaling in embryonic cortical astrocytes: dynamics of calcium and calcium-dependent kinase translocation. J Neurosci 23: 4228-4239.

Zhao M, New L, Kravchenko VV, Kato Y, Gram H, di Padova F, Olson EN, Ulevitch RJ, Han J (1999) Regulation of the MEF2 Family of Transcription Factors by p38. Mol Cell Biol 19: 21-30.

Zhou G, Bao ZQ, Dixon JE (1995) Components of a new human protein kinase signal transduction pathway. J Biol Chem 270: 12665-12669.

Zhu X, Birnbaumer L (1996) G protein subunits and the stimulation of phospholipase C by Gs- and Gi-coupled receptors: Lack of receptor selectivity of Galpha 16 and evidence for a synergic interaction between Gbeta gamma and the alpha ásubunit of a receptor-activated G protein. PNAS 93: 2827-2831.

Zimmermann H, Braun N (1999) Ecto-nucleotidases--molecular structures, catalytic properties, and functional roles in the nervous system. Prog Brain Res 120: 371-385.

Zimmermann J, Lamerant N, Grossenbacher R, Furst P (2001) Proteasome- and p38-dependent Regulation of ERK3 Expression. J Biol Chem 276: 10759-10766.

Zwartkruis FJ, Bos JL (1999) Ras and Rap1: two highly related small GTPases with distinct function. Exp Cell Res 253: 157-165.