Signalling Pathways Mediated by the Bombesin/GRP Receptor

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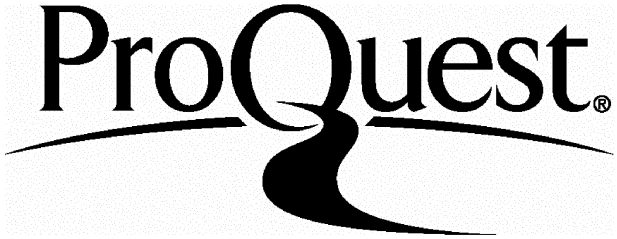

ABSTRACT

Neuropeptides, and in particular peptides of the bombesin family, including gastrin releasing peptide, stimulate DNA synthesis and cell proliferation in cultured cells and are implicated as cellular growth factors in a variety of fundamental biological processes including development, tissue regeneration and tumourigenesis.

Bombesin elicits multiple signalling pathways in various cell types, including activation of phospholipase C, which leads to activation of protein kinase C and elevation of intracellular calcium; tyrosine phosphorylation of intracellular substrates and reorganisation of the actin cytoskeleton, and cell proliferation. It is not clear however whether these responses are mediated by a single receptor subtype or by different subtypes that couple preferentially to specific pathways. To resolve this the mouse bombesin/GRP receptor was transfected into Rat-1 fibroblasts and the pathways activated by bombesin were investigated. Here it is shown that phospholipase C activation, cell growth and tyrosine phosphorylation emanate from a single class of bombesin receptor.

Mitogen Activated Protein Kinase (MAPK) activity is a common point of convergence for mitogenic signals. While the mechanism by which receptor tyrosine kinases activate MAPK is well established, the mechanism by which G protein-coupled receptors activate MAPK is less well understood. The involvement of Ras and Raf in activation of MAPK has not been described in bombesin-induced signalling. This thesis shows that in Rat-1 cells transfected with the bombesin/GRP receptor, bombesin can activate MAPK and Raf-1 in a protein kinase C-independent fashion. Also it is demonstrated for the first time the bombesin stimulates GTP loading of $p21^{ras}$.

Calcium mobilisation from intracellular calcium stores has been observed to be coincident with cell proliferation induced by a variety of growth factors including neuropeptides and especially bombesin. However, a direct role of calcium in this event has not been described. Here it is shown that partial and persistent depletion of intracellular calcium stores, using specific inhibitors of the endoplasmic reticulum calcium pump, thapsigargin and 2,5-di-tert-butylhydroquinone, stimulated reinitiation of DNA synthesis in synergy with either phorbol-12,13-dibutyrate or bombesin in Swiss 3T3 cells. This stimulation of DNA synthesis was dependent on entry of extracellular calcium and hence this suggests a role for calcium in growth control.

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It just leaves me now to say "here Mum and Dad, this is for you."

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ABBREVIATIONS

5-HT	5-hydroxytryptamine (serotonin)
80K/MARCKS	80K/myristoylated alanine rich C-kinase substrate
AA	arachidonic acid
Ach	acetylcholine
ADP	adenosine diphosphate
aPKC	atypical protein kinase C
AR	adrenergic receptor e.g. α_1-AR : α_1 adrenergic receptor
ATP	adenosine triphosphate
βARK	β-adrenergic receptor kinase
BSA	bovine serum albumin
$[Ca^{2+}]_{cyt}$	concentration of cytoplasmic Ca^{2+}
$[Ca^{2+}]_{out}$	concentration of extracellular Ca^{2+}
CaM	calmodulin
CaMK	calmodulin kinase
cAMP	cyclic adensoine 5' monophosphate
CIF	calcium influx factor
cPKC	classical/conventional protein kinase C
CRE	cAMP response element
CREB	cAMP response element binding protein
CTX	cholera toxin
DBHQ	di-*tert*-butylhydroquinone
D-F_5-Phe^6, D-Ala^{11}-Bombesin(6-13)OMe	D-F_5-Phe-Gln-Trp-Ala-Val-D-Ala-His-Leu-OMe
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulphoxide
DTT	dithiothreitol
EC_{50}	concentration that gives half maximum effect
ED_{50}	dose that gives half maximium effect
EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N',-tetraacetic acid
ER	endoplasmic reticulum
ERK	extracellular signal regulated protein kinase
FAK	focal adhesion kinase
FBS	foetal bovine serum

FGF	fibroblast growth factor
FDGF	fibroblast-derived growth factor
Fura-2/AME	fura-2 acetoxymethylester
G protein	guanine nucleotide binding protein
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GPCR	G protein-coupled receptor
GRP	gastrin releasing peptide
GST	glutathione-S-transferase
GTP	guanosine triphosphate
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2ethanesulfonic acid]
IBMX	isobutylmethylxanthine
IC_{50}	concentration that gives half maximum inhibition
Ins	inositol
Ins(1,3,4,5)P_4	inositol 1,3,4,5-tetrakisphosphate
Ins(1,4)P_2	inositol 1,4-bisphosphate
Ins(1,4,5)P_3	inositol 1,4,5-trisphosphate
IPTG	isopropylthio-β-D-galactoside
M-Ach receptor	muscarinic acetylcholine receptor e.g. M_1-Ach receptor : M_1 muscarinc acetylcholine receptor
mAb	monoclonal antibody
MAP-2	microtubule associated protein 2
MAPK	microtubule associated protein 2 kinase/mitogen activated protein kinase
MBP	myelin basic protein
MEK	mitogen activated protein kinase/extracellular signal regulated protein kinase kinase
NGF	nerve growth factor
NmB	neuromedin B
NmC	neuromedin C
nPKC	novel/new protein kinase C
PBS	phosphate buffered saline
PDB	phorbol 12,13-dibutyrate
PDGF	platelet-derivedgrowth factor
PH	pleckstrin homology
PI3K	phosphatidylinositol 3' kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A

PKC	protein kinase C
PKD	protein kinase D
PLA_2	phospholipase A_2
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PS	phosphatidyl serine
PTX	pertussis toxin
SCLC	small cell lung cancer
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SE	standard error
SERCA	sarcoplasmic or endoplasmic reticulum Ca^{2+} ATPase
SIE	sis inducible element
SOS	son of sevenless
SRE	serum response element
SH2	src homology 2
SH3	src homology 3
TG	thapsigargin
TPA	tetradecanoyl phorbol acetate
TSH	thyroid stimulating hormone
Tyr(P)	phosphotyrosyl residue

Chapter 1

INTRODUCTION

1.1. GROWTH REGULATION

Multicellular organisms have developed highly efficient regulatory networks to control cell proliferation. These involve cellular interactions with positive and negative diffusible modulators as well as with the extracellular matrix proteins. In fully mature organisms, the cells of many tissues and organs are maintained in a non-proliferating state (the G_0/G_1 phase of the cell cycle), but can be stimulated to resume DNA synthesis and cell division in response to external stimuli such as hormones, antigens or growth factors. In this manner the growth of individual cells is regulated according to the needs of the whole organism. The regulation of normal cell proliferation is therefore central to many physiological processes including embryogenesis, growth and development, selective cell survival, haemopoeisis, tissue repair and immune responses.

It has become evident that cultured cancer cells which are characterised by unrestrained proliferation acquire complete or partial independence of mitogenic signals in the extracellular environment through different mechanisms (Cross and Dexter, 1991; Westermark and Heldin, 1991). These include production of growth factors that act on the same cells that produced them (autocrine loop) or on adjacent cells (paracrine communication), alterations in the number or structure of cellular receptors and changes in the activity of post-receptor signalling pathways that either stimulate or suppress cell growth (Sager, 1989; Bishop, 1991). For these reasons the identification of the extracellular factors which modulate cell proliferation and the elucidation of the molecular mechanisms involved have emerged as fundamental problems in cancer biology.

The observation that animal cells duplicate their DNA during a discrete interval between cell divisions allowed the cell cycle to be divided into four classical phases: G_1 (growth phase), S (DNA synthetic period), G_2 and M (mitosis). G_1 is the gap between mitosis and the initiation of DNA synthesis, and G2 is the period between S and M (reviewed by Baserga 1976 and Pardee *et al.*, 1978). It was found that, in most cases, cells respond to sub-optimal growth conditions by reversibly arresting in the G_1 or G_0/G_1) phase of the cell cycle (Pardee, 1974; Baserga, 1976). This arrest is observed in normal fibroblasts in culture (Pardee, 1974). Since the crucial control events for the regulation of growth seem to reside in G_1 (Pardee, *et al.*, 1978; Pardee, 1989), elucidation of the mechanisms leading to restimulation of DNA synthesis is therefore a necessary step in the understanding of the control of cell proliferation *in vivo*.

It became evident from the first attempts to propagate animal cells *in vitro* that serum was an essential requirement for the proliferation of most cells. Cells ceased to

proliferate and became quiescent in G_1/G_0 when they depleted serum of its growth promoting activity. Furthermore, addition of fresh serum caused the cells to reinitiate a program of RNA and protein synthesis, DNA synthesis and cell division (reviewed in Pardee, *et al.*, 1978). Many virally and chemically transformed cells were found to have either partially or wholly lost the ability to arrest in G_1 and exhibited a decreased dependence on serum for proliferation. These findings led to the hypothesis that animal sera contained "growth factors" that controlled cellular proliferation (Wolstenholme and Knight, 1971; Temin, *et al.*, 1972; Pardee and Rozengurt, 1975).

1.1.1. GROWTH FACTORS

Within the last decade rapid progress has been made toward the elucidation of the mechanisms of action of the diverse extracellular factors that control the growth of cells. The availability of cell culture in nutrient media together with the purification of various polypeptide growth factors (reviewed in Rozengurt, 1980; Rozengurt and Collins, 1983; James and Bradshaw, 1984) allowed the investigation of mitogen action under chemically defined conditions (Bottenstein, *et al.*, 1979; Shipley and Ham, 1981). From this work it has become clear that a variety of mitogenic factors can regulate the proliferation of normal cells. In this respect the Swiss 3T3 fibroblast has proved a particularly useful system for identification of growth promoting factors (Rozengurt, 1980; Rozengurt, 1986).

Swiss 3T3 cells are a murine fibroblast cell line that was established by repeated sub-culture of disaggregated embryonic cells (Todaro and Green, 1963). Cultures of this 3T3 cell line become "quiescent" in the G_1 to G_0 phase of the cell cycle when the cultures reach confluence at a saturation density determined by the serum concentration (Holley, 1975). This arrest appears therefore to be due to depletion of growth promoting activity in the serum. Readdition of fresh serum to quiescent cultures of these cells stimulates cellular metabolism and after a variable lag period (10 - 15 hours) initiation of DNA synthesis.

The use of Swiss 3T3 cells in culture has a number of advantages for the study of growth control. Identical monolayer cultures of a clonal population of cells can be easily and consistently produced, allowing for reproducibility of results. In addition a chemically-defined nutrient media has been devised for these cells that allows large and reproducible increases in DNA synthesis to be monitored in the presence of growth promoting agents. Also, since most cells are arrested in the same phase of the cell cycle (G_1/G_0), on restimulation they may be regarded as a synchronised cell population.

It should be noted, however, that established cell lines often display abnormal karyotypes and therefore cannot be directly correlated with any cell type *in vivo.* In this respect, the 3T3 cell can be considered as a general model for growth control, but results obtained form this cell line necessarily require further investigation to determine their relevance *in vivo.* However, many mechanisms of growth control identified initially in 3T3 cells have been demonstrated to be of general importance. For example, monovalent ion transport across the plasma membrane (Rozengurt and Heppel, 1975) has since been accepted as a universally important part of growth factor action (Moolenaar, *et al.*, 1983).

The 3T3 cell system has proved particularly useful in two major areas of growth control research. The first is in the identification of agents that modulate the growth state of the cell, and their purification from various biological sources. The second is the elucidation of the mechanisms by which serum, purified growth factors and other mitogens initiate DNA synthesis. Attention has been focused on the early signalling events in mitogenesis as the initial steps in the proliferative response.

It is now recognised that the proliferation of 3T3 cells may be regulated by several classes of mitogen (table 1.I). These include members of a large family of polypeptide growth factors such as PDGF, EGF and insulin-like growth factors and members of a family of small regulatory peptides that classically behave as local hormones or fast acting neurotransmitters. In addition various pharmacological agents including phorbol esters and synthetic diacylglycerols can act as mitogens for these cells (table 1.I).

Both the polypeptide growth factors and the neuropeptides stimulate their growth promoting effects by binding to specific receptors located on the cell surface and activating multiple signalling pathways. At least two major signal transduction pathways initiate the cascades of molecular events stimulated by these two classes of mitogens. The polypeptide growth factors e.g. PDGF and EGF bind to receptors with intrinsic tyrosine kinase activity while the neuropeptides act through receptors coupled to heterotrimeric guanine nucleotide binding proteins (G proteins) to effector activation.

Some experiments presented in this thesis were performed on a 3T3-like cell line, Rat-1 fibroblasts. These cells also retain the ability to quiesce and to reinitiate DNA synthesis when stimulated with growth promoting agents (Higgins, *et al.*, 1992). Because Rat-1 fibroblasts do not respond to as many growth factors as Swiss 3T3 cells, this makes them an ideal candidate for the investigation of exogenous expression of defined receptors.

Table 1.I. Stimulation of Swiss 3T3 fibroblast DNA synthesis by various factors

Class of Growth Factor	Name of Factor	Reference
Polypeptide growth factors	Epidermal Growth Factor	Carpenter and Cohen, 1979
	Insulin-like Growth Factors	Froesch, *et al.*, 1979
	Platelet Derived Growth Factor	Rozengurt, *et al.*, 1983c; Heldin and Westermark, 1984; Lopez-Rivas, *et al.*, 1984
Neuropeptides	Adrenomedullin	Withers, *et al.*, 1996
	Bombesin	Rozengurt and Sinnett-Smith, 1983
	Bradykinin	Woll and Rozengurt, 1988b
	Endothelin	Brown and Littlewood, 1989; Takuwa, *et al.*, 1989
	Vasopressin	Rozengurt, *et al.*, 1979; Dicker and Rozengurt, 1980; Rozengurt and Mendoza, 1980; Rozengurt, *et al.*, 1981a
	Vasoactive Intestinal Contractor	Fabregat and Rozengurt, 1990
	Vasoactive Intestinal Peptide	Zurier, *et al.*, 1988
Activators of Protein kinase C	Phorbol esters, teleocidin, 1-Oleoyl-2-acetylglycerol	Dicker and Rozengurt, 1978; Dicker and Rozengurt, 1980; Dicker and Rozengurt, 1981; Collins and Rozengurt, 1982a; Collins and Rozengurt, 1982b; Rozengurt, *et al.*, 1984
Cyclic nucleotide elevating agents	Cholera toxin, Adenosine agonists, cAMP derivatives, Forskolin, IBMX, Prostaglandin E_1	Rozengurt, 1981; Rozengurt, *et al.*, 1981b; Rozengurt, 1982b; Rozengurt, 1982a; Rozengurt, *et al.*, 1983a; Rozengurt, *et al.*, 1983c
Microtubule disrupting agents	Colchicine, Colemid, Nocodazole, Podolotoxin, Vinblastine	Friedkin and Rozengurt, 1981; Wang and Rozengurt, 1983
Vitamin A derivatives	Retinoic acid	Dicker and Rozengurt, 1979; Dicker and Rozengurt, 1980)
Permeability modulators	Mellitin, Mastoparan	Gelehrter and Rozengurt, 1980; Gil, *et al.*, 1991
Bacterial Toxins	*Pasteurella multocida* toxin	Rozengurt, *et al.*, 1990

1.1.2. SYNERGISTIC SIGNALLING PATHWAYS

The use of Swiss 3T3 cells as a model for the investigation of growth factors has revealed the fundamental concept of synergistic signal transduction pathways. Defined mitogens such as those summarised in table 1.I all exhibit potent synergistic interactions when added to cell cultures maintained in medium devoid of serum (reviewed in Rozengurt, 1986). In contrast to many other mitogens PDGF and bombesin stimulate DNA synthesis and cell division in the absence of any other synergistic factor. Studies with PDGF identified that this growth factor activates PKC (Rozengurt, *et al.*, 1983b) and causes elevation of cAMP (Rozengurt, *et al.*, 1983c). It was predicted that if an agent elicits the same set of early events as PDGF it should act as a growth factor for Swiss 3T3 cells in medium devoid of serum or other mitogens. Agents that elicit part of the early responses stimulated by PDGF should become mitogenic when added in combinations that reconstitute the other early events (Rozengurt, 1986). The availability of a panel of defined mitogens that are biologically active (see table 1.I) provides a tool for elucidating the nature of the regulatory signals and molecular events implicated in these synergistic effects (reviewed in Rozengurt, 1986). For example, a group of agents such as phorbol esters, diacylglycerol and vasopressin elicit a common set of early events; namely they activate PKC but do not alter the basal level of cAMP. Addition of any of these agents either individually or in combination to Swiss 3T3 cells fails to induce a mitogenic response. Agents that increase intracellular cAMP such as prostaglandin E_1, cholera toxin and cAMP derivatives do not activate PKC and do not stimulate DNA synthesis when added singly or in combination. Therefore agents that share a common signalling system cannot act synergistically to stimulate initiation of DNA synthesis.

Crucially the agents mentioned above become potent mitogens when added to quiescent Swiss 3T3 cells in combinations that elicit the generation of both types of signals and thereby reconstitute the complex pattern of signalling events elicited by PDGF. Insulin which can synergise with both groups of extracellular factors at supramaximal concentrations (that is in lieu of insulin-like growth factor) does not act identically to either group of agents. In fact this hormone does not activate PKC, increase intracellular Ca^{2+} or increase the level of cAMP in intact 3T3 cells (Rozengurt, 1986). Similar conclusions can be drawn when EGF is added instead of insulin.

It is important to note at this point that elevation of cAMP and activation of PKC are not the only early signals induced by PDGF and other growth factors and this thesis will describe more recent signalling pathways elicited by G protein-coupled receptors. However the main principle of synergistic pathways leading to mitogenesis, which is illustrated above by PKC and cAMP, remains relevant.

1.1.3. REGULATORY SIGNALS AND OBLIGATORY EVENTS

In line with the hypothesis of growth control discussed above the key events elicited by growth factors in quiescent cells can be broadly divided into two major categories: regulatory signals and obligatory events (Rozengurt, 1986). The former class represent intracellular processes that mediate the action of specific growth-promoting agents; although they are crucial in eliciting biological responses by a given factor they can be bypassed by another group of factors. In contrast, obligatory events are envisaged as molecular steps that must take place for the stimulation of DNA synthesis and cell division to occur regardless of the regulatory signals utilised to activate the cells. This distinction is particularly relevant to section 1.3.2.5. In order to distinguish between regulatory signals and obligatory events one approach is to ascertain the effect of a wide panel of defined mitogenic agents. While regulatory signals are elicited by certain mitogens but not others, obligatory events should be stimulated by all mitogenic combinations. This principle is utilised in section 5.5.

The next section illustrates the mechanism by which receptors with intrinsic tyrosine kinase activity can elicit multiple signals; this is due to their multiple docking sites for signal transduction molecules. The mechanism by which G protein-coupled receptors can elicit multiple signal transduction pathways is more elusive. This aspect of mitogenic signalling will be one of the major subjects of the present thesis. In particular, because bombesin, like PDGF, is a sole mitogen for Swiss 3T3 cells much attention has been given to elucidating its cellular mechanisms of activation. This thesis will describe signalling pathways mediated by the bombesin/GRP receptor which is a seven transmembrane, G protein coupled receptor. However, at points throughout this thesis G protein-coupled receptor signalling shall be compared and contrasted to pathways elicited by receptor tyrosine kinases. The salient points of receptors with intrinsic tyrosine kinase activity shall be briefly described first and then G protein-coupled receptors shall be described in more detail.

1.1.4. RECEPTOR TYROSINE KINASES

Receptor tyrosine kinases bind large polypeptide growth factors such as PDGF and EGF. PDGF is a 30 kDa homo- or heterodimer of A or B chains (reviewed in Ross, *et al.*, 1986). It is not a widely circulating growth factor but it is released by platelets in response to injury and targets mesenchymal cells i.e. smooth muscle and fibroblasts to stimulate their proliferation and migration in to the wound. The role of

PDGF as a growth factor is highlighted by the v-*sis* oncogene from simian sarcoma virus which is homologous to the PDGF-B chain. The inappropriate production of v-*sis* by infected cells may result in autocrine or paracrine stimulation which could contribute to the tumourigenesis observed with this oncogene (Westermark and Heldin, 1991). Unlike PDGF, EGF is ubiquitous being found in nearly all body fluids and the EGF receptor is found in many cell types (Carpenter, 1985). The v-*erbB* oncogene from avian erythroblastosis virus is a truncated EGF receptor which lacks part of the EGF regulated extracellular domain (Downward, *et al.*, 1984).

Receptors for EGF and PDGF contain a large extracellular ligand binding domain, a single transmembrane region and a cytoplasmic portion with a conserved protein tyrosine kinase domain (reviewed in Fantl, *et al.*, 1993; Malarkey, *et al.*, 1995). Upon ligand binding the receptor chains dimerise and this activates their intrinsic protein tyrosine kinase activity. The active receptor autophosphorylates multiple sites by an intermolecular mechanism. The tyrosine residues that become phosphorylated serve as docking sites for multiple signal transduction molecules. This has been best characterised for the PDGFβ receptor. A series of mutations mapped the sites at which the different molecules interact with the intracellular portion of this receptor, see figure 1.1.

The PDGF receptor binds Src, p85, SHPTP-2 Grb2, GAP and PLCγ. Some of these proteins have enzymatic activity such as PLCγ and Src, whilst others appear to have no enzymatic activity but instead serve as linkers or adapters between receptor tyrosine kinases and enzymes, e.g. p85 and Grb2. It is possible that these associations simply serve to localise the signalling molecules to sites near their substrates e.g. phospholipids in membranes are hydrolysed by PLCγ or phosphorylated by PI3K. In addition phosphorylation of substrates may modify the conformation and activity of these molecules (Malarkey, *et al.*, 1995). It has been shown that tyrosine phosphorylation of PLCγ regulates its enzymatic activity (Lee and Rhee, 1995) and see section 1.3.1.

Receptor tyrosine kinases and their associated substrates use protein-protein interactions, mediated by distinct conserved domains, to build up signalling complexes. These protein modules (reviewed in Cohen, *et al.*, 1995) have homology with regions in the non-receptor tyrosine kinase, Src, i.e. Src homology 2 (SH2) and Src homology 3 (SH3) domains. SH2 and SH3 domains are true protein domains: they form compact units that maintain their structure in isolation and each has its N- and C-termini in close apposition so that they can be plugged into the surface of proteins. SH2 domains bind to phosphorylated tyrosine residues. There is some degree of specificity in this interaction which is determined by the three residues immediately carboxyl to the phosphotyrosine (Cohen, *et al.*, 1995). SH3 domains appear to recognise a stretch of about 10 amino acids that are proline rich. It is thought that

like SH2 domains there is specificity between proline rich sequences and SH3 domains determined by variable amino acids that surround the conserved motif (Cohen, *et al.*, 1995). The adaptor molecules Grb2 is composed of several of these modules, SH3—SH2—SH3 (Lowenstein, *et al.*, 1992).

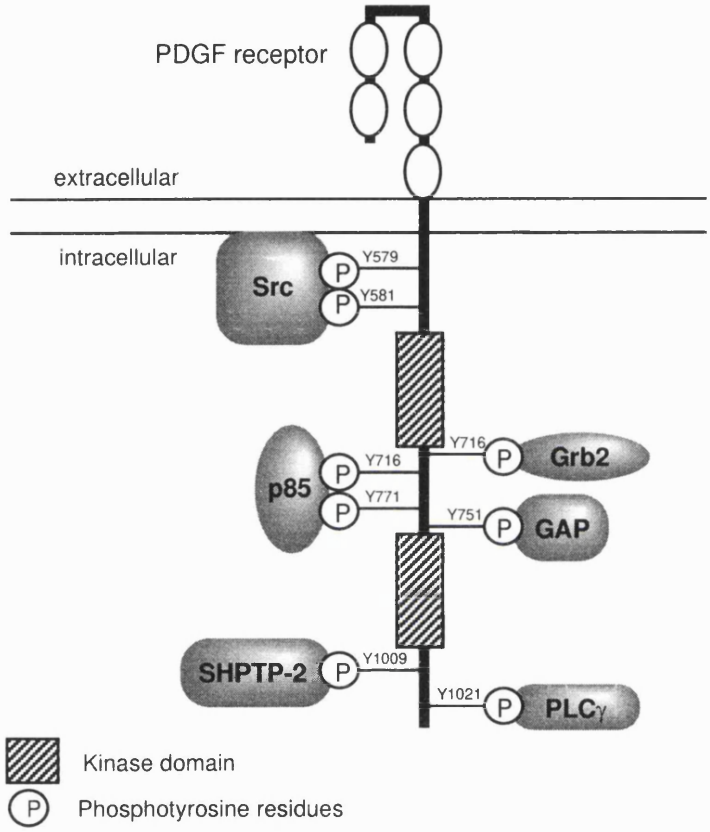

Figure 1.1. Diagram of PDGF receptor with sites of interaction with signal transduction molecules.

Based on Malarkey, *et al.*, (1995). For simplicity only one of the chains is shown. The phosphorylation of the receptor occurs upon ligand induced dimerisation (see text for details).

1.1.5. G PROTEIN-COUPLED RECEPTORS

This class of receptor responds to mitogenic neuropeptides, biogenic amines, glycoproteins and sensory stimuli such as light and odorants. These receptors constitute the classic hormone signalling pathway of receptor - G protein - effector - second messenger. A common theme among this class of receptor is the diversity of receptor subtypes for each ligand which has reached eleven for the biogenic amine, serotonin. Examples of G-protein diversity are shown in table 1.II.

Table 1.II. Examples of G protein coupled receptors and their heterogeneity

Receptor/ligand	Subtype	predominant G protein coupling
muscarinic acetylcholine	M_1, M_3, M_5	$G_{q/11}$
	M_2, M_4	$G_{i/o}$
adenosine	A_1, A_3	$G_{q/11}$
	A_{2A}, A_{2B}	G_s
adrenaline/noradrenaline	α_{1A}, α_{1B}, α_{1D}	$G_{q/11}$
	α_{2A}, α_{2B}, α_{2C}	$G_{q/11}$
	β_1, β_2, β_3	G_s
angiotensin	AT_1	$G_{q/11}$
	AT_2	↓cGMP
bombesin	GRP (BB_2), NmB (BB_1), BRS3	$G_{q/11}$
bradykinin	B_1, B_2	$G_{q/11}$
cholecystokinin/gastrin	CCK_A, CCK_B	$G_{q/11}$
dopamine	D_1, D_5	G_s
	D_2, D_3, D_4	$G_{q/11}$
endothelin	ET_A, ET_B	$G_{q/11}$
glutamate	$mGlu_1$, $mGlu_5$	$G_{q/11}$
	$mGlu_2$, $mGlu_3$, $mGlu_4$, $mGlu_6$, $mGlu_7$	$G_{q/11}$
histamine	H_1	$G_{q/11}$
	H_2	G_s
serotonin	5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}	$G_{q/11}$
	5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}	$G_{q/11}$
	5-HT_4, 5-HT_6, 5-HT_7	G_s
prostanoid	DP, IP, EP_2, EP_4	G_s
	FP, TP, EP_1, EP_3	$G_{q/11}$
	EP_3	(alternatively spliced) $G_{i/o}$
somatostatin	sst_1, sst_2, sst_3, sst_4, sst_5	$G_{i/o}$

NK1 - Substance P receptor, NK2 - Substance K receptor, NK3 - neurokinin B receptor. Table based on TIPS receptor and ion channel nomenclature supplement 1995.

1.1.5.1. GROWTH REGULATION BY G PROTEIN-COUPLED RECEPTORS

Neuropeptides, which are ligands for this class of receptor, have been implicated in normal growth and development and are increasingly recognised as playing a role in sustaining the proliferation of cancer cells (Sethi, *et al.*, 1992). Studies in SCLC cells revealed that these cells have functional receptors for a variety of Ca^{2+} mobilising neuropeptides such as bombesin, GRP, vasopressin, bradykinin, cholecystokinin, gastrin, galanin and neurotensin (Woll and Rozengurt, 1989; Sethi and Rozengurt, 1991a; Sethi and Rozengurt, 1992; Sethi , *et al.*, 1993). Furthermore, these neuropeptides stimulate clonal growth of SCLC cells in semisolid media (Sethi and

Rozengurt, 1991b; Sethi and Rozengurt, 1991a; Sethi and Rozengurt, 1992; Sethi , *et al.*, 1993). It is known that GRP, vasopressin, cholecystokinin and neurotensin are secreted by some SCLC tumours (for refs. see Sethi *et. al.* 1992). Other peptides may be released by a variety of normal cells in the lung, or like bradykinin, produced extracellularly as a result of the proteolytic cleavage of plasma precursors in the damaged tissue surrounding tumours (Steranka, *et al.*, 1989). Collectively these findings support the hypothesis that SCLC growth is sustained by an extensive network of autocrine and paracrine interactions involving multiple neuropeptides which signal through G protein-coupled receptors (Woll and Rozengurt, 1988a; Sethi, *et al.*, 1992).

In addition, receptors that are coupled to G proteins have been shown to transform fibroblasts *in vitro*. The serotonin receptor 5-HT_{1C} was shown to be a conditional oncogene by having agonist-dependent transforming potential (Julius, *et al.*, 1989) and this was also shown for certain muscarinic receptors (Gutkind, *et al.*, 1991). In other studies mutated α_{1B}-AR receptors which were constitutively activated and ligand independent were transforming (Allen, *et al.*, 1991) and activated mutants of the TSH receptor (Parma, *et al.*, 1993) were linked to thyroid hyperplasia. Thus the study of cellular events elicited by G protein-coupled receptors is important for understanding neoplastic growth.

1.1.5.2. STRUCTURE

The main characteristic of G protein-coupled receptors is seven hydrophobic regions of approximately 23 - 28 amino acids and the periodic distribution of the hydrophobicity is consistent with an α-helical conformation. These helices, which contain the areas of highest homology, are postulated to span the plasma membrane and are connected by hydrophilic extracellular and intracellular loops that are less well conserved (Strader, *et al.*, 1995), see figure 1.2.

The arrangement of the helices within the membrane is modelled upon the projection map of rhodopsin (Schertler, *et al.*, 1993) and the probable order deduced from the chemical and physical properties of conserved features of all G-protein linked receptors sequenced at that time (Baldwin, 1993).

The constraints established from detailed sequence analysis were that each helix must be positioned next to its neighbours in the sequence and helices I, IV and V must be the most exposed to lipid and III the least exposed. Helices IV - VII are proposed to be perpendicular to the plasma membrane although conserved prolines may kink them and helices I - III lay slanted in the membrane (Baldwin, 1993), see figure 1.3.

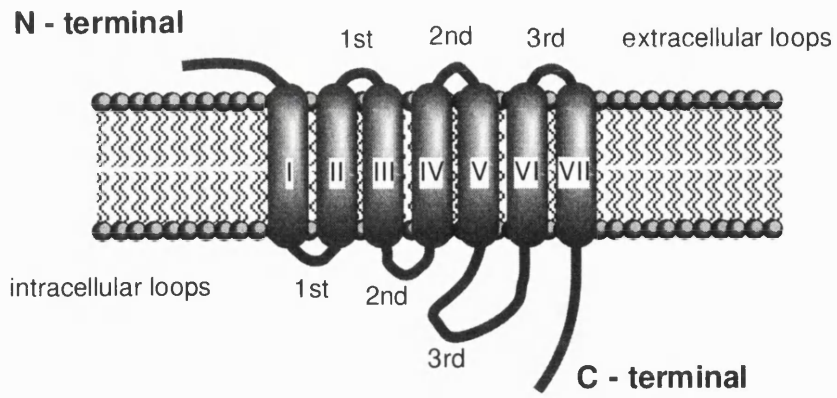

Figure 1.2. Schematic diagram of a seven transmembrane G-protein-coupled receptor

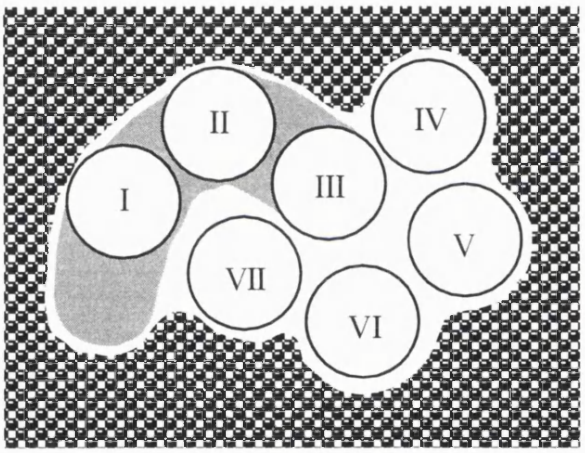

Figure 1.3. Probable arrangement of helices in seven transmembrane domain receptors.

Viewed from the extracellular surface and modelled on rhodopsin projection map. The shaded area between helices I, II and III represents these helices lying at a slant in the membrane.

Biogenic amines bind within the IV - VII pocket. For peptide and protein ligands helix II and III, and the extracellular domains are used as well. For example the C-terminal of substance P binds in the IV - VII pocket and the N-terminal lays across the slanted helix II (Strader, *et al.*, 1995).

Mutational studies have implicated residues at the cytoplasmic ends of all helices and portions of the loops closest to the membrane as critical for overall interaction with the G protein and for specificity determination (Strader, *et al.*, 1994) and especially sequences in the 3rd and 4th intracellular domains. In fact short

synthetic peptides derived from the C-terminal of the 3rd cytoplasmic domain are capable of efficiently stimulating G-proteins *in vitro* (Lefkowitz, *et al.*, 1993). Thus the receptor must shield these peptides from the G protein when in an inactive, ligand free conformation.

The other main feature of G protein-coupled receptors is that they have no enzymatic activity themselves, unlike the receptor tyrosine kinases, but they are nucleotide exchange factors for heterotrimeric G proteins.

1.1.6. HETEROTRIMERIC G-PROTEINS

Seven transmembrane receptors are coupled to heterotrimeric G-proteins that are comprised of an α, a β and a γ subunit. The β and γ subunits can only be separated upon denaturation and so they are generally considered a functional monomer. Molecular cloning has identified 20 α subunits, 5 β subunits and 12 γ subunits to date. See table 1.III for examples of diversity and tissue distribution.

1.1.6.1. STRUCTURE

The structure of heterotrimeric G proteins has been reviewed by Neer (1995). Gα are proteins of 39 - 50 kDa and consist of a GTPase domain and a largely α-helical domain. Areas where the most conformational change occurs upon nucleotide exchange are called switch regions and this is where the β subunit binds to Gα. The receptor binds to the C-terminal end of Gα.

Gβ are approximately 35 kDa and are members of the family of WD-repeat proteins (Neer, *et al.*, 1994) characterised by 4 - 8 recurring highly conserved units ending with Trp-Asp (WD). In β subunits the length of the repeat is about 40 amino acids and so is sometimes known as the WD-40 repeat. Very recently the crystal structure of β has been described (Wall, *et al.*, 1995; Lambright, *et al.*, 1996; Sondek, *et al.*, 1996). The β subunit resembles a 7-bladed propeller with each blade made of 4 antiparallel strands in a β-sheet. The WD-40 repeat comprises the outer strand of one β-sheet together with the 3 inner strands of the next β-sheet. The most variable sequences in the repeats are found in the outer strand and this is consistent with the proposal that WD proteins build multiprotein complexes (Neer, *et al.*, 1994). At the N-terminal is an α-helix which forms a coiled coil with the N-terminal α-helix of the γ subunit.

Gγ is small, about 6 - 9 kDa and shows the most heterogeneity of the G protein subunits and thus has been postulated to determine the specificity of βγ combinations (Neer, 1995). Not all βγ combinations can occur. For example β_1 forms dimers with γ_1 and γ_2 but β_2 does not form a dimer with γ_1. Also β_3 does not dimerise with either γ_1

or γ_2 (Iniguez-Lluhi, *et al.*, 1992; Pronin and Gautam, 1992; Schmidt, *et al.*, 1992), and see section 1.1.6.4.

Table 1.III Examples of heterotrimeric G protein subunits

Subunit		Toxin	Distribution	Typical receptor	Effector
G_s	$\alpha_{s(s)}$	CTX	ubiquitous	β-AR, TSH	stimulate adenylyl cyclase
	$\alpha_{s(l)}$	"	"	"	"
	α_{olf}	"	olfactory neuroepithelium	odorant	"
G_i	α_{i1}	PTX	nearly ubiquitous	M_2-Ach, α_2-AR	inhibit adenylyl cyclase
	α_{i2}	"	ubiquitous	"	"
	α_{i3}	"	nearly ubiquitous	"	"
	α_{oA}	"	brain, others	α_2-AR	"
	α_{oB}	"	brain, others	"	"
	α_{t1}	CTX, PTX	retinal rods	rhodopsin	stimulate cGMP phosphodiesterase
	α_{t2}	CTX, PTX	retinal cones	cone opsin	"
	α_g	CTX(?), PTX	taste buds	taste (?)	?
	α_z		brain, adrenal, platelets	M_2-Ach	inhibit adenylyl cyclase
G_q	α_q		nearly ubiquitous	M_1-Ach, α_1-AR	stimulate PLCβ
	α_{11}		nearly ubiquitous	"	"
	α_{14}		lung, liver, kidney	?	"
	α_{16}		T cells, myeloid cells	?	"
	α_{15}		B cells, myeloid cells	?	?
G_{12}	α_{12}		ubiquitous	?	?
	α_{13}		ubiquitous	?	?
β	β_1		ubiquitous		
	β_2		nearly ubiquitous		
	β_3		"		
	β_4		"		
γ	γ_1		retina, other (?)		
	γ_2		brain, adrenal, other (?)		
	γ_3		brain, testis		
	γ_4		kidney, retina (?)		
	γ_5		widely expressed		
	γ_6		brain, other (?)		
	γ_7		widely expressed		

Based on Hepler and Gilman (1992), additional information from Cali, *et al.* (1992)

1.1.6.2. THE G PROTEIN CYCLE

This has been reviewed by Neer (1995) and Hamm and Gilchrist (1996) , and the main points shall be described. In the inactive state the α subunit of the heterotrimeric G protein has GDP bound and is associated with the receptor. Upon ligand binding the receptor activates Gα. This is thought to occur by changes in the receptor conformation which may expose a peptide at the C-terminus of the 3rd intracellular loop (Lefkowitz, *et al.*, 1993). Active Gα has lower affinity for GDP which then dissociates. Because the cellular concentration of GTP is higher than GDP, GTP binds to the guanine nucleotide binding site of Gα. This causes a conformational

change in the switch region of Gα at the βγ binding site and causes dissociation of the complex into Gα, Gβγ and receptor. The crystal structure of βγ does not change when Gα is dissociated (Lambright, *et al.*, 1996; Sondek, *et al.*, 1996). It has been proposed that the activity of βγ is due to the active binding site being exposed (Clapham, 1996). Gα has intrinsic GTPase activity and is therefore deactivated after a certain time. Phospholipase C can enhance the intrinsic GTPase activity of $G\alpha_q$ (Berstein, *et al.*, 1992). When GTP is hydrolysed to GDP, Gα reassociates with βγ and the receptor. Thus the activity of both α and βγ is limited by the hydrolysis of GTP (see figure 1.4) (Neer, 1995; Hamm and Gilchrist, 1996).

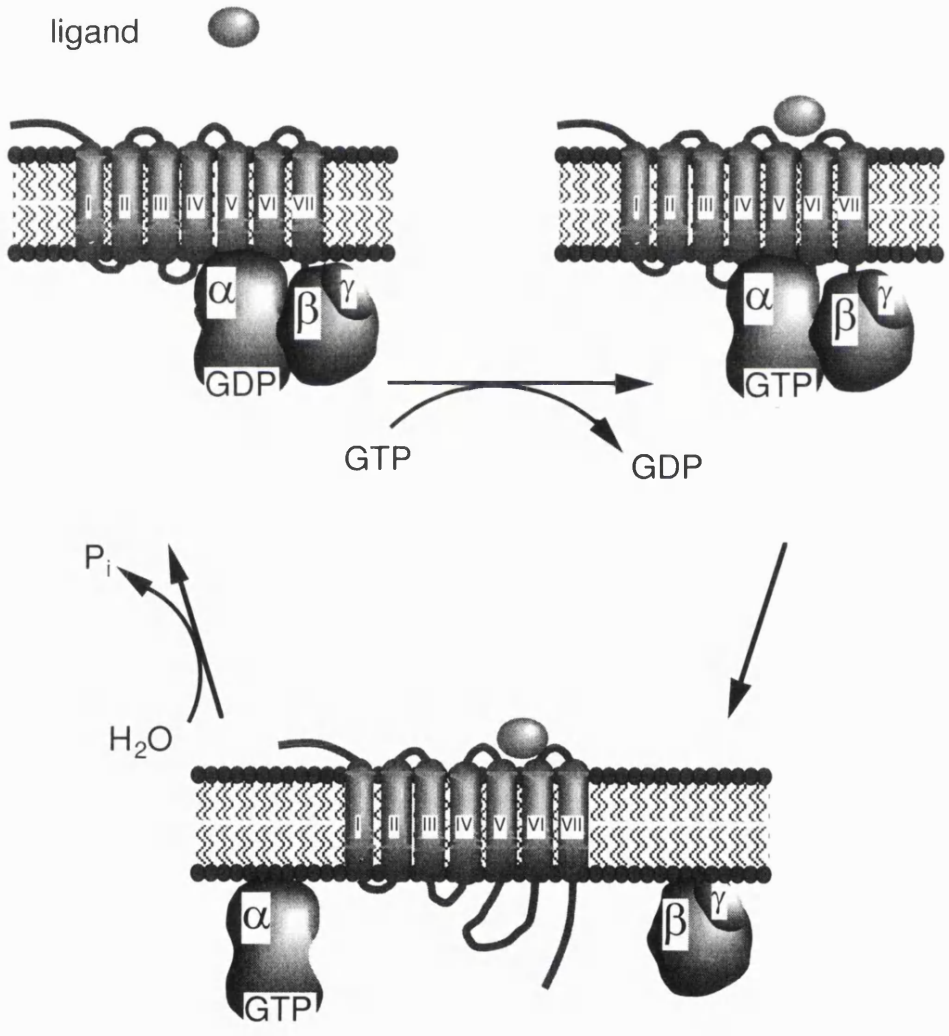

Figure 1.4. G protein cycle.

When ligand binds to receptor Gα exchanges GDP for GTP. In this activated state it dissociates from receptor and βγ subunit. Both Gα and Gβγ stimulate effectors.

The assembled receptor-G protein complex results in a high affinity state of the receptor for its ligand. However, when the G protein in its GTP bound state has dissociated, the receptor agonist complex is less stable and the receptor changes to a lower affinity state. Thus, in membrane preparations in the presence of GTP there is a decrease in the affinity of the receptor for its ligand (Spiegel, 1988). Use has been made use of this observation to identify receptor coupling to G proteins (e.g. see Sinnett-Smith, *et al.*, 1990 and section 1.2.1).

1.1.6.3. CLASSES OF G PROTEIN

Although βγ transduce signals also (Clapham and Neer, 1993) (see below) it is the α subunit which determines the functional class of G protein. These fall into four groups (see table 1.III): G_s because they stimulate adenylyl cyclase, G_i because they inhibit adenylyl cyclase, G_q which activates PLCβ and G_{12} for which the direct effector is unknown although recent work has implicated the G_{12} family in Na^+/H^+ exchange and stress fibre assembly (Dhanasekaran, *et al.*, 1994; Voyno-Yasenetskaya, *et al.*, 1994a; Buhl, *et al.*, 1995). Gα regulation of these effectors will be described in later sections.

It was originally thought that receptors only coupled to one species of Gα but this now appears not to be the case. This will be discussed further in Chapter 3.

1.1.6.4. βγ SUBUNITS

The role of βγ was originally thought to be as a chaperone or negative regulator to guide the α subunit back to the receptor. However in recent years it has been shown that βγ subunits can activate effectors themselves (Clapham and Neer, 1993). The effectors for which direct association has been shown are adenylyl cyclase and PLCβ. Another important effector of βγ are G protein receptor kinases (GRK) that desensitise seven transmembrane receptors (Inglese, *et al.*, 1993). They were originally identified for β-adrenergic receptors (βARK). The involvement of βγ subunits in cellular events has been demonstrated experimentally by βARK and $G\alpha_t$ which act as inhibitors by sequestering the βγ subunits (Inglese, *et al.*, 1995). βγ subunits bind to the PH domain of βARK (Gibson, *et al.*, 1994; Luttrell, *et al.*, 1995). PH domains were first defined as two repeats of approximately 100 residues in pleckstrin, the major PKC substrate in platelets. Now PH domains are identified in a wide variety of signalling and cytoskeletal proteins many of which are found associated with plasma or organella membranes (reviewed in Gibson, *et al.*, 1994; Cohen, *et al.*, 1995). Although the primary sequence homology is low between individual PH domains the tertiary structure of this domain is virtually the same. It is not clear what peptide sequences PH domains bind to although Gβγ subunits will bind to carboxyl portions of some PH domains (Gibson, *et al.*, 1994; Luttrell, *et al.*, 1995). Also it is suggested that

the amino terminal portion of the PH domain binds specifically to PIP_2 (Harlan, *et al.*, 1994) in keeping with the majority of PH domain-containing proteins being found localised to cellular membranes.

It was mentioned above that not all βγ combinations can occur. This could indicate that specific βγ combinations are required for coupling to distinct receptors or for activating distinct effectors. During this thesis aspects of βγ signalling shall be discussed, therefore some evidence regarding βγ specificity shall be presented here.

A series of experiments in which selected endogenous subunits were targeted by microinjection of antisense oligonucleotides revealed some degree of specificity. It was shown that in neuronal cells the M_4 muscarinic receptor couples to $G\alpha_{o1}\beta_3\gamma_4$ whereas a somatostatin receptor couples to $G\alpha_{o2}\beta_1\gamma_3$ (Kleuss, *et al.*, 1991; Kleuss, *et al.*, 1992; Kleuss, *et al.*, 1993). More recently Yan, *et al.* (1996) have used the yeast two hybrid system to show differential ability of β and γ subunits to form the G protein βγ complex. All isoforms were tested (β_1-β_5 and γ_1-γ_5 and γ_7) and it was demonstrated that β_1 and β_2 can form tight complexes with most γ except (γ_1 and γ_2) and that there is wide heterogeneity between the other combinations. This shows that there could be specific βγ combinations but does not reveal anything about the selection of specific signalling pathways.

The effect of βγ subunits on various enzymes and signalling pathways has been the subject of many studies. Table 1.IV shows some events which specific βγ subunits regulate. It should be noted that not all βγ combinations have been assessed. In general the combinations which have been tested so far show similar activities. Some βγ combinations appear to show specificity: $\beta_1\gamma_1$, the retinal βγ for $G\alpha_t$ (Iniguez-Lluhi, *et al.*, 1992; Ueda, *et al.*, 1994) is recognised to be different (see Clapham and Neer, 1993) and $\beta_2\gamma_2$ which does all the signalling described for other βγ subunits except it does not activate the tyrosine kinases, Tsk and Btk. Some aspects of specificity have been shown in other ways. Luttrell, *et al.* (1995) looked at the effect of several PH domains on the signalling mediated by βγ subunits from G_q and found that it was only affected by the PH domain from PLCγ. This suggested some specificity in PH domain recognition of βγ. It should be noted that most of the above studies are performed with ectopic expression of βγ subunits which may compromise specificity. To conclude, there is some evidence for βγ specificity but this is not well documented at the functional level.

Table 1.IV. Function of βγ subunits

		exist	PTX	AC	PLC	MAPK	shc	Tsk	Btk	actin
β_1	γ_1	+	-	-	-/+	+		-		
	γ_2	+	+	+	+	+	+	+	+	-
	γ_3	+	+	+	+	+		+	+	-
	γ_4	+								
	γ_5	+	+	+	+					
	γ_7	+	+	+	+					
β_2	γ_1	-	-		-	-		-		
	γ_2	+	+	+	+	+	+	-	-	-
	γ_3	+	+	+	+	+		+	+	-
	γ_5	+	+	+	+					
	γ_7	+	+	+	+					
β_3	γ_1	-			-	-				
	γ_2	-			-	-				
	γ_3				-	-				
β_4	γ_1				-	-				
	γ_2				-	-				
	γ_3				-	-				
refs.		a	b	c	d	e	f	g	h	i

The table shows a summary of the documented effects of βγ subunits, (+) denotes an effect, (-) denotes no effect. **exist**: not all βγ combinations can exist. (+) shows that they form a physical dimer and (-) shows they do not; **PTX**: enhancement of pertussis toxin ADP ribosylation of G_i or G_o; **AC**: regulation of adenylyl cyclase. This includes both inhibition and stimulation of the relevant subtypes as described in the introduction, both effects have been grouped; **PLC**: activation of PLCβ isoforms; **MAPK**: activation of MAP kinase; **shc**: tyrosine phosphorylation of shc; **Tsk**: activation of Tsk; **Btk**: activation of Btk; **actin**: formation of stress fibres. refs.: **a** (Iniguez-Lluhi, *et al.*, 1992; Kleuss, *et al.*, 1992; Pronin and Gautam, 1992; Schmidt, *et al.*, 1992; Kleuss, *et al.*, 1993); **b** (Ueda, *et al.*, 1994); **c** (Iniguez-Lluhi, *et al.*, 1992; Ueda, *et al.*, 1994); **d** (Ueda, *et al.*, 1994; Hawes, *et al.*, 1995); **e** (Faure, *et al.*, 1994; Hawes, *et al.*, 1995); **f** (Touhara, *et al.*, 1995; van Biesen, *et al.*, 1995); **g** and **h** (Langhans-Rajasekaran, *et al.*, 1995); **i** (Buhl, *et al.*, 1995).

1.1.6.5. MODIFYING TOXINS

Bacterial toxins have been used to help dissect G protein-mediated signal transduction pathways such as cholera toxin from *Vibrio cholerae* which activates $G\alpha_s$ through inhibition of its GTPase activity (Cassel and Selinger, 1977). This is achieved by ADP-ribosylating a specific arginine residue (van Dop, *et al.*, 1984b). Another useful

tool is pertussis toxin from *Bordetella pertussis.* This toxin ADP-ribosylates a specific cysteine residue near the C-terminal of G_i and G_o (West, *et al.*, 1985). G_s and G_q do not have the necessary cysteine and are insensitive to pertussis toxin. The preferred substrate of pertussis toxin is the trimeric complex rather than just the α subunit which means that $\beta\gamma$ is no longer liberated from G_i. Thus cholera toxin locks G_s in the GTP state whereas pertussis toxin locks G_i in the GDP state and is used as an inhibitor of G_i (van Dop, *et al.*, 1984a; Spiegel, 1988).

1.2. BOMBESIN

Bombesin is a bioactive tetradecapeptide that was originally discovered in extracts from the skin of the European toad *Bombina bombina* (Anastasi, *et al.*, 1971) which had pleiotrophic pharmacological effects in a range of different mammals (for general review see Tache, *et al.*, 1988). Bombesin produced a rise in systemic blood pressure and had stimulant action on isolated smooth muscle preparations of the intestine, colon, uterus and bladder. When administered centrally it affected thermoregulation, gastric function and behaviour. Bombesin also caused pancreatic secretions of digestive enzymes (e.g. amylase), insulin and glucagon (Tache, *et al.*, 1988), and the release of several gut hormones (Ghatei, *et al.*, 1982) Bombesin-like immunoreactivity is found in the brain, spinal cord, in nerve fibres along the gastrointestinal tract, where it is thought to act as a neurotransmitter, and in neuroendocrine cells of the lung and thyroid (for refs. see Solcia, *et al.*, 1988; Sunday, 1988).

The search for mammalian counterparts to bombesin led to the isolation of a gastrin releasing peptide from porcine gastric tissue (McDonald, *et al.*, 1979) and two other peptides from porcine spinal cord, neuromedin B (NmB) (Minamino, *et al.*, 1983) and neuromedin C (NmC) (Minamino, *et al.*, 1984), which had contractile activity on uterine tissue. Bombesin-like peptides fall into 3 groups on the basis of their primary structure, pharmacological activity and receptor affinity. The bombesin subfamily is characterised by the C-terminal tetrapeptide Gly-His-Leu-Met-NH_2, the litorin/ranatensin subfamily by Gly-His-Phe-Met-NH_2 and the phyllitorin subfamily by Gly-Ser-Phe(Leu)-Met-NH_2 (examples relevant for this thesis are shown in figure 1.5).

Bombesin subfamily

GRP	Ala- Pro- Val- Ser- Val- Gly- Gly-Gly-Thr- Val- Leu- Ala- Lys- Met-Tyr- Pro-Arg-Gly-Asn- His-Trp-Ala- *Val*-**Gly-His-*Leu*-Met-NH_2**
GRP-10 or Neuromedin C	Gly-Asn- His-Trp-Ala- *Val*-**Gly-His-*Leu*-Met-NH_2**
Bombesin	pGlu-Gln-Arg- Leu-Gly-Asn-Gln-Trp-Ala- *Val*-**Gly-His-*Leu*-Met-NH_2**

Litorin subfamily

Litorin	pGlu- Gln-Trp-Ala- *Val*-**Gly-His-*Phe*-Met-NH_2**
Neuromedin B	Gly-Asn- Leu-Trp-Ala-*Thr*-**Gly-His-*Phe*-Met-NH_2**

Figure 1.5. Amino acid sequence of mammalian and amphibian bombesin-like peptides

Amino acids in bold show the C-terminal tetrapeptide that identify the subfamily. Amino acids in *italics* show where NmB and litorin differ from bombesin and GRP.

Bombesin, litorin, ranatensin and phyllitorin are all amphibian peptides. GRP, NmB and NmC are mammalian peptides. GRP and NmC have 9 of the last 10 amino acids identical to bombesin and NmC is also known as GRP-10 or GRP_{18-27}. NmB belongs to the litorin subfamily and although the first found had 9 amino acids a large NmB of 32 amino acids has also been described (Minamino, *et al.*, 1988). By submitting 27 bombesin-like peptides to parallel bioassay it was shown that the minimum amino acid sequence required for bombesin-like activity was the C-terminal heptapeptide, the tryptophan residue being of crucial importance. Full activity was seen with the C-terminal nonapeptide (Erspamer, *et al.*, 1988).

Bombesin immunoreactivity was found in the endocrine cells of the developing human lung (Wharton, *et al.*, 1978) and in small-cell lung carcinoma (SCLC) (Moody, *et al.*, 1981; Wood, *et al.*, 1981; Erisman, *et al.*, 1982) a cancer of pulmonary neuroendocrine cells characterised by a rapid growth rate, high metastatic potential and ectopic hormone production (Greco and Oldham, 1979). This raised the possibility that bombesin could participate in the control of cell proliferation and indeed it was shown that bombesin was a sole mitogen for Swiss 3T3 cells in culture (Rozengurt and Sinnett-Smith, 1983). This was followed by the observation that bombesin/GRP was a growth factor for normal bronchial epithelial cells (Willey, *et al.*, 1984). SCLC cells release bombesin-like peptides (for refs. see Moody and Korman, 1988) and it was shown by use of antibodies towards bombesin (Cuttitta, *et al.*, 1985) and selective stimulation by bombesin of SCLC lines (Carney, *et al.*, 1987) that

bombesin acted as an autocrine growth factor for SCLC. In addition [Leu^{13}-$\psi(CH_2NH)Leu^{14}$]bombesin, a specific bombesin/GRP receptor antagonist (Woll, *et al.*, 1988), was shown to inhibit the growth of SCLC cell lines *in vitro* and inhibit xenographt formation in nude mice (Mahmoud, *et al.*, 1991). Since then bombesin/GRP has been implicated in other tumours such as pancreatic (Avis, *et al.*, 1993) and gastric tumours (Qin, *et al.*, 1994) and has also been shown to be a growth factor for gastric carcinoma cell lines (Bold, *et al.*, 1994).

1.2.1. BOMBESIN/GRP RECEPTOR

Bombesin/GRP mediates its effects through specific receptors first characterised on pancreatic acinar cells (Jensen, *et al.*, 1978). The bombesin/GRP receptor in Swiss 3T3 cells is a 75 - 80 kDa cell surface glycoprotein that was identified by affinity crosslinking (Zachary and Rozengurt, 1987) with a protein core of 43 kDa (Sinnett-Smith, *et al.*, 1988) and functionally coupled to a GTP binding protein (Fischer and Schonbrunn, 1988; Sinnett-Smith, *et al.*, 1990) demonstrated by the ability of GTP or GTP analogues to reduce the affinity of bombesin for its receptor (see section 1.1.6.2). Two types of bombesin receptor were pharmacologically distinguished in rat oesophagus, a bombesin/GRP preferring receptor (K_i bombesin = 4 nM, K_i NmB = 156 nM) and a NmB preferring receptor (K_i NmB = 0.3 nM, K_i bombesin = 2 nM) (von Schrenck, *et al.*, 1989). Bombesin and GRP differ from NmB by 2 amino acids in the C-terminal heptapeptide. One of these differences, the Phe of NmB compared to the Leu of bombesin and GRP at the penultimate residue, is also found in litorin (see figure 1.5), but litorin has equal potency to bombesin in smooth muscle preparations and in Swiss 3T3 cells (Erspamer, *et al.*, 1988; Millar and Rozengurt, 1990b; Sinnett-Smith, *et al.*, 1990). The residue that differs between NmB and bombesin, GRP or litorin is the Thr of NmB five residues from the C-terminal end, see figure 1.5, and this could be the key residue that confers receptor specific recognition.

Molecular cloning has identified both the bombesin/GRP receptor (Spindel, *et al.*, 1990; Battey, *et al.*, 1991; Corjay, *et al.*, 1991) and the NmB preferring receptor (Corjay, *et al.*, 1991; Wada, *et al.*, 1991). Sequence analysis revealed that these receptors did belong to the superfamily of G-protein linked receptors. More recently another receptor has been cloned that shows sequence similarity to the bombesin/GRP receptor but the physiological ligand has not yet been defined (Gorbulev, *et al.*, 1992; Fathi, *et al.*, 1993).

1.3. BOMBESIN-MEDIATED SIGNAL TRANSDUCTION

Binding of bombesin/GRP to its receptor initiates a cascade of intracellular signals (summarised in figure 1.6) culminating in DNA synthesis 10 - 15 hours later. These signals shall be described in this next section.

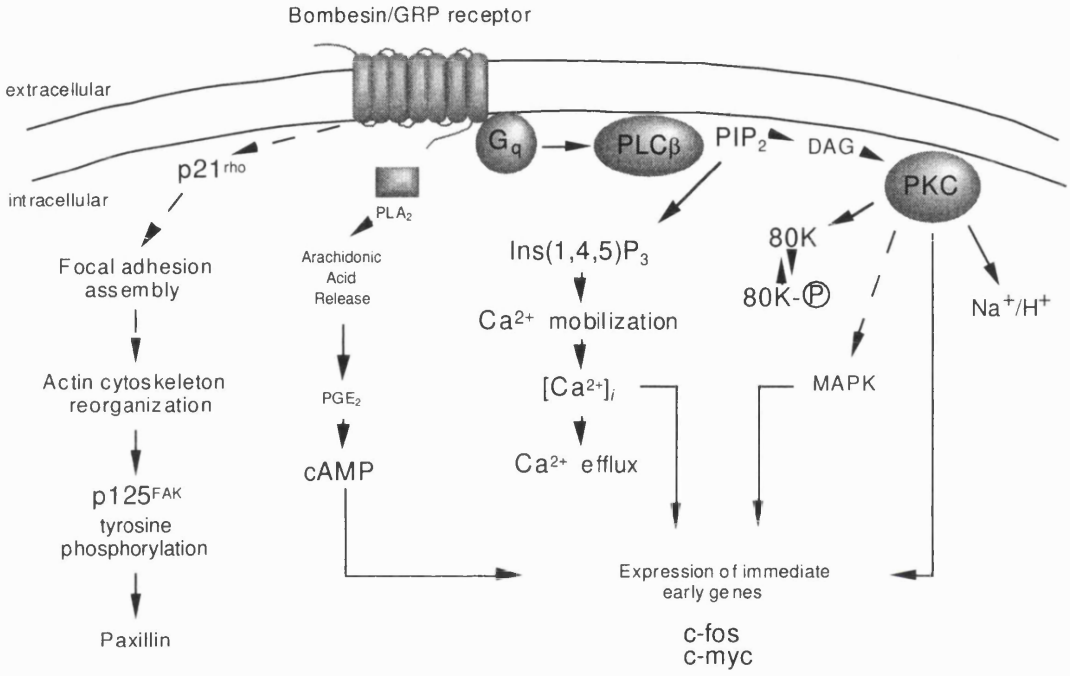

Figure 1.6. Bombesin-stimulated signal transduction pathways

1.3.1. PHOSPHOLIPASE C

One of the earliest events to occur after the binding of bombesin to its receptor is the rapid stimulation of phospholipase C (PLC) catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in the plasma membrane (Mendoza, *et al.*, 1986; Lopez-Rivas, *et al.*, 1987; Nanberg and Rozengurt, 1988). This reaction produces inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (Berridge, 1984), the functions of which are described below.

Phosphatidylinositol specific PLC has multiple isoforms which fall into 3 families, β, γ and δ (reviewed in Exton, 1994; Lee and Rhee, 1995). PLC isozymes all contain the highly homologous X and Y regions which are believed to be the catalytic domain. In addition they all contain an N-terminal PH domain and PLCγ also contains

additional PH, SH2 and SH3 domains between the X and Y regions (Lee and Rhee, 1995). There are 4 isoforms of $PLC\beta_{(1-4)}$ and these are activated by members of the $G\alpha_q$ family. In addition $PLC\beta_{(1-3)}$ are also activated by $G\beta\gamma$. This could explain why receptors that are coupled to G_i proteins which are classically linked to inhibition of adenylyl cyclase, can cause PIP_2 hydrolysis in a pertussis toxin-sensitive fashion (Abdel-Baset, *et al.*, 1992). The different isozymes of PLCβ are activated to different extents by $G\alpha_q$ or $G\beta\gamma$. α_q activates $PLC\beta_1$ the most and $PLC\beta_2$ the least, whereas $\beta\gamma$ activate $PLC\beta_3$ the most and $PLC\beta_1$ the least (Exton, 1994). α and $\beta\gamma$ subunits of heterotrimeric G proteins bind to different areas on PLCβ, α_q binds to the C-terminal region (Lee, *et al.*, 1993b) and $\beta\gamma$ appears to bind to the Y region (Kuang, *et al.*, 1996). The hydrolysis of PIP_2 in response to various agonists that signal through G protein-coupled receptors depends upon the cellular expression of the different isozymes.

PLCγ binds to activated phosphorylated growth factor receptors via its SH2 domain, and is then phosphorylated on tyrosine residues which lie between the X and Y regions (Kim, *et al.*, 1991). Tyrosine phosphorylated PLCγ is the active form (Lee and Rhee, 1995). The mechanism of activation of PLCδ is not yet known (Exton, 1994).

Bombesin stimulates phosphatidylinositol specific PLC in a pertussis toxin-insensitive manner in Swiss 3T3 cells (Zachary, *et al.*, 1987) suggesting that bombesin does not couple to members of the $G_{i/o}$ family. Indeed it has been shown that addition of bombesin to Swiss 3T3 cells induces [α-^{32}P]GTP azidoanilide photolabelling of G_q and G_{11} (Offermanns, *et al.*, 1994a).

As well as being a substrate for PLC, PIP_2 is recognised to play a role in actin cytoskeletal regulation (Janmey, 1994) and serve as a membrane attachment site for proteins with PH domains (Harlan, *et al.*, 1994). Thus PIP_2 depletion by PLC-mediated hydrolysis could also have important consequences for the cell (Lee and Rhee, 1995) including initiation of cytoskeletal rearrangement (Divecha and Irvine, 1995).

1.3.2. INOSITOL 1,4,5-TRISPHOSPHATE AND Ca^{2+} MOBILISATION

Hydrolysis of PIP_2 produces $Ins(1,4,5)P_3$ which, as a second messenger, binds to an intracellular receptor and induces the release of Ca^{2+} from internal stores (Berridge, 1993) and below. The $Ins(1,4,5)P_3$ signal produced by receptor-mediated PLC activation is switched off by two mechanisms which are described in detail by Majerus (1992) and Shears (1992). The first is due to dephosphorylation by inositol polyphosphate 5-phosphatase which converts $Ins(1,4,5)P_3$ to $Ins(1,4)P_2$, the latter of which has no Ca^{2+} mobilising ability. $Ins(1,4)P_2$ is further dephosphorylated to Ins by phosphatases that are inhibited by Li^+. The second mechanism is phosphorylation to

$Ins(1,3,4,5)P_4$ by $Ins(1,4,5)P_3$ 3-kinase. There are some reports which suggest that $Ins(1,3,4,5)P_4$ is also involved in Ca^{2+} fluxes, this is further described in section 1.3.2.3. $Ins(1,3,4,5)P_4$ can also be dephosphorylated by 3-phosphatases and 5-phosphatases and eventually back to Ins. It is thought that some of these metabolites have biological functions although it is not clear what these are (Berridge and Irvine, 1989; Menniti, *et al.*, 1993).

Bombesin causes a rapid increase in cytoplasmic $Ins(1,4,5)P_3$ which co-incides with a transient increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) and with Ca^{2+} efflux from the cells (Mendoza, *et al.*, 1986; Lopez-Rivas, *et al.*, 1987; Nanberg and Rozengurt, 1988). This efflux of Ca^{2+} lasts for approximately 2 min, after which there is a 50% depletion of total cellular Ca^{2+} which is persistent, lasting for at least 1 h (Mendoza, *et al.*, 1986). This pattern of Ca^{2+} signalling is also seen with other mitogens, for example, addition of serum or vasopressin to fibroblasts loaded with $^{45}Ca^{2+}$ causes the efflux of $^{45}Ca^{2+}$ from an intracellular pool followed by depletion in total cellular $^{45}Ca^{2+}$ that persists for more than 1 h (Lopez-Rivas and Rozengurt, 1983; Lopez-Rivas and Rozengurt, 1984).

The early transient increase in $[Ca^{2+}]_{cyt}$ upon bombesin stimulation is due to release from intracellular stores. There is also a second phase to the Ca^{2+} signal which is the sustained elevation of $[Ca^{2+}]_{cyt}$ which is dependent upon extracellular Ca^{2+}. These two phases shall be described in detail below.

1.3.2.1. CALCIUM HOMEOSTASIS

Calcium concentration in blood plasma is about 3 mM of which about half is ionised Ca^{2+} (Carafoli, 1987). In contrast, the amount of ionised Ca^{2+} in the cytoplasm is 100 - 200 nM even though the amount of calcium in intracellular organelles such as the endoplasmic reticulum (ER) can reach millimolar concentrations. This results in a large electrochemical gradient across the plasma membrane. Ionised Ca^{2+} can be kept at these low concentrations by two mechanisms: (i) binding to proteins that can act as Ca^{2+} buffers, and (ii) by active transport across cellular membranes. The cytoplasm contains many proteins which bind Ca^{2+} with high affinity but some of these are Ca^{2+} regulated proteins rather than Ca^{2+} buffers. $[Ca^{2+}]_{cyt}$ is kept low mainly by removal from the cell across the plasma membrane or by uptake into intracellular organelles such as the ER or mitochondria, the former of which is believed to be the hormonally regulated Ca^{2+} store (Carafoli, 1987).

Hormonally Regulated Intracellular Ca^{2+} Store

The main characteristic of a hormone-responsive Ca^{2+} store is the co-localisation of low affinity, high capacity Ca^{2+} binding proteins and the $Ins(1,4,5)P_3$ receptor. The lumen of the ER contains several major proteins including Grp78 (BiP or

immunoglobulin heavy chain binding protein), protein disulphide isomerase (PDI), endoplasmin and reticulin. Endoplasmin and reticulin have been estimated to bind, in the mM range, about 10 and 20 molecules of calcium per molecule, respectively (Koch, 1990). All four molecules have the KDEL retention signal of the ER preceded by clusters of acidic residues, characteristic of Ca^{2+} binding proteins. It is proposed that Ca^{2+} binding to these proteins contributes both to Ca^{2+} storage and to facilitate the binding of the KDEL sequence to its receptor and thus control the retention of protein in the lumen of the ER (Milner, *et al.*, 1992).

Ins(1,4,5)P_3 only releases about 40% of cellular Ca^{2+} compared to the amount released by a Ca^{2+} ionophore suggesting that there are multiple intracellular Ca^{2+} pools some of which could be mitochondria or nuclei (Gill, *et al.*, 1992). Subcellular fractionation shows that the Ins(1,4,5)P_3-releasable Ca^{2+} store is in microsomes that are not mitochondria or nuclei. Within the microsome population there is heterogeneity in their ability to sequester and release Ca^{2+} (Meldolesi, *et al.*, 1990). It has been proposed that the Ca^{2+} store is an organelle called a "calciosome". This may or may not be distinct from the ER, however the current feeling is that the ER is not homogenous and discrete areas could function specifically as the calciosome (Koch, 1990; Meldolesi, *et al.*, 1990; Lytton and Nigam, 1992). Indeed it has been documented that endoplasmin is not distributed uniformly throughout the ER supporting the idea of separate functional areas (Koch, 1990).

Calcium Pumps

Calcium is actively pumped into the stores by the sarcoplasmic/endoplasmic reticulum Ca^{2+}ATPase (SERCA), reviewed in Thomas and Hanley (1994). Five SERCA isoforms have been identified that are derived from 3 genes. SERCA 1 and 2 are alternatively spliced with isoform 2b being considered the main ER-type Ca^{2+} pump of non-muscle cells. The role these pumps play in Ca^{2+} homeostasis has been investigated using specific inhibitors., which are are thapsigargin, di-*tert*-butylhydroquinone (DBHQ) and, to a lesser extent, cyclopiazonic acid. The first two inhibitors have been used in this thesis and they shall be described.

Thapsigargin — Thapsigargin was originally isolated from the Western Mediterranean plant *Thapsia garganica* and identified as the major active component when the plant was used as a treatment for muscle and joint inflammation (Rasmussen, *et al.*, 1978). Its skin irritation properties were linked to histamine secretion from mast cells (Rasmussen, *et al.*, 1978). Thapsigargin was then shown to activate other cells in the inflammatory response (Ali, *et al.*, 1985). Because thapsigargin had structural similarities with TPA this led to its identification as a tumour promoter, but it was not a phorbol ester type tumour promoter (Hakii, *et al.*,

1986). The mechanism of action of thapsigargin was linked to Ca^{2+} fluxes (Ali, *et al.*, 1985) and this was demonstrated to be via increasing cytoplasmic Ca^{2+} through the discharge of intracellular stores (Thastrup, *et al.*, 1989) without the generation of inositol phosphates (Jackson, *et al.*, 1988). This was achieved by specifically inhibiting SERCA pumps (Thastrup, *et al.*, 1990; Sagara and Inesi, 1991). Thapsigargin is very potent, it is effective in subnanomolar concentrations, and specific, it has no action on the plasma membrane Ca^{2+}ATPase or any other ion-motive ATPase and appears to form an irreversible dead-end complex with SERCA pumps (Thomas and Hanley, 1994).

DBHQ — Investigation into quinone toxicity in hepatocytes revealed that DBHQ impaired microsomal Ca^{2+} sequestration. It was shown to inhibit ATP-dependent Ca^{2+} sequestration and Ca^{2+}-stimulated ATPase activity of microsomal fractions but not plasma membrane or mitochondrial fractions (Moore, *et al.*, 1987). Like thapsigargin, DBHQ could rapidly elevate cytosolic Ca^{2+} without producing inositol phosphates or altering the passive efflux of Ca^{2+} from Ca^{2+} loaded microsomes (Kass, *et al.*, 1989). DBHQ is less potent than thapsigargin, IC_{50} for inhibition of Ca^{2+}ATPase is about 1 μM (Moore, *et al.*, 1987; Kass, *et al.*, 1989) and like thapsigargin, DBHQ stabilises the non-Ca^{2+} bound state of the enzyme (Thomas and Hanley, 1994).

Addition of SERCA inhibitors to cells results in the elevation of $[Ca^{2+}]_{cyt}$ within 15 s - 2 min, followed by a sustained elevation for many minutes. This reveals that Ca^{2+} homeostasis is in a constant dynamic state that balances Ca^{2+} uptake with a constant passive leak from internal stores, see figure 1.7. Thus the Ca^{2+} pump must be working continuously against a native high Ca^{2+} permeability of the ER membrane (Thomas and Hanley, 1994). The leak "channel" has not been identified.

Calcium Channel

The Ca^{2+} release channel is the Ins(1,4,5)P_3 receptor (Mikoshiba, 1993). This is a large protein (known as P_{400}) and upon cloning it was shown to have a large N-terminal cytoplasmic domain and a C-terminal transmembrane domain. The cDNA encoded a protein that bound Ins(1,4,5)P_3 and had Ca^{2+} channel activity, hence the Ins(1,4,5)P_3 receptor is an Ins(1,4,5)P_3-gated Ca^{2+} channel. The transmembrane domain contains 6 or 8 membrane spanning regions as predicted from the hydropathy profile of the amino acid sequence. The functional Ins(1,4,5)P_3 receptor is a homotetramer which is regulated by Ca^{2+}. With increasing amounts of Ca^{2+} the Ins(1,4,5)P_3 receptor Ca^{2+} channel is activated in a positive feedback manner but when $[Ca^{2+}]_{cyt}$ gets high this inhibits the Ins(1,4,5)P_3 receptor in a negative feedback manner (Taylor and

Marshall, 1992). Ins(1,4,5)P_3 receptor distribution is mainly on the ER supporting the role of the ER as the Ca^{2+} store (Mikoshiba, 1993).

1.3.2.2. CALCIUM RELEASE FROM INTRACELLULAR STORES

At rest $[Ca^{2+}]_{cyt}$ is low due to removal by the plasma membrane ATPase and uptake into the ER by SERCA. Upon agonist stimulation PLCγ or PLCβ hydrolyses PIP_2 to yield Ins(1,4,5)P_3 which binds to the Ins(1,4,5)P_3 receptor on the ER. The intrinsic Ca^{2+} channel opens and Ca^{2+} flows down the concentration gradient resulting in a large increase in intracellular Ca^{2+} from about 0.1 to 0.5 - 1 μM (reviewed by Berridge, 1993; Petersen, *et al.*, 1994). This increased $[Ca^{2+}]_{cyt}$ is transient and Ca^{2+} is removed by reuptake into the ER and extrusion through the plasma membrane. This is the Ca^{2+} efflux observed (Lopez-Rivas and Rozengurt, 1983; Lopez-Rivas and Rozengurt, 1984). Agonists activate the plasma membrane pump both by Ca^{2+}-dependent (calmodulin) and independent (phosphorylation by PKA or PKC) mechanisms (Carafoli, 1992). The ER Ca^{2+}ATPase is also stimulated by an increase in $[Ca^{2+}]_{cyt}$ and inhibited when the ER stores are full (IC_{50} = 300 μM) (Petersen, *et al.*, 1994).

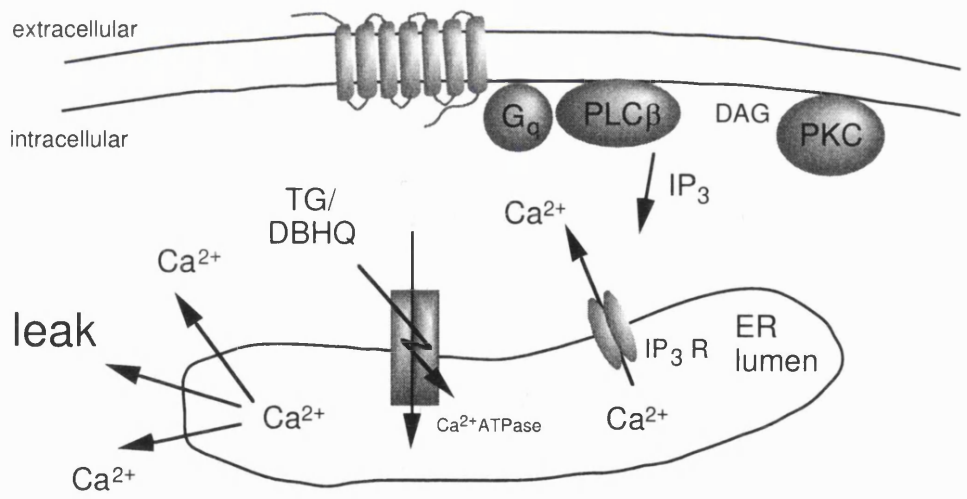

Figure 1.7. Calcium Homeostasis and Release

Calcium homeostasis is a balance between the Ca^{2+}ATPase and the passive Ca^{2+} leak from the ER. Thapsigargin (TG) and DBHQ can interrupt this balance by inhibiting the Ca^{2+}ATPase. Upon agonist stimulation of e.g. the bombesin/GRP receptor, PLCβ produces Ins(1,4,5)P_3 which binds to its receptor on the ER causing the release of Ca^{2+}. The ER store is replenished by the Ca^{2+}ATPase.

Calcium in Single Cells

The use of patch clamp electrophysiological techniques and video or confocal fluorescent microscopy has shown that Ca^{2+} signals within a single cell are more complex than just a single transient elevation. Cytosolic Ca^{2+} can oscillate and spread throughout the cell in waves. This is believed to be due to the biphasic nature of Ca^{2+} on the regulation of the Ins(1,4,5)P_3 receptor (Taylor and Marshall, 1992), and see below.

Ca^{2+} mobilising agents can evoke Ca^{2+} oscillations in several exocrine glands, fibroblasts, hepatocytes, endothelial cells, macrophages and oocytes (Tepikin and Petersen, 1992). The spatiotemporal behaviour of Ca^{2+} observed in a variety of cells varies from whole-cell oscillations or repetitive spiking to intracellular target patterns and spiral waves (Clapham and Sneyd, 1995). There are basically two types of oscillations that can be produced by various agonists, low frequency baseline spikes and higher frequency sinusoidal oscillations superimposed on an elevated baseline, but is not known how different agonists can produce these different oscillatory profiles (Tepikin and Petersen, 1992; Putney and Bird, 1993). A variety of models have been proposed to explain the basic oscillatory phenomena (see Clapham and Sneyd, 1995) including oscillations of Ins(1,4,5)P_3 (Ciapa, *et al.*, 1994), although currently favoured models are explained by the biphasic effect of Ca^{2+} on the Ins(1,4,5)P_3 receptor (Clapham and Sneyd, 1995; Petersen, *et al.*, 1994). The principles are as follows.

Agonists cause the production of Ins(1,4,5)P_3 which activates receptors on a subset of the ER resulting in a low level of Ca^{2+} release. When the Ca^{2+}ATPase has filled the ER store the luminal [Ca^{2+}] inactivates the pump. The low level of Ca^{2+} release increases the local $[Ca^{2+}]_{cyt}$ until the threshold is reached where Ca^{2+} synergises with Ins(1,4,5)P_3 to open all the Ins(1,4,5)P_3 sensitive channels. This results in an explosive release of Ca^{2+}. However as Ca^{2+} increases, Ca^{2+} will slowly bind to an inactivating domain on the receptor inhibiting the Ca^{2+} flux. This results in a net uptake of Ca^{2+} back into the ER by the Ca^{2+}ATPase and Ca^{2+} is extruded by the plasma membrane ATPase. There would be a refractory period as $[Ca^{2+}]_{cyt}$ is lowered and the Ins(1,4,5)P_3 receptor becomes activated again. This could explain oscillations if constant Ins(1,4,5)P_3 was present. Also the positive effect of Ca^{2+} on its own release is the reason for the propagation of waves in a 2-dimensional spatial model.

To compensate for the extrusionof Ca^{2+} from the cytoplasm Ca^{2+} entry is required and this will be discussed in the next section. However this compensation is not complete and in prolonged stimulation by an agonist there is a balance between Ca^{2+} release, extrusion and influx. This results in the stores being maintained in a

depleted state in Swiss 3T3 cells after prolonged stimulation by serum or vasopressin (Lopez-Rivas and Rozengurt, 1983; Lopez-Rivas and Rozengurt, 1984).

Note that the above events can only be measured in single cells. Populations of cells in suspension will not oscillate synchronously.

1.3.2.3. CALCIUM INFLUX FROM THE EXTRACELLULAR MEDIUM

It has been known for a long time that the Ca^{2+} signal is biphasic (Putney, *et al.*, 1981; Putney and Bird, 1993). There is a transient increase in $[Ca^{2+}]_{cyt}$ from intracellular stores, followed by a prolonged influx from the extracellular medium. The Ca^{2+} channels activated in this process operate with extracellular Ca^{2+} in the mM range (Mauger, *et al.*, 1984; Poggioli, *et al.*, 1985). The mechanism of activation of these channels is still controversial. Originally it was thought that it was the increase in $[Ca^{2+}]_{cyt}$ which activated the influx (von Tscharner, *et al.*, 1986), or that the inositol trisphosphate metabolite, $Ins(1,3,4,5)P_4$, was the intracellular messenger for this process (Irvine and Moor, 1986; Irvine and Moor, 1987). This mechanism was revived as a potential candidate more recently when patch-clamp techniques were used (Luckhoff and Clapham, 1992; Hashii, *et al.*, 1993). Also a combination of both Ca^{2+} and inositol phosphates has been proposed (Pittet, *et al.*, 1989). It was thought that the mechanism of action could involve a conformational coupling proposed by (Putney, 1986) by which the $Ins(1,4,5)P_3$ receptor could join the ER and plasma membranes, a process regulated by inositol phosphates. Once anchored by the $Ins(1,4,5)P_3$ receptor the plasma membrane calcium channel could join to the Ca^{2+}ATPase of the ER forming a structure analogous to that seen in a gap junction (Irvine and Moor, 1987). This would mean that Ca^{2+} entry would bypass a journey through the cytoplasm. There is now evidence against this model and it is now accepted that Ca^{2+} crosses the cytoplasm first (Putney and Bird, 1993).

The currently favoured mechanism for Ca^{2+} influx is that the empty state of the Ca^{2+} store regulates the influx and this has been described as Capacitative Calcium Influx (Putney, 1986). This was supported later in a study by Takemura and Putney (1989). These investigators used agonists to stimulate the cells in the absence of extracellular Ca^{2+} followed by antagonists to stop this stimulation thus bringing inositol phosphate levels back to the resting state. Ca^{2+} influx was monitored on replacing extracellular Ca^{2+} in the absence of raised inositol phosphates. Capacitative calcium influx was substantiated when thapsigargin and DBHQ were used to deplete the ER store because these agents do not cause the release of inositol phosphates (Takemura, *et al.*, 1989; Robinson, *et al.*, 1992). The Ca^{2+} influx activated by agonists, or by depletion of agonist sensitive stores with thapsigargin or DBHQ, was indistinguishable supporting the role of the filling state of the store as the regulator of

Ca^{2+} influx (Demaurex, *et al.*, 1992; Schilling, *et al.*, 1992; Breittmayer, *et al.*, 1993; Montero, *et al.*, 1993a).

How does the empty store activate influx? It was proposed that a small signalling molecule is released from the ER (rather than a metabolite of $Ins(1,4,5)P_3$) that activates calcium influx because thapsigargin and DBHQ do not cause release of inositol phosphates but they do activate Ca^{2+} influx (Takemura, *et al.*, 1989). This putative signalling molecule has become the focus of intense investigation. Cytochrome *P*-450 was suggested to have a role in production of this messenger (Alvarez, *et al.*, 1991) which was estimated to have a half life of 7 s (Montero, *et al.*, 1992). However, it is now thought that *P*-450 inhibitors block the influx channel itself rather than blocking the production of the messenger (Vostal and Fratantoni, 1993). Parekh, *et al*, (1993) then described a diffusible messenger which could be washed away in patch clamp studies with a half life of 10 s and was deactivated by an okadaic acid-sensitive phosphatase. At the same time Randriamampita and Tsien (1993) prepared a crude Jurkat cell extract which had Ca^{2+} influx properties when applied extracellularly to several cell types and called the active agent(s) CIF (calcium influx factor). It had a phosphate group which was consistent with the observation of Parekh, *et al.* and M_r of under 500 (Randriamampita and Tsien, 1993). This extract was further investigated by different groups who showed it to be a heterogeneous mix of intra- and extra-cellular acting factors (Gilon, *et al.*, 1995; Thomas and Hanley, 1995). Thomas and Hanley (1995) applied the extracts intracellularly by microinjection of oocytes then purified the extract further to obtain 3 calcium mobilising molecules one of which fulfilled criteria for a CIF (Kim, *et al.*, 1995).

However other observations suggest that CIF is not the only regulator of Ca^{2+} influx. PKC activation was shown to greatly enhance the influx induced by thapsigargin in RINm5F insulin secreting cells (Bode and Goke, 1994) but PKC activation in neutrophils, megakaryoblastic cells and Jurkats inhibited thapsigargin-induced Ca^{2+} influx (Montero, *et al.*, 1993b; Conroy, *et al.*, 1994; Xu and Ware, 1995). Tyrosine phosphorylation has also been proposed as a mechanism for Ca^{2+} influx. In human foreskin fibroblasts and lymphocytes tyrosine kinase inhibitors blocked Ca^{2+} influx but not release (Lee, *et al.*, 1993a; Tepel, *et al.*, 1994). A role for GTP hydrolysis was described in the initiation of Ca^{2+} influx in lachrymal acinar cells and a leukaemic mast cell line (Bird and Putney, 1993; Fasolato, *et al.*, 1993). Also metabolic poisons which decrease ATP levels, and hence GTP levels, can inhibit influx (Gamberucci, *et al.*, 1994). Pertussis toxin was shown to inhibit Ca^{2+} influx (Fernando and Barritt, 1994). This was substantiated by the observation that antibodies to $G\alpha_{i1-2}$ and peptides against $G\alpha_{i2}$ but not $G\alpha_{i3}$ blocked Ca^{2+} influx (Berven, *et al.*, 1995).

Thus the mechanism of calcium influx is hotly debated and a unifying hypothesis has not been presented yet. This could mean that there are multiple mechanisms to Ca^{2+} influx (Neher, 1992) which may differ subtly in their biological function and the experimental procedure used may bias for the measurement of one pathway over the other. Alternatively these differences could reflect variation between cell types (Thomas and Hanley, 1994) or even steps in a sequential process for the production or action of CIF.

1.3.2.4. CALCIUM GRADIENTS

It is important to point out that free Ca^{2+} is a very localised signal. In the cytoplasm there are many buffering proteins which will limit the range of the Ca^{2+} ion. It has been estimated that whereas the diffusion constant is 24 μm for $Ins(1,4,5)P_3$, that for unbuffered Ca^{2+} is 0.1 μm and buffered Ca^{2+} is 5 μm. Therefore, in cells smaller than 20 μm, $Ins(1,4,5)P_3$ is a global messenger whereas Ca^{2+} acts in restricted domains (Allbritton, *et al.*, 1992). Thus within the cell there are Ca^{2+} gradients (Petersen, *et al.*, 1994). Micro-gradients can occur within a spatial dimension of 1 - 10 μm and over a concentration range of 0.1 - 1 μM and can be visualised with current Ca^{2+} imaging techniques. Also Petersen, *et al.* (1994) suggest the possibility of nano-gradients which could appear around Ca^{2+} channels and may reach concentrations of 100 μM. This may occur if Ca^{2+} mobilisation through the channel is faster than diffusion through the cytoplasm and may only last milli seconds depending on the state of the Ca^{2+} channel. Nano-gradients cannot be visualised with current Ca^{2+} imaging techniques.

1.3.2.5. CALCIUM IN GROWTH CONTROL

It has been known for more than 20 years that transformed cells require less extracellular calcium for growth than non-transformed cells (Balk, 1971; Balk, *et al.*, 1973; Boynton, *et al.*, 1977; Paul and Ristow, 1979; Nicholson, *et al.*, 1984). This indicated that the effect of calcium is regulatory rather than permissive and that a Ca^{2+}-dependent step could be bypassed in transformed cells. The ability of cells to take up Ca^{2+} from the extracellular medium is crucial for normal cell proliferation. This has been studied using Ca^{2+}-deficient culture medium or blood plasma in parathyroidectomised rats, or by using Ca^{2+} channel antagonists (reviewed by Takuwa, *et al.* (1995). The importance of Ca^{2+} influx through non-voltage sensitive channels is further illustrated by the clinical investigation of a blocker of these channels in patients with cancer (Kohn, *et al.*, 1996). However, these studies show that Ca^{2+} is essential but it is more difficult to demonstrate that Ca^{2+} is a regulatory signal rather than an obligatory event, see section 1.1.3.

The intracellular calcium chelator dmBAPTA has been used to quench increases in $[Ca^{2+}]_{cyt}$ induced by serum to show a causal relationship between increases in $[Ca^{2+}]_{cyt}$ and re-entry to the cell cycle (Wahl and Gruenstein, 1993). However, it is difficult to distinguish between permissive and regulatory effects of $[Ca^{2+}]_{cyt}$, also dmBAPTA chelates other ions and this introduces non-specific effects. Takuwa, *et al.* (1991a) showed that bombesin required extracellular Ca^{2+} to reinitiate DNA synthesis in Swiss 3T3 cells. However this study did not distinguish between permissive and regulatory requirements for Ca^{2+} either.

Because many early signalling events induced by bombesin were independent of the $[Ca^{2+}]_{out}$ (Takuwa, *et al.*, 1991a) it was concluded that Ca^{2+} has an obligatory role at G_1/S and/or G_2/M (Takuwa, *et al*, 1995). Ca^{2+} is now recognised to be essential at various points throughout the cell cycle including G_1/S and G_2/M but it has been difficult to ascribe anything more than a supporting or housekeeping role to this ion (Means, 1994; Berridge, 1995; Takuwa, *et al.*, 1995).

The role of Ca^{2+} in initiating the transition between G_0/G_1 is not clear, with the exception of lymphocytes. Changes in Ca^{2+} fluxes in lymphocytes have been identified as stimulating proliferation but this is because an increase in $[Ca^{2+}]_{cyt}$ induces the IL-2 gene which then acts as an autocrine growth factor (Crabtree, 1989). The role of Ca^{2+} in initiating fibroblast proliferation has not been defined, however, several observations indicate that Ca^{2+} could be important.

1. Neuropeptides such as bombesin, vasopressin, endothelin and bradykinin, which are potent mitogens for Swiss 3T3 cells (Rozengurt, 1986), induce a rapid and transient increase in $[Ca^{2+}]_{cyt}$ followed by a persistent depletion of Ca^{2+} from stores in the ER (Lopez-Rivas and Rozengurt, 1984; Mendoza, *et al.*, 1986; Lopez-Rivas, *et al.*, 1987; Nanberg and Rozengurt, 1988).

2. Receptors that are coupled to G_q have oncogenic potential. These include the conditional oncogenes 5-HT_{1C} receptor (Julius, *et al*, 1989) and M_1-, M_3- and M_5-Ach receptors (Gutkind, *et al.*, 1991) which all require ligand activation to induce transformation. Also mutants of the α_{1B}-AR that constitutively activate G_q enhance mitogenesis and transform fibroblasts (Allen, *et al.*, 1991).

3. M_2- and M_4-Ach receptors which couple to G_i are not transforming (Gutkind, *et al.*, 1991). Interestingly transfected 5-HT_{1A} receptors which couple to pertussis toxin-sensitive G_i proteins were shown to be conditionally transforming, although activation of these receptors did induce PIP_2 hydrolysis (Abdel-Baset, *et al.*, 1992), probably mediated by $\beta\gamma$ subunits as described earlier.

4. Constitutively activated G_q mutants transform fibroblasts (Kalinec, *et al.*, 1992).

5. Microinjection of antibodies raised against G_q blocks the mitogenic activity of bradykinin (LaMorte, *et al.*, 1993a).

6. A bacterial toxin from *Pasteurella multocida* which causes a striking activation of inositol phosphate production and Ca^{2+} mobilisation, also promotes cell proliferation including anchorage-independent growth (Rozengurt, *et al.*, 1990; Higgins, *et al.*, 1992; Murphy and Rozengurt, 1992).

7. Microinjection of PLC into quiescent NIH 3T3 induces entry into S phase (Smith, *et al.*, 1989).

Thus Ca^{2+} mobilisation is coincident with mitogenic stimulation. But this does not prove a role for Ca^{2+} fluxes in the mitogenic activation of quiescent fibroblasts because hydrolysis of PIP_2 also generates DAG which activates PKC. The importance of PKC in cellular proliferation has been previously described (Rozengurt, *et al.*, 1984; Nishizuka, 1988 and see section 1.3.3). Furthermore, insulin and EGF can stimulate DNA synthesis in Swiss 3T3 cells without inducing Ca^{2+} mobilisation (Rozengurt, *et al.*, 1983b; Vara and Rozengurt, 1985). Similarly, mutant receptors for platelet-derived growth factor and fibroblast growth factor defective in PLCγ activation have been shown to retain mitogenic activity upon ligand activation (Mohammadi, *et al.*, 1992; Peters, *et al.*, 1992; Valius, *et al.*, 1993; Valius and Kazlauskas, 1993). Also activated Gα mutants that do not stimulate PIP_2 hydrolysis are transforming such as α_{12} and α_{13} (Xu, *et al.*, 1993; Voyno-Yasenetskaya, *et al.*, 1994b) and α_i (*gip*2 oncogene), although the latter is only weakly transforming and only in certain fibroblasts (Pace, *et al.*, 1991; Gupta, *et al.*, 1992). Thus, the contribution of Ca^{2+} to mitogenic signalling remains unclear.

Another approach for assessing the role of Ca^{2+} in the transition from G_0 into DNA synthesis, is the use of specific inhibitors of the Ca^{2+}ATPase of the ER to create an isolated Ca^{2+} signal. Addition of these compounds to intact cells induces mobilisation of Ca^{2+} from internal stores bypassing PLC-mediated formation of $Ins(1,4,5)P_3$.

This has advantages over the use of the Ca^{2+} ionophores A23187 or ionomycin which increase $[Ca^{2+}]_{cyt}$ by permeablising the plasma, ER and mitochondrial membranes to Ca^{2+} and uncouple oxidative phosphorylation (Reed and Lardy, 1972; Liu and Herman, 1978). Ca^{2+} homeostasis is therefore disrupted in non-specific manner so it is not suprising that induction of DNA synthesis in fibroblasts has not been described for the Ca^{2+} ionophores. Thapsigargin and DBHQ will selectively deplete the hormonally-responsive store causing a situation which resembles an agonist response more closely.

Previous studies have shown that the addition of either thapsigargin or DBHQ, at micromolar concentrations, profoundly inhibited cell proliferation or induced cytotoxic effects (Ghosh, *et al.*, 1991; Short, *et al.*, 1993). These findings are difficult

to reconcile with the fact that mitogenic neuropeptides and other growth promoting factors also cause persistent depletion of Ca^{2+} from the ER and that thapsigargin has been shown to be a tumour promoter.

The experiments presented in Chapter 5 were designed to assess the effect of gradual depletion of Ca^{2+} from the ER on the ability of quiescent cells to exit from G_0 and enter DNA synthesis.

1.3.2.6. CALCIUM EFFECTORS

The main Ca^{2+} binding protein in the cytoplasm is the 17 kDa ubiquitous multifunctional calmodulin (CaM) that binds Ca^{2+} with a K_d in the μM range consistent with activation at high $[Ca^{2+}]_{cyt}$ (reviewed in Gnegy (1993). CaM acts as a Ca^{2+}-dependent regulator of cyclic nucleotide metabolism, Ca^{2+} transport, protein phosphorylation and dephosphorylation cascades, ion transport, cytoskeletal function and cell proliferation. Takuwa, *et al.* (1995) describes the evidence that CaM is the major mediator of Ca^{2+}-dependent proliferation. In particular CaM levels start to rise in late G_1 and S phase and reach maximum in late G_2. CaM may mediate its effects in the nucleus during S phase. Also a variety of transformed cells and tumour tissues show elevated levels of CaM and overexpression of CaM shortens G_1 and increases cell density at growth saturation (Takuwa, *et al.*, 1995).

At the protein level CaM also binds to a number of other, predominately cytoskeletal, proteins including MAP-2, MARCKS, adducin and tubulin (reviewed in Gnegy (1993). Interestingly many of the cytoskeletal CaM binding proteins can be phosphorylated by PKC. CaM also activates isozymes of adenylyl cyclase, cyclic nucleotide phosphodiesterase and several protein kinases including myosin light chain kinase, which is involved in muscle contraction, and multifunctional CaM kinase (CaMK).

Multifunctional CaMK, like CaM, is ubiquitous and as the name suggests it phosphorylates many substrates. These are involved in many cellular processes such as neurotransmitter release, muscle contraction, glycogen-, fatty acid- and carbohydrate metabolism, microtubule assembly and signal transduction (reviewed in Braun and Schulman (1995), including the cAMP response element binding protein, CREB, see section 1.3.9.2. An interesting aspect to CaMK activity has been proposed recently in which it acts as Ca^{2+} spike frequency detector (Hanson, *et al.*, 1994). CaMK exists as a multimeric enzyme of 8 - 10 monomers that are arranged like spokes on a wheel with the kinase domain at the periphery (Braun and Schulman, 1995). The subunits are active when Ca^{2+}/CaM is bound and in this state they can phosphorylate the neighbouring subunit (which also needs Ca^{2+}/CaM bound to be an effective substrate). This autophosphorylation within the holoenzyme converts CaMK to a Ca^{2+}/CaM-independent state. Computer simulations predict that if $[Ca^{2+}]_{cyt}$ oscillates

at a higher than threshold frequency, the rate at which Ca^{2+}/CaM associates with CaMK is greater than the rate of dissociation of Ca^{2+}/CaM and subsequent dephosphorylation of CaMK subunits. Higher frequency Ca^{2+} spikes will increase the likelihood of having Ca^{2+}/CaM on two neighbouring subunits: the "kinase" and the "substrate". In these conditions effective intersubunit phosphorylation can occur and hence Ca^{2+}/CaM-independent CaMK activity. This model suggests that CaMK could act as a frequency decoder that becomes activated in response to agonists that induce Ca^{2+} oscillations above a certain frequency (Hanson, *et al.*, 1994).

1.3.3. PROTEIN KINASE C

PLC-mediated hydrolysis of PIP_2 also generates 1,2-diacylglycerol (DAG). DAG, which can also be generated from other sources such as phosphatidylcholine hydrolysis, acts as a second messenger in the activation of protein kinase C (PKC) by multiple extracellular stimuli (Nishizuka, 1988; Nishizuka, 1995) including bombesin (Erusalimsky, *et al.*, 1988). In accord with this, bombesin strikingly increases the phosphorylation of an acidic, myristoylated protein that migrates with an apparent molecular mass of 80 kDa which has been identified as a prominent substrate for PKC in cultured cells and several tissues, (Rozengurt, *et al.*, 1983b; Erusalimsky, *et al.*, 1991) see section 1.3.3.3. PKC activation can vary depending on the mitogen. For example PKC activation by bradykinin shows a transient phosphorylation of 80K which is almost back to basal levels after 2 min (Issandou and Rozengurt, 1990), whereas bombesin-stimulated 80K phosphorylation remains elevated for at least 10 min (Zachary, *et al.*, 1986).

Calcium-dependent protein kinase (PKC) was originally identified as a phospholipid-, diacylglycerol- and Ca^{2+}-dependent protein kinase which phosphorylates serine residues. It has long been implicated in growth regulation because it is the site of action of the potent tumour-promoting phorbol esters (Nishizuka, 1984). Phorbol esters stimulate DNA synthesis and cell division in synergy with insulin and other growth factors (Dicker and Rozengurt, 1978; Dicker and Rozengurt, 1980). Also the addition of the synthetic DAG, 1-oleoyl-2-acetylglycerol (OAG) mimics the action of phorbol esters in stimulating reinitiation of DNA synthesis and cell division (Rozengurt, *et al.*, 1984).

An approach to testing the role of PKC in biological responses is to exploit the selective removal of PKC caused by prolonged pretreatment of the cells with phorbol ester. Chronic exposure to phorbol esters leads to a marked decrease in the number of specific phorbol ester-binding sites (Collins and Rozengurt, 1984) and to the disappearance of measurable PKC activity in cell-free preparations (Rodriguez-Pena

and Rozengurt, 1984). Accordingly the cells become desensitised to the mitogenic effects elicited by phorbol esters (Collins and Rozengurt, 1982b; Collins and Rozengurt, 1984) or OAG (Rozengurt, *et al.*, 1984).. When cells are pretreated with PDB to downregulate PKC, DNA synthesis stimulated by bombesin alone is abolished but in the presence of insulin DNA synthesis is unaffected (Rozengurt and Sinnett-Smith, 1987).

1.3.3.1. STRUCTURE AND ACTIVATION

To date there are 10 PKC isoforms which fall into 3 categories, classical or conventional PKC (cPKC), new or novel PKC (nPKC) and atypical PKC (aPKC) see table 1.V.

Table 1.V. PKC isoforms in mammalian tissues

	Subspecies	Activators	Tissue expression
cPKC	α	Ca^{2+}, DAG, PS	universal
	βI	"	some tissues
	βII	"	many tissues
	γ	"	brain only
nPKC	δ	DAG, PS	universal
	ε	DAG, PS, PIP_3	brain and others
	η(L)	DAG, PS, PIP_3	skin, lung, heart
	θ	?	muscle, hemopoetic cells
aPKC	ζ	PS, PIP_3	universal
	λ(ι)	?	many tissues

From Nishizuka (1995) PS - phosphatidylserine; PIP_3 - phosphatidylinositol 3,4,5-trisphosphate.

All PKCs require phosphatidylserine (PS) for activation. This is an acidic phospholipid which is located exclusively on the cytoplasmic face of membranes. There is variation between the other co-activators and this is reflected in differences in the domain structure (C1 - C4) of the different classes of PKCs (reviewed in Nishizuka, 1995) see figure 1.8.

The C1 domain comprises an auto-inhibitory pseudosubstrate sequence followed by two tandem repeats of a cysteine rich, zinc finger motif which is responsible for DAG and phorbol ester binding. The aPKC group lack one cysteine rich domain and do not bind DAG and phorbol esters. The C2 domain, which is conserved among other proteins and is known as the CalB domain, is only present in cPKCs and is required for Ca^{2+}-dependent phospholipid binding. Hence the classical

PKCs are Ca^{2+} dependent. The C3 region contains the ATP binding consensus sequence and the C4 domain binds the protein substrate.

The C-terminal catalytic domain is separated from the N-terminal regulatory domain via a flexible hinge region. Substrate binding displaces the pseudosubstrate region from the substrate binding site of the catalytic domain. This was deduced from the increased susceptibility to proteolysis of the pseudosubstrate region upon substrate binding. Cleavage of the pseudosubstrate sequence or antibodies directed against it result in constitutively activated PKC enzymes (Newton, 1995).

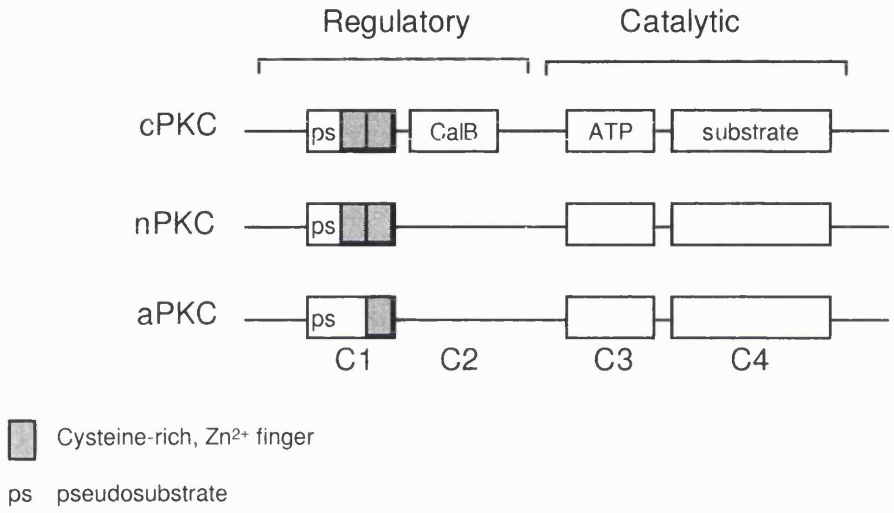

Figure 1.8. Structure of PKC isozymes

Swiss 3T3 cells contain PKCs α, δ, ε and ζ. Prolonged treatment of Swiss 3T3 cells with phorbol esters therefore down regulates PKCs α, δ and ε (Olivier and Parker 1992).

1.3.3.2. SUBSTRATES

It is believed that the function of different isozymes of PKC is to phosphorylate different substrates although the affinity of PKCs for known substrates and rate of phosphorylation do not differ much between isozymes. Attention has now turned to the location within the cell to explain specificity and it appears that PKCs are targeted to distinct subcellular locations (Jaken, 1996). It has been widely observed that PKC translocates from the cytoplasm to the membrane upon activation (Dekker and Parker, 1994), and DAG increases the affinity of PKC for PS (Nishizuka, 1995). These observations are consistent with the suggested importance of localisation of the enzymes. Substrates for PKC are 80K/MARCKS, vinculin, talin, adducins and annexins

which are involved in attaching the cytoskeleton with the cellular membranes; G protein-coupled receptors such as muscarinic receptors and receptor tyrosine kinases such as the EGF receptor; and enzymes involved in metabolism and signal transduction (Liu, 1996).

1.3.3.3. 80K/MARCKS

One of the earliest events occurring within seconds after phorbol ester and growth factor stimulation of cells is the phosphorylation of an acidic 80 kDa protein termed "80K" (Rozengurt, *et al.*, 1983b). This was shown to be a specific PKC substrate and hence a useful tool for assaying PKC activity (Rodriguez-Pena and Rozengurt, 1986). Subsequently, 80K was purified and cloned (Brooks *et al.*, 1990; Erusalimsky, *et al.*,(1991). Further studies revealed that 80K is the murine homologue of the bovine myristoylated alanine rich C-kinase substrate, MARCKS (Herget, *et al.*, 1992). The function of 80K/MARCKS is not entirely clear. Upon agonist-stimulated phosphorylation it translocates from the membrane to the cytosolic fraction (Herget and Rozengurt, 1994) and then it is down regulated by a post-transcriptional mechanism (Brooks, *et al.*, 1991; Brooks, *et al.*, 1992). Also the expression of 80K/MARCKS is very low when cells are dividing and its re-expression coincides with growth arrest in G_0 (Herget, *et al.*, 1993). MARCKS has actin and calmodulin binding sites and is implicated in regulation of the cytoskeleton (Aderem, 1992). Recently it was shown that 80K/MARCKS deficiency in mice lead to abnormal brain development and perinatal death (Stumpo, *et al.*, 1995) suggesting an important role for 80K/MARCKS in the regulation of growth and development.

1.3.3.4. PROTEIN KINASE D

PKC enzymes are not the only targets of the second messenger DAG. Recently a novel serine/threonine protein kinase has been cloned (Valverde, *et al.*, 1994) that binds and is activated by PDB (Valverde, *et al.*, 1994: van Lint, *et al.*, 1995) and has been named protein kinase D (PKD). This kinase has a catalytic domain that is most similar to myosin light chain kinase and the Ca^{2+}/CaM regulated kinases. In particular PKD exhibits only a low degree of similarity to the highly conserved regions of PKCs and thus PKD represents a distinct class of protein kinases. PKD has two cysteine rich domains that are responsible for binding phorbol esters (Valverde, *et al.*, 1994), furthermore phorbol esters and synthetic diacylglycerols activate PKD in the presence of PS (van Lint, *et al.*, 1995). The identification of another target for phorbol esters raises the possibility that some of the cellular actions of phorbol esters could be mediated by PKD.

1.3.4. MONOVALENT ION FLUXES

Growth factors stimulate monovalent cation fluxes (Rozengurt and Ober, 1988). This was first observed by Rozengurt and Heppel (1975) who found that addition of serum rapidly stimulated the influx rate of $^{86}Rb^+$ (a K^+ tracer) into quiescent Swiss 3T3 cells. Most of the increased $^{86}Rb^+$ uptake was inhibited by ouabain indicating that it was mediated by the plasma membrane Na^+-K^+ pump. Subsequently, a variety of agents which are mitogenic in Swiss 3T3 cells were also shown to stimulate Na^+-K^+ pump activity. These include PDGF, FDGF, vasopressin, phorbol esters and bombesin. This was also observed for a wide variety of mitogens in a range of cell types (reviewed in Rozengurt and Ober, 1988).

The Na^+-K^+ pump activity is limited by the availability of intracellular Na^+ and changes in the rate of Na^+ entry therefore play a primary role in controlling the rate of Na^+-K^+ pump activity. Studies with Li^+ uptake identified a second Na^+-specific transport system which was activated by serum and was amiloride-sensitive (Smith and Rozengurt, 1978) suggesting that this Na^+ influx was mediated by the Na^+/H^+ antiport. Since Na^+/H^+ exchange can operate in either direction, the stimulation of Na^+ entry via the Na^+/H^+ antiport by mitogenic agents could result from at least two alternative mechanisms. Growth factors could directly activate the antiport system leading to an increase in Na^+/H^+ exchange and subsequent increase in pH_i. Alternatively the enhancement of Na^+/H^+ antiport activity by mitogens could represent a compensatory mechanism to reduce cytoplasmic acidification caused by excess protons generated by growth factor-enhanced cellular metabolism. Schuldiner and Rozengurt (1982) demonstrated that incubation of Swiss 3T3 cells in the presence of EGF, vasopressin and insulin increases the pH_i and this was found to be a general response to mitogenic stimulation.

The fluxes of Na^+, H^+ and K^+ across the membrane of Swiss 3T3 cells and other cell types can be envisaged as a "Na^+ cycle". Stimulation of the Na^+/H^+ antiport by mitogens increases Na^+ influx and H^+ efflux, raising intracellular Na^+ and pH. The resulting increase in intracellular Na^+ stimulates the Na^+-K^+ pump, elevating intracellular K^+ and restoring the electrochemical gradient for Na^+ (Rozengurt and Ober, 1988).

Several Na^+/H^+ exchangers have been cloned recently (NHE1, NHE2, NHE3 and NHE4) but only NHE1 is ubiquitously expressed, the others appear to have a specialised distribution in epithelia (Bianchini and Pouyssegur, 1994). It is thought that NHE1 is regulated by phosphorylation or by a regulatory protein that may also be phosphorylated (reviewed in Bianchini and Pouyssegur, 1994). The involvement of PKC is implicated because phorbol esters and OAG enhance both ouabain-sensitive

$^{86}Rb^+$ uptake and amiloride-sensitive $^{22}Na^+$ entry (Vara, *et al.*, 1985). However, bombesin-stimulated monovalent ion fluxes is only partially inhibited after down regulation of PKC by prolonged treatment with phorbol ester, suggesting the presence of PKC-independent pathways (Mendoza, *et al.*, 1986). In support of this CaM has been proposed to have a role in activating NHE1 (Bianchini and Pouyssegur, 1994), and more recently, the newly identified Gα subunits α_{12} and α_{13} have been implicated in control of monovalent ion fluxes. Overexpression of activated mutants of α_q, α_{12} and α_{13} cause intracellular alkalisation (Dhanasekaran, *et al.*, 1994; Voyno-Yasenetskaya, *et al.*, 1994a). α_q and α_{13} were dependent upon PKC for this effect but α_{12}-induced intracellular alkalisation was PKC-independent. Thus, it appears that Na^+/H^+ can be regulated by multiple mechanisms.

Monovalent ion fluxes have been proposed to play a role in the initiation of cell proliferation. Inhibition of ion fluxes prevents the initiation of DNA synthesis and increased ion fluxes are associated with initiation of DNA synthesis (reviewed in (Rozengurt and Ober, 1988).

1.3.5. PHOSPHOLIPASE A_2 AND ARACHIDONIC ACID RELEASE

While bombesin stimulates DNA synthesis in the absence of other growth factors, vasopressin is mitogenic for Swiss 3T3 cells only in synergistic combination with other factors (Rozengurt, *et al.*, 1979; Rozengurt and Sinnett-Smith, 1983). Binding of vasopressin to its distinct receptor on quiescent cultures of Swiss 3T3 cells causes a rapid production of Ins(1,4,5)P_3, mobilisation of Ca^{2+} from intracellular stores and sustained activation of PKC via a G-protein linked transduction pathway (reviewed in Rozengurt, 1991b). Since the initiation of DNA synthesis is triggered by independent signal-transduction pathways that act synergistically in mitogenic stimulation, the ability of bombesin to act as a sole mitogen could be due to activation of a signalling pathway not stimulated by vasopressin.

Recently, bombesin, but not vasopressin, has been shown to induce a marked, biphasic release of arachidonic acid into the extracellular medium (Millar and Rozengurt, 1990a; Domin and Rozengurt, 1993). A first phase involves rapid activation of phospholipase A_2 (PLA_2). These results showed a clear difference in the pattern of early signals induced by the neuropeptides bombesin and vasopressin in Swiss 3T3 cells. The stimulation of arachidonic acid release by bombesin is likely to contribute to bombesin-induced mitogenesis because externally applied arachidonic acid potentiates mitogenesis induced by agents that stimulate PIP_2 hydrolysis but not arachidonic acid release, for example vasopressin (Millar and Rozengurt, 1990a). Arachidonic acid release in Swiss 3T3 cells stimulated by bombesin is biphasic with a

rapid phase which lasts for 10 min followed by a sustained release which lasts for at least 60 min (Takuwa, *et al.*, 1991b). Bombesin-induced activation of PLA_2 involves multiple mechanisms, including intracellular Ca^{2+} release for the first phase, and PKC activation and Ca^{2+} influx for the second phase. However there also appears to be other unidentified mechanisms involved (Takuwa, *et al.*, 1991b).

Phospholipase A_2 (PLA_2) enzymes can generally be divided into two major groups, secretory PLA_2 ($sPLA_2$) which are non-specific in their hydrolysis of phospholipid substrate and cytoplasmic PLA_2 ($cPLA_2$) which specifically hydrolyse phospholipids that contain arachidonic acid (Glaser, *et al.*, 1993). $cPLA_2$ contains a CalB domain which mediates its Ca^{2+}-dependent translocation to membrane vesicles (Clark, *et al.*, 1991). Possible signals involved in the activation of PLA_2 include Ca^{2+} fluxes, PKC activation and activation of $G_{i/o}$ proteins (Piomelli, 1993). It is thought that PKC mediates its effect by activating MAPK which has been shown to phosphorylate and activate $cPLA_2$ *in vitro* (Lin, *et al.*, 1993). After hydrolysis free arachidonic acid can either diffuse out of the cell, be recycled back into phospholipids or be metabolised to prostaglandins, prostacyclins or thromboxanes. These metabolites may act as intracellular second messengers or as paracrine/autocrine factors which bind to specific seven transmembrane prostanoid receptors (Piomelli, 1993) and see table 1.II).

Arachidonic acid released by bombesin is believed to be converted to E-type prostaglandins which acting in an autocrine and paracrine manner enhance cAMP accumulation in the cell (Millar and Rozengurt, 1988; Millar and Rozengurt, 1990a). Since elevated cAMP levels constitute a mitogenic signal for Swiss 3T3 cells, see table 1.I, at least one consequence of arachidonic acid release may be the modulation of intracellular cAMP levels. However, other arachidonic acid metabolites may also play a role in mitogenic signal transduction by bombesin.

1.3.6. ADENYLYL CYCLASE, CAMP AND PKA

Bombesin causes enhancement of forskolin stimulated adenylyl cyclase activity (Millar and Rozengurt, 1988) that is believed to be due to arachidonic acid release (see previous section), which is partially dependent on PKC. The intracellular levels of cAMP are controlled by membrane bound adenylyl cyclase which converts intracellular ATP to cAMP (reviewed in Taussig and Gilman, 1995). At present there are 7 full length cloned adenylyl cyclases all approximately 120 kDa and all activated by $G\alpha_s$ and the diterpene forskolin. However they differ in their localisation and finer regulatory mechanisms. For instance $G\beta\gamma$ can stimulate type II and IV adenylyl cyclase but inhibit type I. Ca^{2+}/CaM can activate types I, VIII and III whereas the others are insensitive to

Ca^{2+}/CaM, and types V and VI are inhibited by Ca^{2+} ions. In a similar fashion to the PLCβ isozymes in section 1.3.1 the cAMP response to various agonists depends upon which adenylyl cyclases are expressed (Taussig and Gilman, 1995).

The downstream effector of cAMP is the cAMP-dependent protein kinase (PKA). This consists of two inhibitory regulatory subunits and two catalytic subunits in the inactive state. When cAMP binds to the regulatory subunit, the catalytic subunits dissociate and their inhibition is released. PKA has many substrates (see Walsh and van Patten (1994) a number of which are involved in the breakdown of glycogen for metabolic energy. Genes which are responsive to increases in cellular cAMP have a cAMP response element (CRE) in their promoter to which the cAMP response element binding protein (CREB) binds upon phosphorylation by PKA (Karin and Hunter, 1995), for example see section 1.3.9.2.

1.3.7. TYROSINE PHOSPHORYLATION

1.3.7.1. TYROSINE PHOSPHORYLATION OF MULTIPLE SUBSTRATES

Recently it was shown that agonists which signal through G protein-coupled receptors increase protein tyrosine phosphorylation. These include the peptides bombesin, angiotensin II, endothelin, vasopressin and bradykinin (Huckle, *et al.*, 1990; Force, *et al.*, 1991; Leeb-Lundberg and Song, 1991; Tsuda, *et al.*, 1991; Zachary, *et al.*, 1991a), the protease thrombin (Force, *et al.*, 1991) and the biological amines acetylcholine, serotonin and norepinephrine (Tsuda, *et al.*, 1991; Gutkind and Robbins, 1992). They all rapidly stimulated the tyrosine phosphorylation of bands that ran diffusely on SDS-PAGE around 60 - 80 kDa and 110 - 130 kDa. In addition, increases in tyrosine kinase activity were also shown (Zachary, *et al.*, 1991b; Huckle, *et al.*, 1992). These substrates appeared to be a subset of those stimulated by EGF (Huckle, *et al.*, 1990; Force, *et al.*, 1991) but distinct from those of PDGF such as PLCγ, ras-GAP or PI3K (Zachary, *et al.*, 1991a; Gutkind and Robbins, 1992).

It was shown that there was no tyrosine kinase activity in Swiss 3T3 membrane preparations stimulated with bombesin even though they exhibited specific bombesin binding that could be modulated by guanine nucleotides. In contrast addition of PDGF to Swiss 3T3 membranes did stimulate tyrosine kinase activity (Zachary, *et al.*, 1991a). The cell surface M_r 75 - 85 kDa receptor for bombesin which had been affinity labelled with ^{125}I-GRP was not immunoprecipitated by anti-phosphotyrosine residues. These results indicate that stimulation of tyrosine phosphorylation is not a consequence of receptor associated kinase activity (Zachary, *et al.*, 1991a) consistent with bombesin binding to a seven transmembrane receptor.

It was unclear at first whether tyrosine phosphorylation was downstream of PLC because all these agonists were acting on receptors which were coupled to PI hydrolysis. In rat liver epithelial cells angiotensin II stimulated tyrosine phosphorylation in a Ca^{2+} dependent manner and Ca^{2+} mobilising agents such as ionophores and thapsigargin were able to mimic the effects of agonist in smooth muscle cells and glomerula mesangial cells, although to a lesser extent (Huckle, *et al.*, 1990; Force, *et al.*, 1991; Tsuda, *et al.*, 1991). This effect of Ca^{2+} was not seen in all cell types (Zachary, *et al.*, 1991a). Also activation of PKC by phorbol ester produced a similar pattern of tyrosine phosphorylated proteins, but again to a lower level than receptor agonist stimulation (Force, *et al.*, 1991; Tsuda, *et al.*, 1991; Zachary, *et al.*, 1991a) or not at all (Huckle, *et al.*, 1990; Leeb-Lundberg and Song, 1991). The role of PKC was examined in agonist stimulation of events. The PKC inhibitors H7 and staurosporine or down regulation of phorbol ester-sensitive isoforms did not affect agonist-induced protein tyrosine phosphorylation although they did abolish phorbol ester-induced tyrosine phosphorylation and tyrosine kinase activity (Force, *et al.*, 1991; Leeb-Lundberg and Song, 1991; Zachary, *et al.*, 1991b). PKC and Ca^{2+} therefore did not account for the full effects of tyrosine phosphorylation. Thus tyrosine phosphorylation of 60 - 80 kDa and 110 - 130 kDa represented a new pathway stimulated by G protein-coupled receptors which was not downstream of PLC-mediated events.

The identification of these bands was aided by studies in v-src transformed cells in which a number of proteins were phosphorylated on tyrosine. To identify these putative v-src substrates, monoclonal antibodies were raised against the proteins that were tyrosine phosphorylated (Glenney and Zokas, 1989; Kanner, *et al.*, 1990) and the proteins they recognised were affinity purified. This procedure resulted in the isolation of two new proteins both localised to focal adhesions, paxillin (Turner, *et al.*, 1990) and a novel tyrosine kinase, p125 Focal Adhesion Kinase (FAK) (Schaller, *et al.*, 1992). Subsequently some of the heterogeneity regarding Ca^{2+} stimulation of the tyrosine phosphorylation of a 120 kDa band, e.g. by angiotensin, has been explained by the demonstration that it is in fact not FAK but an unidentified protein (Earp, *et al.*, 1995). Before describing FAK and paxillin in more detail focal adhesions shall be explained.

1.3.7.2. FOCAL ADHESIONS

Cells in culture adhere strongly to the substratum at places called focal contacts or focal adhesions (reviewed by Jockusch, *et al.*, 1995). These structures are where actin bundles, also known as stress fibres, of the cytoskeleton are anchored to the extracellular matrix and they are essential *in vivo* for cellular attachment in tissue

formation during embryogenesis and wound healing. This link between intra- and extracellular regions is mediated by integrins.

Integrins are heterodimers consisting of an α and a β subunit. There are many isoforms of each subunit which could in theory associate to give more than 100 integrin heterodimers but the actual diversity appears much more restricted. Specific αβ combinations bind to specific extracellular matrix proteins such as collagen or fibronectin (reviewed in Hynes, 1992). Integrins are transmembrane proteins and have a cytoplasmic tail which interacts with proteins found in focal adhesions. The most abundant protein involved in the construction of the cytoplasmic face of the focal adhesion is actin from stress fibres which terminate there. These are actin filaments in an antiparallel arrangement which are thick enough to be clearly visible using fluorescence microscopy. Other high abundance structural proteins found in focal adhesions are α-actinin, filamin, talin and vinculin which bind and cross link actin. α-actinin also binds to β_1-integrin, vinculin and zyxin. Vinculin, as well as binding actin and α-actinin, will also bind talin and paxillin (Jockusch, *et al.*, 1995).

Proteins in focal adhesions each interact with many other proteins to build these large structures, hence a regulatory element is built in to regulate their rapid assembly and disassembly. This is described further in later sections. Zyxin and paxillin are substrates for phosphorylation and can bind α-actinin and vinculin respectively. Kinases associated with focal adhesions include tyrosine kinases, src and FAK and serine kinases, PKCα and PKCδ (Jockusch, *et al.*, 1995).

The dynamics of these focal adhesions have been the focus of much attention due to transformed cells having reduced adhesion to the extracellular matrix, showing fewer focal adhesions and stress fibres, and being morphologically altered to a rounder and less flattened state (Ben-Ze'ev, 1985; Vasiliev, 1985). Similarly cells with low metastatic ability have well developed focal contacts and actin bundles, while high metastatic cells have fewer and smaller contacts and less actin bundles (Ben-Ze'ev, 1985; Vasiliev, 1985). The link between cell adhesion and cell proliferation has been recognised for many years. Untransformed cells will not grow in suspension but require a solid substrate for growth - this was described as anchorage-dependent growth. Transformation of these fibroblasts with polyoma virus negated the requirements for a solid support and this was described as anchorage-independent growth (Stoker, *et al.*, 1968). Thus the regulation of adhesion and cellular morphology could play an important role in cancer.

1.3.7.3. STRESS FIBRES

Focal adhesions are where actin stress fibres are anchored to the extracellular matrix. Quiescent cells and the majority of cells *in vivo* do not contain stress fibres

but they appear upon mitogenic stimulation *in vitro* and in instances *in vivo* such as wound healing in blood vessels (Ben-Ze'ev, 1985; Vasiliev, 1985; Jockusch, *et al.*, 1995). To co-ordinate stress fibre assembly the diffuse actin filament network must be broken down by Ca^{2+} and phospholipid dependent gelsolin and profilin and repolymerised in thick bundles. The first clue to a stress fibre regulator was the observation that the bacterial exoenzyme C3 from *Clostridium botulinum*, when introduced into cells, caused them to round up due to disappearance of actin microfilaments. This was attributed to the ADP-ribosylation of the small GTP-binding protein, $p21^{rho}$ (Rubin, *et al.*, 1988; Chardin, *et al.*, 1989), a member of the ras superfamily (Hall, 1990). When an activated mutant of Rho was injected into cells it caused the appearance of stress fibres (Paterson, *et al.*, 1990). Injection of C3 inhibited serum-, LPA- and bombesin-induced focal adhesion formation (measured by vinculin redistribution) and stress fibre assembly in quiescent cells which was consistent with a role for rho in these processes (Ridley and Hall, 1992). Like Ras (see section 1.3.8.5), Rho is regulated by guanine nucleotides exchange factors (GEFs) and GTPase activating proteins (GAPs) (Takai, *et al.*, 1995).

1.3.7.4. SRC

The first protein tyrosine kinase to be discovered was the v-src oncoprotein. There are many kinases closely related to src most showing restricted distribution to haematopoetic cells, only Src, Fyn, and Yes are widely expressed (Erpel and Courtneidge, 1995). Members of the src family are activated in response to a variety of stimuli in many cell types and are required for a mitogenic response to PDGF in fibroblasts (Erpel and Courtneidge, 1995). More recently thrombin and angiotensin have been shown to activate Src activity (Chen, *et al.*, 1994; Sadoshima and Izumo, 1996).

All members of the src family are associated with cellular membranes by myristic acid attachment and they contain an SH2, an SH3 and a catalytic domain, and C-terminal (tail) regulatory sequences (Erpel and Courtneidge, 1995; Superti-Furga and Courtneidge, 1995). The catalytic activity of Src is repressed by the C-terminal Src kinase (Csk) which phosphorylates a specific tyrosine residue in the tail. The current model suggests that in the inactivated state the phosphorylated tyrosine in the tail binds intramolecularly to the SH2 domain and that this interaction is stabilised by the SH3 domain. Activation of Src could occur by dephosphorylation of the inhibitory residue, or high affinity competition for the SH2 or SH3 domain. Activation by phosphatase activity appears to occur in lymphocytes with lymphocyte specific proteins, and in fibroblasts overexpression of protein tyrosine phosphatase α will activate Src. An additional tyrosine is present within the catalytic domain of c-Src and

autophosphorylation of this residue stimulates kinase activity (Erpel and Courtneidge, 1995; Superti-Furga and Courtneidge, 1995).

1.3.7.5. FOCAL ADHESION KINASE

In cells transformed with v-src it was noticed that src was associated with the cytoskeleton. When investigators looked for the substrates of src they found a major tyrosine phosphorylated protein of 120 kDa in v-src transformed chick embryo fibroblasts (Kanner, *et al.*, 1990). Upon cloning, the deduced amino acid sequence revealed it to be a distinctive protein tyrosine kinase (Schaller, *et al.*, 1992). Compared to other protein tyrosine kinases it had low amino acid identity in the catalytic domain and no homology outside the catalytic domain. In particular there are no SH2 or SH3 domains although there are two proline rich areas in the C-terminal half. In addition there are no hydrophobic regions indicative of membrane spanning potential and no acylation sites. Thus p125 is a cytosolic protein tyrosine kinase. The overall structure is unique in that the catalytic domain is flanked by large N- and C-terminal domains. Immunostaining of chick embryo fibroblasts show that p125 was localised with tensin and hence focal adhesions so it was called focal adhesion kinase (FAK). Localisation was shown to depend on a focal adhesion targeting (FAT) sequence at the C-terminal end of FAK (Hildebrand, *et al.*, 1993).

A protein of 120 kDa that was tyrosine phosphorylated in response to cross linking integrins or plating cells on fibronectin (Guan, *et al.*, 1991; Kornberg, *et al.*, 1991) was identified as FAK (Burridge, *et al.*, 1992; Lipfert, *et al.*, 1992). Also the 110 - 130 kDa band that was rapidly tyrosine phosphorylated in response to mitogenic neuropeptides was identified as containing FAK (Zachary and Rozengurt, 1992; Sinnett-Smith, *et al.*, 1993).

1.3.7.6. p130^{CAS}

p130 was also identified as a major tyrosine phosphorylated protein in v-src transformed cells (Kanner, *et al.*, 1990) and it was subsequently shown to be tyrosine phosphorylated in response to bombesin and other neuropeptides (Zachary, *et al.*, 1992). p130 was cloned by virtue of being a major v-crk associated substrate in v-crk transformed cells, thus p130^{cas} is implicated as being a common mediator of v-crk and v-src (Sakai, *et al.*, 1994).

1.3.7.7. PAXILLIN

Glenney and Zokas (1989) also made monoclonal antibodies to tyrosine phosphorylated proteins in v-src transformed cells. One antibody identified a protein which co-localised with vinculin to focal adhesions. This protein was affinity purified and shown to bind to vinculin but not to talin, filamin, α-actinin or actin and was

named paxillin (Turner, *et al.*, 1990). Tyrosine phosphorylation of paxillin, like FAK, had been linked to src activation and was subsequently shown to be concurrently tyrosine phosphorylated with FAK in integrin-mediated cell adhesion (Burridge, *et al.*, 1992), chick development (Turner, *et al.*, 1993) and upon stimulation of quiescent fibroblasts with mitogenic neuropeptides (Zachary, *et al.*, 1992; Zachary, *et al.*, 1993). The co-ordinate tyrosine phosphorylation of FAK and paxillin suggested that paxillin could be a substrate of FAK and this was substantiated by showing that FAK binds to and phosphorylates paxillin (Hildebrand, *et al.*, 1995; Schaller and Parsons, 1995). This association appears to be constitutive because the interaction can be detected in cells in suspension and therefore in the absence of focal adhesions (Hildebrand, *et al.*, 1995).

1.3.7.8. BOMBESIN-INDUCED FAK AND PAXILLIN TYROSINE PHOSPHORYLATION

Tyrosine phosphorylation of FAK and paxillin represented a new pathway stimulated by bombesin. This new pathway was especially interesting because it could be dissociated from both PKC activation and Ca^{2+} mobilisation (Sinnett-Smith, *et al.*, 1993; Zachary, *et al.*, 1993). The lines of evidence were:

1 Bombesin-induced phosphorylation of FAK is consistently greater than the maximum effect elicited by PDB or Ca^{2+} mobilising agents.
2 Bombesin stimulates FAK tyrosine phosphorylation more rapidly than PDB.
3 Bombesin stimulates FAK tyrosine phosphorylation at concentrations considerably lower than those required to activate PKC as judged by phosphorylation of 80K/MARCKS or to mobilise Ca^{2+}.
4 Downregulation of PKC by chronic pretreatment of cells with PDB abrogates the subsequent effect of PDB but has no substantial effect on the response to either low or high concentrations of bombesin.
5 A highly selective inhibitor of PKC blocked the increase in FAK tyrosine phosphorylation induced by PDB but did not impair the response to bombesin.
6 Neither Ca^{2+} ionophore nor thapsigargin caused a Ca^{2+}-dependent increase in FAK phosphorylation
7 Depletion of the intracellular Ca^{2+} pool by treating cells with thapsigargin blocked the mobilisation of Ca^{2+} by a subsequent addition of bombesin but had no effect on FAK phosphorylation.

Because FAK localised to focal adhesions which are where stress fibres terminate it was thought that the actin cytoskeleton could be important in bombesin-induced tyrosine phosphorylation. Using cytochalasin D to disrupt the actin network did indeed prevent bombesin-induced FAK phosphorylation (Sinnett-Smith, *et al.*,

1993). Because tyrosine phosphorylation was dependent on the actin cytoskeleton and Rho was upstream of actin stress fibre formation it was hypothesised that Rho was also upstream of neuropeptide-induced tyrosine phosphorylation. This was confirmed when C3 treated cells were stimulated with bombesin and endothelin and the resulting tyrosine phosphorylation of FAK and paxillin was inhibited (Rankin, *et al.*, 1994). Thus Rho was placed upstream of stress fibre assembly, focal adhesion formation and tyrosine phosphorylation of FAK and paxillin.

1.3.7.9. ROLE OF FAK AND PAXILLIN TYROSINE PHOSPHORYLATION

The above sections show that FAK and paxillin are implicated in integrating signals from cell adhesion, activated oncogenes and mitogenic neuropeptides (Zachary and Rozengurt, 1992). The formation of focal adhesions requires co-oordinated assembly of multiple proteins, thus the identification of proteins able to interact with FAK and paxillin has been the focus of much attention. Recently FAK has been demonstrated to bind multiple proteins *in vitro* and *in vivo*. These include $p130^{cas}$ (Polte and Hanks, 1995), talin (Chen, *et al.*, 1995), the SH2 domains of src and fyn (Cobb, *et al.*, 1994) and Csk (C-terminal Src kinase) (Bergman, *et al.*, 1995).

Similarly, studies with paxillin showed association with multiple proteins. Paxillin binds to the SH3 domain of Src (Weng, *et al.*, 1993) and when phosphorylated on tyrosine, paxillin will associate with the SH2 domain of v-Crk (Birge, *et al.*, 1993). It was subsequently shown that phosphorylation of paxillin by FAK creates the binding site for Crk (Schaller and Parsons, 1995). These investigators also demonstrated the ability of Src and Csk to phosphorylate paxillin *in vitro* and that the SH2 domains of Src and Csk can bind to phosphorylated paxillin.

Upon molecular cloning, the primary amino acid sequence of paxillin revealed the presence of one LIM domain (3) and 3 LIM-like (1,2,4) domains (Turner and Miller, 1994; Salgia, *et al.*, 1995) and see figure 1.9. LIM domains were identified as a cysteine rich motif found in the transcription factors *lin-11*, *isl-1* and *mec-3* (and also in *ceh-14*) where two copies were found in tandem (Freyd, *et al.*, 1990; Karlsson, *et al.*, 1990) and are thought to be involved in protein-protein interactions, similar to dimerisation of other transcription factors. Another gene, CRIP (cysteine rich intestinal peptide), contains just 1 LIM domain and no homeodomain, thus LIM domains can be found in proteins other than transcription factors (Freyd, *et al.*, 1990; Karlsson, *et al.*, 1990).

In addition paxillin has a proline rich region indicative of a potential SH3 binding domain, five tyrosines which have homology to SH2 binding motifs for Src, v-Crk, PLCγ and p85, see figure 1.9. Paxillin also had consensus binding sites for PKC (Turner and Miller, 1994; Salgia, *et al.*, 1995).

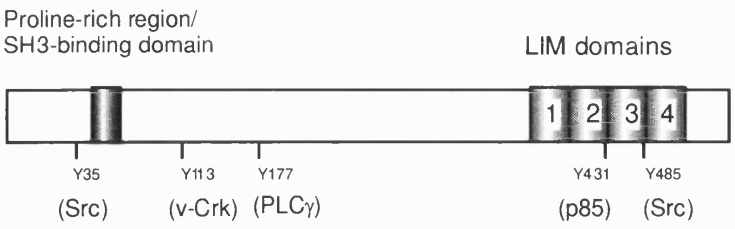

Figure 1.9. Linear diagram of paxillin structure

Shown are potential tyrosine phosphorylation sites and the corresponding recognition by SH2 containing proteins, an SH3-binding region and LIM-like domains.

Thus FAK and paxillin interact with a large number of proteins but the physiological relevance of all these interactions is unknown. It has been suggested that paxillin functions as an adaptor or scaffold onto which many proteins can assemble. The sequential events in the assembly of the focal adhesion is also unknown. FAK kinase activity does not appear to be required for the assembly of focal adhesions (Wilson, *et al.*, 1995). However it does appear that FAK promotes the unstability of focal adhesions, because FAK-deficient mice have focal adhesions but the migration of cells during development is severely retarded (Ilic, *et al.*, 1995).

1.3.8. THE MAPK PATHWAY

1.3.8.1. OVERVIEW

The MAPK pathway is a point of convergence for a variety of mitogenic factors and the oncogene products v-Raf, v-Ras and v-Src. It has a central role in relaying extracellular signals to cellular responses. MAPKs are activated by a variety of mitogens (Rossomando, *et al.*, 1989) and by oncogenic ras (Leevers and Marshall, 1992; Wood, *et al.*, 1992). Because dominant negative N17ras (Feig and Cooper, 1988) abrogated MAPK activity stimulated by PDGF, insulin and NGF (de Vries-Smits, *et al.*, 1992; Robbins, *et al.*, 1992; Thomas, *et al.*, 1992; Wood, *et al.*, 1992), this suggested that MAPK was downstream of ras in a signalling pathway. MEK was identified as a MAPK activator (Gomez and Cohen, 1991; Kosako, *et al.*, 1992; Matsuda, *et al.*, 1992; Nakielny, *et al.*, 1992a; Nakielny, *et al.*, 1992b), and was activated by phosphorylation by a serine/threonine kinase. Thus Ras, MEK and MAPK were put on the same pathway. This was confirmed by reconstitution in a permeabilised cell system (Dent, *et al.*, 1993).

Raf-1 had also been linked to a Ras pathway through work with oncogenic forms of Ras. Antibodies against Ras or a dominant negative Ras did not inhibit v-raf

induced cell transformation (Smith, *et al.*, 1986; Feig and Cooper, 1988). On the contrary dominant negative or antisense Raf could block transformation by Ras (Kolch, *et al.*, 1991). Also, Ras was required for NGF-stimulated Raf phosphorylation and activation (Troppmair, *et al.*, 1992; Wood, *et al.*, 1992). This suggested that Raf-1 was downstream of Ras.

Thus Raf was a candidate for linking Ras and MEK and this was confirmed by showing that v-Raf activated MAPK independently of Ras, and both c-Raf and v-Raf could phosphorylate and activate MEK (Dent, *et al.*, 1992; Howe, *et al.*, 1992; Kyriakis, *et al.*, 1992). The MAPK pathway or MAPK cascade was put together as Ras-Raf-MEK-MAPK.

The importance of this pathway in mitogenic signalling is demonstrated by the ability of v-Ras (Barbacid, 1987) and v-Raf (Rapp, *et al.*, 1983) and constitutively active mutants of MEK (Cowley, *et al.*, 1994; Mansour, *et al.*, 1994) to transform fibroblasts. Also, interfering mutants of, or antibodies against, Ras (Smith, *et al*, 1986; Feig and Cooper, 1988), Raf (Kolch, *et al.*, 1991), MEK (Cowley, *et al.*, 1994), and MAPK (Pages, *et al.*, 1993; Troppmair, *et al.*, 1994) inhibit cell proliferation in response to mitogenic stimuli such as oncogenes and growth factors. This pathway is not only involved in proliferation, prolonged stimulation of the MAPK cascade induces differentiation in PC12 cells (Cowley, *et al.*, 1994; Marshall, 1995). The activation of the individual components shall be described in detail.

1.3.8.2. MAPK

For over a decade it has been observed that bands of 42 and 44 kDa have been phosphorylated on tyrosine in response to polypeptide growth factors, such as PDGF, EGF and IGF; the proteases thrombin and trypsin and phorbol esters (Hunter and Cooper, 1985). Rossomando, *et al.* (1989) proposed this to be equivalent to a serine/threonine kinase, also ≈40 kDa and phosphorylated on tyrosine (Ray and Sturgill, 1988), that was activated by insulin, EGF, TPA and serum (Ray and Sturgill, 1987; Hoshi, *et al.*, 1988) and which phosphorylated MAP-2, myelin basic protein and S6 kinase II (also known as $pp90^{rsk}$ or RSK) (Ray and Sturgill, 1987; Sturgill, *et al.*, 1988; Erickson, *et al.*, 1990). It was called MAPK because it was a MAP2 kinase and a mitogen activated protein kinase (Rossomando, *et al.*, 1989).

The molecular cloning of MAPK revealed a family of protein kinases with homology to the yeast kinases FUS3 and KSS1 which mediate the yeast response to pheromones. This was evidence of an evolutionary conserved pathway which was regulated in response to extracellular signals to promote entry into the cell cycle. Therefore the proteins encoded by the cDNAs isolated were called extracellular signal-regulated kinase, ERK1 and ERK2, and these corresponded to $pp44^{mapk}$ and $pp42^{mapk}$ respectively (Boulton, *et al.*, 1990; Boulton, *et al.*, 1991). MAPK is phosphorylated on

threonine and tyrosine residues in response to mitogens (Ray and Sturgill, 1988) and both of these residues are required to be phosphorylated for MAPK activity (Anderson, *et al.*, 1990). They were identified as Thr 183 and Tyr 185 (Payne, *et al.*, 1991).

1.3.8.3. MEK

MAPK activators were purified from fractions of stimulated cell lysates that caused the phosphorylation of MAPK on Thr and Tyr (Ahn, *et al.*, 1991; Gomez and Cohen, 1991). Because recombinant MAPK could autophosphorylate at a very low level (Seger, *et al.*, 1991) it was thought that MAPK activator could be enhancing the autophosphorylation activity. MAPK activator was shown to be a kinase itself by removing the kinase activity of MAPK by either a kinase inhibitor (5'-p-fluorosulfonylbenzoyladenosine) (Adams and Parker, 1992) or by kinase inactive mutants (Nakielny, *et al.*, 1992b; Seger, *et al.*, 1992a). Kinase deficient MAPK was still phosphorylated demonstrating that the activator was a kinase itself. This was confirmed upon molecular cloning of MAPK kinase (Crews, *et al.*, 1992; Seger, *et al.*, 1992b) and the protein product was called MAPK/ERK kinase, MEK. MEK phosphorylates MAPK on thr and tyr residues demonstrating that it is a "dual specificity" kinase (Kosako, *et al.*, 1992; Nakielny, *et al.*, 1992a; Seger, *et al.*, 1992a). MEK itself is a phosphoprotein and dephosphorylation by protein phosphatase 2A, a serine/threonine phosphatase, inactivates it (Gomez and Cohen, 1991; Matsuda, *et al.*, 1992).

1.3.8.4. RAF

Identification of the MEK activator came from the observation that both Raf-1 and MAPK were downstream of Ras (see section 1.3.8.1). The activation of MAPK and MEK by Raf was looked for directly. MAPK and MEK activities were stimulated in cells infected with viruses containing v-Raf (Dent, *et al.*, 1992; Howe, *et al.*, 1992; Kyriakis, *et al.*, 1992) and also v-Raf activated MEK *in vitro* (Dent, *et al.*, 1992; Kyriakis, *et al.*, 1992). In addition immunoprecipitated Raf-1 from phorbol ester-stimulated cells activated MEK *in vitro* (Howe, *et al.*, 1992). Activation was shown to be due to phosphorylation of MEK by Raf-1 on two serine residues between subdomains VII and VIII of the kinase region (Huang, *et al.*, 1993; Alessi, *et al.*, 1994).

The activation of Raf-1 itself is not so well defined. (Moodie, *et al.*, 1993) were looking for proteins which bound to immobilised Ras and they identified Raf-1 and MEK which bound to Ras in a GTP-dependent manner. Similarly (Koide, *et al.*, 1993) added Ras.GTP to cell lysates and co-immunoprecipitated Raf-1. Because Raf-1 was localised to the plasma membrane in cells with active Ras, Leevers, *et al.* (1994) and Stokoe, *et al.* (1994) put a membrane localisation signal on Raf-1 thus bypassing the requirement for Ras. Membrane associated Raf-1 was activated independently of Ras,

but could also be stimulated further by treating the cells with EGF (Leevers, *et al.*, 1994). Thus active Ras functions by bringing Ras to the plasma membrane where other event(s) take place.

These activation event(s) appear to be phosphorylation of Raf. Morrison, *et al.* (1988) observed that in mitogen treated cells Raf-1 was phosphorylated on serine and threonine residues, and also on tyrosine residues in v-src transformed cells or after stimulation by PDGF. A more detailed analysis revealed that Ser 43, Ser 259 and Ser 621 were the major sites of serine phosphorylation in PDGF stimulated cells (Morrison, *et al.*, 1993) and phosphorylation of Tyr 340 and Tyr 341 in response to src, lck and the activated PDGF receptor were important for activity (Fabian, *et al.*, 1993; Marais, *et al.*, 1995). An *in vitro* study with plasma membranes from serum stimulated, v-ras or v-src transformed cells also showed that there was ras-dependent and ATP-dependent step(s) for Raf-1 activation (Dent and Sturgill, 1994).

PKC has been suggested to be the activator of Raf-1. Co-expression of cPKCs and Raf-1 in insect cells resulted in the phosphorylation and activation of Raf-1 (Sozeri, *et al.*, 1992). In NIH 3T3 cells PKCα induced the phosphorylation of Raf-1 on Ser 499. This serine was essential for activation by PKCα but not activation by Ras and the tyrosine kinase, lck (Kolch, *et al.*, 1993). Similarly Carroll and May (1994) showed PKC-mediated phosphorylation of Ser 497 and Ser 619. The relevance of the Ser 497/499 phosphorylation is unclear because these residues are not phosphorylated in PDGF stimulated cells (Morrison, *et al.*, 1993). Raf-1 activation could be dependent on tyrosine phosphorylation, serine phosphorylation or both depending upon the mitogenic stimulation. Morrison, *et al.* (1988) was unable to detect tyrosine phosphorylation of Raf-1 from cells treated with phorbol ester, EGF, FGF or H-ras.

It has been suggested there are two mechanisms of activation of Raf-1. One is due to membrane association and another due to mitogenic stimulation (Leevers, *et al.*, 1994) or the tyrosine kinase v-src (Marais, *et al.*, 1995). Consistent with this is the suggestion that lipids play a role in regulating Raf-1 activity (Cai, *et al.*, 1993; Ghosh, *et al.*, 1996).

Other activators of Raf-1 could be the 14-3-3 proteins. These are a family, α-η, of proteins that were originally isolated by systematic analysis of brain proteins. Subsequently they have been shown to exist as dimers and are thought to be regulators in signal transduction or phosphorylation mechanisms (Aitken, *et al.*, 1992). Using yeast two hybrid screens or co-immunoprecipitation, 14-3-3β and 14-3-3ζ were identified as Raf-1 binding proteins which also increased Raf-1 activity (Aitken, 1995; Burbelo and Hall, 1995). The crystal structure reveals that 14-3-3 are right angled structures and they dimerise to form a clamp or a channel. The amino-terminal helices of the two subunits contact one another and form the floor of the channel, while the carboxy-terminal helices form the walls. It is suggested that because

heterodimers form 14-3-3 can act as a scaffold to bring signalling molecules together (Bax and Jhoti, 1995). In this context it is interesting that 14-3-3 have been shown to regulate PKC activity (Aitken, *et al.*, 1992; Aitken, 1995) and to bind to PI3K 110kDa catalytic subunit (14-3-3τ) (Bonnefoy-Berard, *et al.*, 1995) and Bcr (14-3-3β) (Braselmann and McCormick, 1995). Because Raf-1 does not interact with Bcr itself but does in the presence of 14-3-3, these investigators propose that 14-3-3 acts as a linker or scaffold. Consistent with this (Luo, *et al.*, 1995) describe that truncation of the 14-3-3 amino terminal domain does not prevent 14-3-3 interaction with Raf-1 but there is reduced dimerisation of 14-3-3, and Raf-1 bound to these monomeric C-terminal domains is inactive.

Thus there appears to be multiple mechanisms of Raf-1 activation, and this could reflect the variety of factors that signal through Raf-1.

There are three characterised mammalian Raf proteins, c-Raf or Raf-1, A-Raf and B-Raf. They share three highly conserved regions (CR1-3) surrounded by variable sequences, CR1 and CR2 play a regulatory role and CR3 contains the catalytic kinase domain (Daum, *et al.*, 1994). CR1 consists of the binding domain for Ras and a cysteine rich region with a zinc finger motif. CR2 is rich in serine and threonine residues some of which are regulatory phosphorylation sites but these sites differ between the isoforms and can result in different regulatory mechanisms. For example in PC12 cells TPA transiently activates Raf-1 which is partially PKC-dependent, but gives a prolonged activation of A-Raf which is completely PKC-dependent (Bogoyevitch, *et al.*, 1995; Erhardt, *et al.*, 1995). Also it is well documented that there are Raf-1 independent mechanisms of ERK activation (Chao, *et al.*, 1994; Vaillancourt, *et al.*, 1994; Zheng, *et al.*, 1994; Erhardt, *et al.*, 1995; Faure and Bourne, 1995) There are other MEK activators which are not yet fully characterised. These include a 40-50 kDa MEK activator in NIH 3T3 cells which is stimulated by PDB, thrombin and PDGF (Reuter, *et al.*, 1995) and a 45-50 kDa MEK activator in PC12 cells after NGF stimulation (Pang, *et al.*, 1995).

1.3.8.5. RAS

The viral oncogenes of certain acute transforming retroviruses were the first ras genes to be identified. There are three functional ras genes: H-ras (v-H-ras of Harvey murine sarcoma virus), K-ras (of v-K-ras of Kirsten murine sarcoma virus) and N-ras (transforming gene of neuroblastoma cell line), reviewed by Lowy and Willumsen (1993). Ras undergoes a series of postranslational modifications including farnesylation and C-terminal methylation resulting in association with the inner face of the plasma membrane. Mutant proteins with alterations in this residue are cytosolic and transformation defective.

$p21^{ras}$ is a low molecular weight monomeric GTP binding protein. Similar to the Gα subunit of heterotrimeric G proteins, Ras is inactive when GDP is bound and is activated upon the binding of GTP. Ras can be activated in two ways:

1. The intrinsic rate of GDP dissociation is very low and this rate can be increased by guanine nucleotide exchange factors. This allows the more abundant GTP to bind and activate Ras. Examples of these proteins are the drosophila son of sevenless (SOS) and its mammalian counterpart (mSOS).

2. Ras possesses intrinsic GTPase activity that limits the life time of the active Ras.GTP species and this can be activated by GTPase activating proteins, such as p120ras-GAP, and neurofibromin 1. These proteins are therefore negative regulators of Ras and so Ras can be activated by the inhibition of GAPs (Lowy and Willumsen, 1993).

A point mutation at position 17 for asparagine (N17ras) results in a lower affinity for GTP. This Ras mutant acts in a dominant negative fashion and is often used to inhibit endogenous Ras activity by sequestering upstream regulatory proteins (Feig and Cooper, 1988).

Several groups showed that stimulating various cell types with PDGF or EGF or introducing v-src all increased the amount of GTP bound to Ras (Gibb, *et al.*, 1990; Satoh, *et al.*, 1990a; Satoh, *et al.*, 1990b). Although receptor tyrosine kinases bind and phosphorylate GAP (Molloy, *et al.*, 1989) they do not change GAP activity (Zhang, *et al.*, 1992; Buday and Downward, 1993b). Instead in EGF-stimulated cells there is an increase in the nucleotide exchange rate (Buday and Downward, 1993b; Medema, *et al.*, 1993). The clue for the mechanism of activation of Ras in fibroblasts by growth factors came from genetic analysis of eye development in *Drosophila melanogaster* and vulval development in *Caenorhabditis elegans.*

Son of Sevenless (Sos), a GEF that activated Ras, was identified downstream from the drosophila receptor tyrosine kinase, Sevenless (Simon, *et al.*, 1991; Bonfini, *et al.*, 1992). Then Lowenstein, *et al.* (1992), looking for EGF receptor binding proteins, isolated an adaptor molecule of 23 kDa which consisted of SH3-SH2-SH3 and called it growth factor receptor bound protein 2 (Grb2). Grb2 bound to specific phosphotyrosine residues of activated PDGF and EGF receptors via its SH2 domain but it was not tyrosine phosphorylated itself. By analogy with the structurally similar Sem-5 from *C. elegans*, Grb2 was shown to potentiate Ras-induced DNA synthesis upon co-microinjection and mutants of Grb2 or isolated Grb2 domains abolished Ras induced DNA synthesis. This showed that intact Grb2 was important for Ras function (Lowenstein, *et al.*, 1992).

Another adaptor molecule, Shc (src-homology and collagen), has a C-terminal SH2 domain and an adjacent proline rich motif with regions of homology with

collagen. Shc is tyrosine phosphorylated upon cellular stimulation by EGF (Pelicci, *et al.*, 1992), or transformation by v-src (McGlade, *et al.*, 1992). Co-immunoprecipitation showed that Grb2, via its SH2 domain, binds to tyrosine phosphorylated Shc. Overexpression of Shc is transforming in fibroblasts and induces Ras-dependent neurite outgrowth in PC12 cells. This suggested that a Shc/Grb2 complex regulates Ras activity (Rozakis-Adcock, *et al.*, 1992).

Grb2 was then shown to bind constitutively to Sos, or its mammalian counterpart mSos, by direct interactions between both SH3 domains of Grb2 and the proline-rich regions in Sos (Buday and Downward, 1993a; Chardin, *et al.*, 1993; Egan, *et al.*, 1993; Gale, *et al.*, 1993; Li, *et al.*, 1993; Rozakis-Adcock, *et al.*, 1993). In addition, upon growth factor stimulation, the GEF activity of Sos does not change but there is a translocation from the cytosol to the membrane (Buday and Downward, 1993a).

Thus, in the resting state the Grb2/mSos complex is present in the cytosol. Upon activation, growth factor receptors become tyrosine phosphorylated and this creates a specific binding site for Grb2. Grb2 associates with the receptor at the membrane creating a receptor/Grb2/mSos complex. In this manner, mSos is translocated to the membrane where its substrate, Ras, is localised (McCormick, 1993). In addition the tyrosine phosphorylation of Shc by the activated EGF receptor could also act as a docking site for Grb2/mSos and cause the activation of Ras (Egan, *et al.*, 1993; Rozakis-Adcock, *et al.*, 1993). This is summarised in figure 1.10.

1.3.8.6. EFFECTS OF MAPK

The activation of MAPK has pleiotrophic effects. Upon activation, ERK1 and ERK2 (but not MEK) enter the nucleus within 5 min (Chen, *et al.*, 1992; Lenormand, *et al.*, 1993). Substrates for ERK phosphorylation are transcription factors and nuclear proteins (Elk-1); upstream components of the MAPK pathway for feed back regulation such as the EGF receptor, SOS, Raf-1, MEK, and downstream kinases such as RSK and MAPKAP kinase 2; cytoskeletal elements such as the microtubule associated proteins (MAPs); and other signal transduction molecules such as PLA_2 (Malarkey, *et al.*, 1995).

1.3.8.7. ACTIVATION OF THE MAPK PATHWAY BY G PROTEIN-COUPLED RECEPTORS

The MAPK pathway described above was elucidated for growth factors which signal through receptor tyrosine kinases such as EGF, PDGF, NGF and insulin, however ligands which stimulate seven transmembrane receptors also activate MAPK. Tyrosine phosphorylation of 40 - 45 kDa bands were first reported for thrombin (Hunter, *et al.*, 1985) and MAPK activity was shown for bombesin (Takuwa, *et al.*, 1991a) followed by endothelin (Wang, *et al.*, 1992), bradykinin (Ahn, *et al.*, 1992) and angiotensin (Duff, *et al.*, 1992; Ishida, *et al.*, 1992). Receptors coupled to G_i, G_s and G_q have been shown to

stimulate MAPK activity but the mechanism of activation differs between the classes. Mechanisms of MAPK activation shall be described for G_i and G_q-coupled receptors.

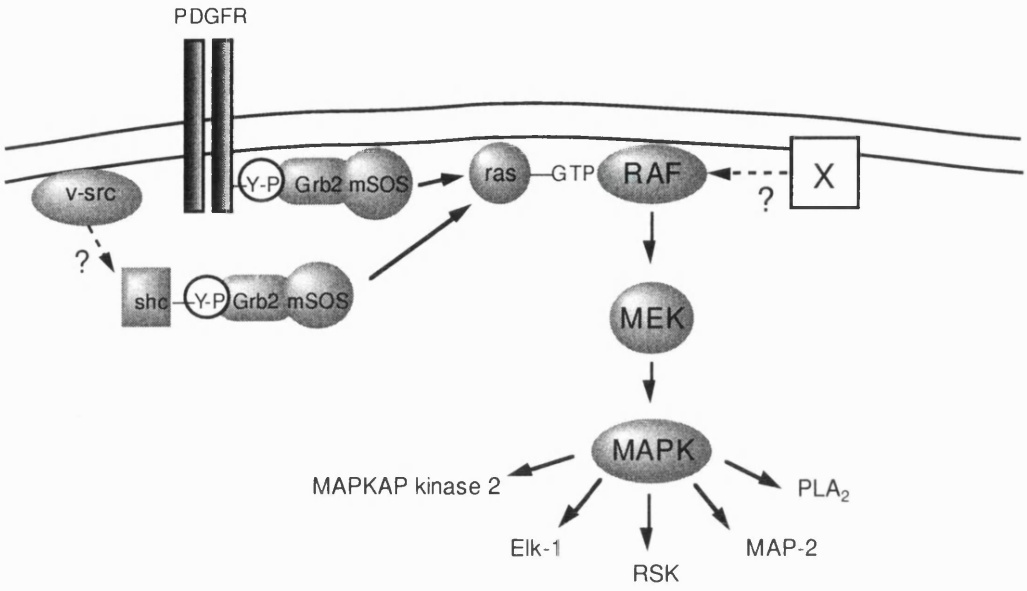

Figure 1.10. Receptor tyrosine kinase activation of the MAPK pathway

A kinase cascade is initiated by growth factor activating their receptor tyrosine kinase or in cells transformed by tyrosine kinases such as v-src. GTP binds to ras which causes the association with Raf-1 at the plasma membrane where Raf-1 is activated by an unknown mechanism. Raf-1 phosphorylates and activates MEK which phosphorylates and activates MAPK.

Receptors coupled to G_i

M_2-Ach-Ach and α_1-AR receptors were shown to stimulate Raf-1, MEK and MAPK activity (Alblas, *et al.*, 1993; Winitz, *et al.*, 1993), and LPA and thrombin were shown to increase the proportion of GTP bound to Ras (Van Corven, *et al.*, 1993). In addition, thrombin-stimulated mitogenesis was blocked by microinjection of N17ras or anti-ras antibodies (LaMorte, *et al.*, 1993b). Activation of Ras, Raf-1 and MAPK were all shown to be pertussis toxin-sensitive consistent with G_i-mediated signal transduction pathways.

However, this was not mediated by $G\alpha_i$ as overexpression of activated mutants did not significantly enhance MAPK activity, instead overexpression of β and γ subunits together increased MAPK activity (Crespo, *et al.*, 1994b; Faure, *et al.*, 1994). Also endogenous $\beta\gamma$ could be sequestered by introduction of $G\alpha_t$ or the PH domain of βARK and this inhibited $\beta\gamma$ and agonist stimulation of MAPK (Crespo, *et al.*, 1994b;

Faure, *et al.*, 1994; Koch, *et al.*, 1994). N17ras inhibited agonist- and βγ-stimulated MAPK activity (Crespo, *et al.*, 1994b) and agonist-stimulated Ras.GTP loading was inhibited by βARK (Koch, *et al.*, 1994). It can be concluded that receptors which couple to G_i activate the MAPK pathway via βγ-mediated ras activation, followed by activation of Raf-1, MEK and MAPK. Thus G_i coupled receptors use the same signalling pathways as receptor tyrosine kinases. This extends to the activation of Ras, disruption of the Grb2/Sos complex with truncated versions of Sos without GEF activity prevented agonist and βγ stimulated MAPK activity (van Biesen, *et al.*, 1995). It was also shown that βγ subunits caused the phosphorylation of Shc which co-immunoprecipitated with Grb2 and mSos (Touhara, *et al.*, 1995; van Biesen, *et al.*, 1995). Thus Shc may represent the link between βγ activation of a tyrosine kinase and Ras activation. This is summarised in figure 1.11.

Receptors coupled to G_q

Receptors coupled to G_q do not generally activate the MAPK pathway through stimulation of Ras. Bombesin, endothelin and M_1-Ach do not cause GTP loading of Ras in Rat-1 and Swiss 3T3 cells (Satoh, *et al.*, 1990b; Van Corven, *et al.*, 1993; Winitz, *et al.*, 1993) and α_1-AR-stimulated MAPK is not inhibited by N17ras (Hawes, *et al.*, 1995).

Activation of the Raf-1 isoform is unclear: stimulation of CCK_B and M_1-Ach receptors activate Raf-1 in Rat-1 and COS 7 cells (Crespo, *et al.*, 1994a; Seufferlein, *et al.*, 1995) and α_1-AR-stimulated MAPK is inhibited in COS 7 by a dominant negative Raf-1 (Hawes, *et al.*, 1995). In contrast, it has been shown that M_1-Ach does not stimulate Raf-1 in Rat-1 cells (Winitz, *et al.*, 1993; Russell, *et al.*, 1994) and bombesin does not stimulate Raf-1 activity in Swiss 3T3 cells (Mitchell, *et al.*, 1995; Seufferlein, *et al.*, 1996).

Activation of seven transmembrane receptors that are coupled to G_q leads to stimulation of PKC activity (see section 1.3.3). Activation of MAPK is largely PKC-dependent for a variety of agonists such as bombesin in Swiss 3T3 cells (Pang, *et al.*, 1993; Seufferlein, *et al.*, 1996), α_1-AR in COS 7 (Hawes, *et al.*, 1995), M_1-Ach in NIH 3T3 cells (Crespo, *et al.*, 1994a), endothelin in mesangial cells (Wang, *et al.*, 1992), and M_1-Ach in COS 7 (Crespo, *et al.*, 1994b).

Transfection of activated α_q subunits into COS 7 cells activates MAPK (Faure, *et al.*, 1994). Consistent with this is the demonstration that α_1-AR stimulated MAPK is not inhibited by the βARK PH domain (Koch, *et al.*, 1994; Hawes, *et al.*, 1995) and bombesin-stimulated MAPK is not inhibited by $G\alpha_t$ (Faure, *et al.*, 1994).

Thus G_q-coupled receptors activate MAPK by an α_q- and PKC-dependent but βγ- and Ras-independent pathway (Hawes, *et al.*, 1995) and see figure 1.11, although it is unclear whether the MAPK kinase kinase involved is Raf-1.

The mechanism by which PKC activates the MAPK pathway is still unclear. In section 1.3.8.4 it was described that phorbol esters cause the activation of Raf-1 (Morrison, *et al.*, 1988; Howe, *et al.*, 1992; Kyriakis, *et al.*, 1993) and PKC may activate Raf-1 directly (Sozeri, *et al.*, 1992; Kolch, *et al.*, 1993; Carroll and May, 1994). Studies with dominant negative Raf have showed that Raf-1 is necessary for phorbol ester-stimulated MAPK activity and/or cell proliferation (Kolch, *et al.*, 1991; Troppmair, *et al.*, 1994). Therefore, in general, PKC can activate Raf-1 but there are exceptions, Chao, *et al.* (1994) showed that PKC activation of MAPK is not dependent on Raf-1. However there are multiple enzymes that function at the level of Raf-1. These are summarised in figure 1.12.

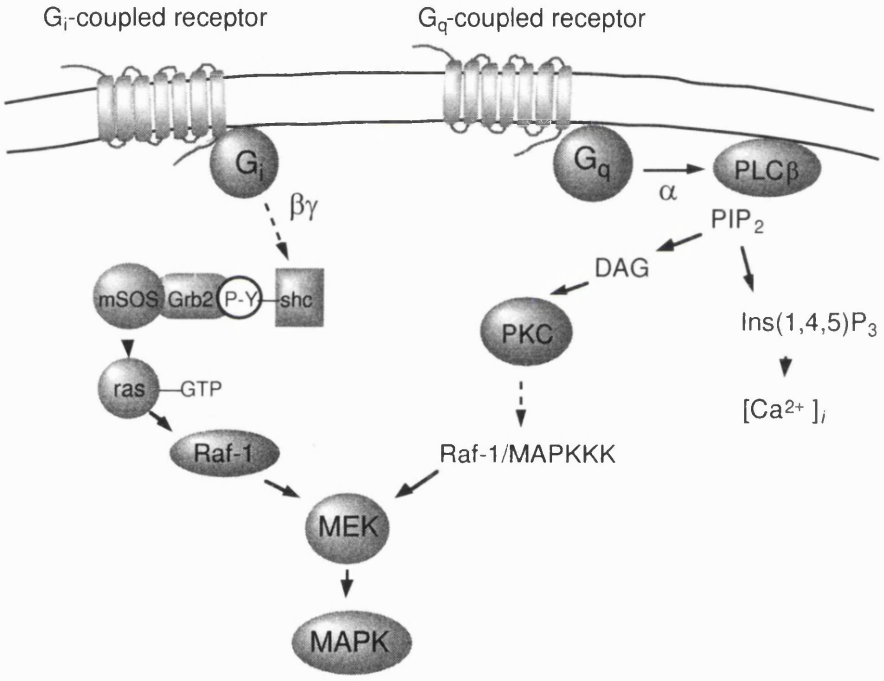

Figure 1.11. Mechanism of MAPK activation by G protein coupled receptors

1.3.8.8. BOMBESIN-INDUCED MAPK ACTIVATION

The mechanism for MAPK activation by bombesin in Swiss 3T3 cells is consistent with the model for general G_q-coupled receptor activation. Addition of bombesin to Swiss 3T3 cells induces a rapid activation of MAPK that is maximum at 5 min and then declines to a plateau of a low level of MAPK activity which persists for several hours (Withers, *et al.*, 1995). After prolonged treatment with phorbol ester to down regulate phorbol ester-sensitive isoforms, bombesin-induced MAPK is abolished

showing complete dependence on PKC (Pang, *et al.*, 1993; Seufferlein, *et al.*, 1996). Bombesin does not increase Ras.GTP loading in Swiss 3T3 cells (Satoh, *et al.*, 1990b; Mitchell, *et al.*, 1995), nor does it activate Raf-1 (Mitchell, *et al.*, 1995; Seufferlein, *et al.*, 1996). Thus bombesin activates MAPK via a PKC-dependent pathway. Consistent with this is the observation that in COS 7 cells bombesin-stimulated MAPK was not dependent on $\beta\gamma$ subunits (Faure, *et al.*, 1994).

1.3.8.9. CELLULAR CONTEXT

Some studies indicate that Ras could be involved in MAPK activation by G_q-coupled receptors. Activation of the M_1-Ach, 5-HT_{2B} or α_{1B}-AR receptor stimulates Ras.GTP loading (Koch, *et al.*, 1994; Mattingley, *et al.*, 1994; Launay, *et al.*, 1996) and addition of cholecystokinin to rat pancreatic acinar cells activates Ras (Duan, *et al.*, 1995). Similarly, M_1-Ach-stimulated MAPK is inhibited by N17ras and $G\alpha_t$ (Crespo, *et al.*, 1994b). MAPK activation mediated by stimulation of the CCK_B receptor is PKC-independent in Rat-1 cells (Seufferlein, *et al.*, 1995). This could describe a pathway that is PKC-independent and $\beta\gamma$- and Ras-dependent (Crespo, *et al.*, 1994a; Crespo, *et al.*, 1994b).

These differences can be attributed to several factors such as receptor identity and experimental procedure. However an important aspect is the cellular context in which the MAPK pathway is activated. A study by Faure and Bourne (1995) demonstrates the variability in activating the MAPK cascade in three cell types, Swiss 3T3, COS-7 and Rat-1. These investigators show that EGF and LPA, which signal via receptor tyrosine kinase and G_i-coupled receptor respectively, proportionally activate Raf-1, MEK and $p42^{mapk}$ to different extents between the three cell types. Also the effect of elevating cellular levels of cAMP inhibited these kinases to different levels such that MAPK and MEK were inhibited in Rat-1 cells but not in COS-7 or Swiss 3T3 cells.

Another study compared cellular context of G_q-coupled receptors by use of the M_1-Ach receptor in COS-7 cells versus CHO cells (van Biesen, *et al.*, 1996). It was demonstrated that in COS-7 cells M_1-Ach receptors activate MAPK via a PKC-dependent and $\beta\gamma$- and Ras-independent pathway that was pertussis toxin-insensitive whereas in CHO cells M_1-Ach activation of MAPK was sensitive to this toxin. This was attributed to M_1-Ach coupling to $G\alpha_o$ in CHO cells. Interestingly, the α_1-AR retains COS-7-like G_q coupling characteristics in CHO cells. To conclude, the mechanism by which G_q-linked receptors activate MAPK can vary according to receptor identity and cellular context.

1.3.8.10. FUNCTION OF Gβγ

Activation of tyrosine kinases

G protein-coupled receptor activation of Ras requires the Grb2/mSos interaction (van Biesen, *et al.*, 1995). Also, βγ subunits (Touhara, *et al.*, 1995; van Biesen, *et al.*, 1995) and agonists for the receptors LPA, α_{2A}-AR, angiotensin II, thrombin and gastrin (van Biesen, *et al.*, 1995; Chen, *et al.*, 1996; Sadoshima and Izumo, 1996; Seva, *et al.*, 1996) increase the phosphorylation of Shc on tyrosine. The model proposed is that Grb2 binds to the phosphotyrosine residue of Shc to bring mSos in contact with Ras (Touhara, *et al.*, 1995; van Biesen, *et al.*, 1995).

So what tyrosine kinases do βγ activate? It has been shown that βγ together with another membrane component activate the tyrosine kinases Btk and Tsk (Langhans-Rajasekaran, *et al.*, 1995) both of which contain PH domains. Btk and Tsk are found in B and myeloid cells and T cells respectively but there may be an analogous situation in fibroblasts. The possibility that Raf-1 or Shc were substrates of Tsk or Btk was not tested (Langhans-Rajasekaran, *et al.*, 1995). In the pheromone signalling pathway of *Saccharomyces cerevisiae* a serine kinase, Ste20p, has been identified to be activated by βγ (Wu, *et al.*, 1995a).

Other tyrosine kinases could be the EGF receptor or Src. Kinase competent EGF receptor chains were necessary for the activation of MAPK by thrombin, endothelin and LPA although βγ subunits were not tested on this model (Daub, *et al.*, 1996). Thrombin and angiotensin activate src family kinases (Chen, *et al.*, 1994; Sadoshima and Izumo, 1996) although activation of Src by βγ has not been demonstrated.

Recently βγ have been shown to activate novel isoforms of PI3K in platelets, neutrophils and U937 cells that are distinct from the type that is activated by receptor tyrosine kinases (Stephens, *et al.*, 1994; Thomason, *et al.*, 1994; Stoyanov, *et al.*, 1995) and βγ stimulation of Shc phosphorylation is inhibited by the PI3K inhibitor wortmannin (Touhara, *et al.*, 1995), although these investigators do point out that PI3K can associate with Src family kinases independently of agonist stimulation and the effect of wortmannin may be disrupting Src activity. This would imply that Src is involved in βγ-mediated Shc phosphorylation as suggested above. Hu, *et al.* (1996) also shows α_1-AR activation of PI3K and wortmannin inhibition of Ras.GTP loading. Similarly wortmannin inhibits LPA and α_2-AR stimulated Ras.GTP loading (Hawes, *et al.*, 1996). These observations have parallels to Ras activation described by Valius and Kazlauskas (1993). These investigators demonstrated that PDGF receptor mutants that only had the tyrosine residue specific for binding the p85 regulatory subunit of PI3K, but not Grb2, were able to activate Ras and induce DNA synthesis.

Construction of multimeric complexes

It was described in section 1.1.6.4 that a function of the β subunit could be to build multiprotein complexes. βγ appears to localise the tyrosine kinases Btk and Tsk to the membrane (Langhans-Rajasekaran, *et al.*, 1995) and initiate the formation of the Shc/Grb2/Sos complex by stimulating the tyrosine phosphorylation of Shc.

Raf is complexed with 14-3-3 and Bcr (Braselmann and McCormick, 1995) the latter of which has a PH domain and therefore could interact with βγ. Section 1.3.8.4 describes how 14-3-3 proteins could be involved in activation of Raf-1 by acting as linkers between signal transduction molecules. Thus 14-3-3 and/or βγ may contribute to building the complex required for Raf-1 activation at the membrane. Consistent with this is the demonstration that βγ are found in complexes with Ras, Raf and MEK (Wang and Durkin, 1995) and shown to bind Raf-1 (Pumiglia, *et al.*, 1995).

In conclusion G protein βγ subunits are implicated in many protein-protein interactions but further work is needed to clarify their role in activation of Ras and the MAPK pathway.

1.3.8.11. STRESS-ACTIVATED PROTEIN KINASES

The MAPK pathway described above is activated by mitogens, however there are other MAPK subtypes which are activated by homologous pathways in response to stress (Cano and Mahadevan, 1995; Cobb and Goldsmith, 1995). The first of these alternative cascades culminates in the activation of MAPKs called JNK (c-Jun N-terminal kinase) or SAPK (stress-activated protein kinase) and these are generally activated in response to UV irradiation and protein synthesis inhibitors. The other cascade culminates in the activation of p38/RK which shows the highest homology to yeast high-osmolarity glycerol response 1 (HOG1) suggesting it plays a part in mammalian osmotic sensors. The p38 cascade is also activated by heat shock. These cascades have their respective MAPK kinases and MAPK kinase kinases to make distinct MAPK modules although there does appear to be cross talk between the pathways at the level of MAPKKK (Cano and Mahadevan, 1995; Cobb and Goldsmith, 1995). These are summarised in figure 1.12.

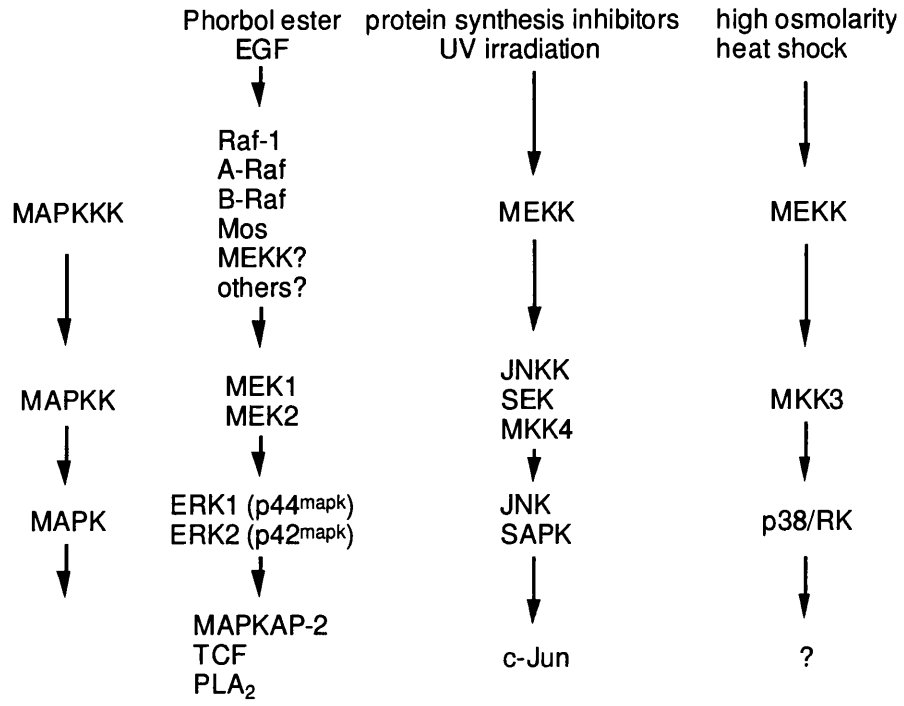

Figure 1.12. Multiple mammalian MAPK modules

1.3.9. INDUCTION OF EARLY GENES

In addition to the events in the membrane and cytosol described above, bombesin rapidly and transiently induces the expression of the cellular oncogenes c-*fos* and c-*myc* in quiescent fibroblasts (Palumbo, *et al.*, 1986; Rozengurt and Sinnett-Smith, 1987). Since these cellular oncogenes encode nuclear proteins, it is plausible that their transient expression may play a role in the transduction of the mitogenic signal to the nucleus (Lewin, 1991). The demonstration that the product of the proto-oncogene c-*jun*, identified as a major component of the transacting factor AP-1, forms a tight complex with Fos protein is consistent with a role of c-*fos* in the regulation of gene transcription (Lewin, 1991).

1.3.9.1. AP-1

Activator protein-1 (AP-1) was first identified as a transcription factor that binds to an essential cis element of the human metallothionein II_A promoter (Angel and Karin, 1991). The binding site for AP-1 was recognised as the TPA response element (TRE) of several cellular genes including collagenase, stromelysin and interleukin-2 whose transcription is induced after 8 - 12 hours in response to treatment of cultures cells with phorbol ester. AP-1 consists of Jun proteins (c-Jun, JunB and JunD) and Fos proteins (c-Fos, FosB, Fra1 and Fra2). Jun proteins can form

homo- and heterodimers whereas Fos proteins do not associate with each other. As dimerisation is a prerequisite for DNA binding, Fos does not stimulate transcription in the absence of Jun. However, Fos proteins can associate with any of the Jun proteins to generate stable heterodimers that have higher DNA binding activity than Jun homodimers. As well as being responsive to PKC, activation of the AP-1 complex serves as a nuclear target for signalling pathways elicited by mitogenic stimulation or acute expression of various oncoproteins such as Src, H-ras, Raf or Mos (Angel and Karin, 1991).

1.3.9.2. INDUCTION OF AP-1 COMPONENTS

c-*fos*

c-*fos* transcription is increased within minutes in response to various stimuli such as serum, EGF, PDGF, phorbol ester, cAMP, neurotransmitters, TSH and Ca^{2+} ionophore (Curran, *et al.*, 1985; Angel and Karin, 1991). c-*fos* is under the control of several upstream enhancer elements, the *sis* inducible element (SIE) at -345, the serum response element (SRE) at -300, and the cAMP response element (CRE) at -60 (see figure 1.13) (Janknecht, *et al.*, 1995). The SIE is responsive to cytokines and some growth factors such as PDGF (v-sis), but this shall not be described here. The SRE mediates serum induction of c-*fos* and also that of individual growth factors such as PDGF, EGF and phorbol ester. The SRE is recognised by the serum response factor (SRF) whose binding results in the recruitment of a ternary complex factor (TCF), which could be Elk-1 or SAP-1, which cannot bind to the SRE by itself. Following mitogenic stimulation, Elk-1 is rapidly phosphorylated by the ERK class of MAPKs which increase its transcriptional activity (Karin, 1995).

The CRE represents a binding site for CREB and related transcription factors. CREB is a well known substrate for PKA and phosphorylation increases its transactivation activity. Also CREB is shown to be phosphorylated by CaMKs activated by an increase in Ca^{2+} (Janknecht, *et al.*, 1995).

It is unclear *in vivo* whether these elements act independently because signals which activate the SIE and CRE also activate the SRE. In Swiss 3T3 cells elevation of cAMP is a mitogenic signal but does not induces c-*fos*, similarly treatment with Ca^{2+} ionophores does not induce this proto-oncogene. However both cAMP and Ca^{2+} elevation can synergise with PKC activation to induce c-*fos* expression (Mehmet and Rozengurt, 1991).

c-*jun*

Unlike c-Fos, cells have a higher basal levels of c-Jun. Also the induction of c-Jun is not as transient as that of c-Fos (Angel and Karin, 1991). The c-*jun* promoter is simpler than the c-*fos* promoter in that it contains just one major cis acting element

which is a modified TRE. c-Jun binds to this promoter upon dimerisation with ATF2 and this complex is activated when c-Jun and ATF2 becomes phosphorylated by active JNK (Karin, 1995).

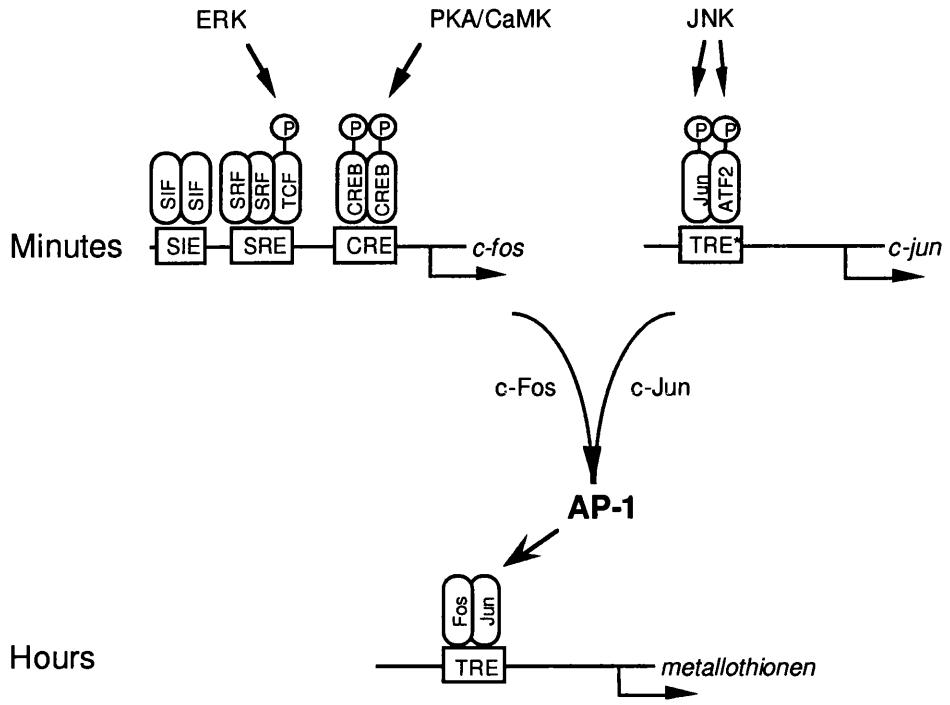

Figure 1.13. Activation of AP-1 transcription factor

The MAPK pathway culminates in ERK-dependent phosphorylation of Elk-1 (TCF) which activates transcription of the *c-fos* gene. The JNK pathway phosphorylates and activates pre-existing c-Jun and ATF2 which induce transcription of c-*jun*. c-Fos then dimerises with c-Jun to form the AP-1 complex which activates the transcription of early response genes. (TRE*, modified TRE)

There is evidence implicating PKC activation in the sequence of events linking bombesin receptor occupancy and proto-oncogene induction (Rozengurt and Sinnett-Smith, 1988b). Accordingly, bombesin-induced oncogene expression is markedly reduced by down regulation of PKC. PKC activation leads to the activation of MAPK (see section 1.3.8) which directly phosphorylates transcription factors resulting in the increased expression of c-*fos*. However, neither direct activation of PKC by phorbol esters nor addition of vasopressin evoke a maximal increase in c-*fos* mRNA levels. It is likely that the induction of c-*fos* by bombesin is mediated by the coordinated effects of

PKC activation, Ca^{2+} mobilisation and an additional pathway dependent on arachidonic acid release (Rozengurt, 1991a).

1.4. COUPLING TO MULTIPLE PATHWAYS

Signal transduction pathways mediated by the bombesin/GRP receptor is attributed to the G_q-mediated events of PIP_2 hydrolysis, PKC activation and Ca^{2+} mobilisation. However, section 1.3.7.8 describes the evidence that protein tyrosine phosphorylation, stimulated by bombesin, is independent of PKC and Ca^{2+}. The mechanism by which bombesin stimulates tyrosine phosphorylation is unknown particularly events immediately subsequent to receptor stimulation. Milligan (1993) describes three models for the mechanism for signal bifurcation by G protein coupled receptors:

Model 1 Although pharmacologically currently indistinguishable, multiple receptor subtypes are co-expressed.

Model 2 A single receptor activates multiple G proteins, each of which is able to regulate a separate effector.

Model 3 A single receptor interacts with a single G protein. Bifurcation of signals is subsequently produced by the regulation of different effectors by the G protein α and $\beta\gamma$ subunits.

In the context of bombesin-induced tyrosine phosphorylation the first model has been proposed several times. Cirillo, *et al.* (1986) observed that bombesin stimulated the tyrosine phosphorylation of a protein of 115 kDa. This was attributed to the then unknown bombesin receptor being a novel receptor tyrosine kinase. The interpretation of these results is now judged to be incorrect and it has been convincingly demonstrated that the bombesin/GRP receptor has no phosphotyrosine activity (Zachary, *et al.*, 1991b). Recently Bold, *et al.* (1994) reported that bombesin antagonists which inhibited cytoplasmic Ca^{2+} increases did not block cell proliferation and bombesin agonists which did increase Ca^{2+} did not stimulate cell growth, however bombesin-induced cell proliferation was inhibited by a tyrosine kinase inhibitor, tyrophostin. They concluded that one bombesin receptor couples to Ca^{2+} mobilisation and a distinct receptor couples to tyrosine phosphorylation and cell growth, see figure 1.14. However, no direct evidence was presented in support of this.

Recently a new bombesin-like receptor, BRS-3 has been identified by molecular biology techniques (Gorbulev, *et al.*, 1992; Fathi, *et al.*, 1993) although the physiological ligand is currently unknown. This raises the possibility that there could be other unidentified bombesin receptors.

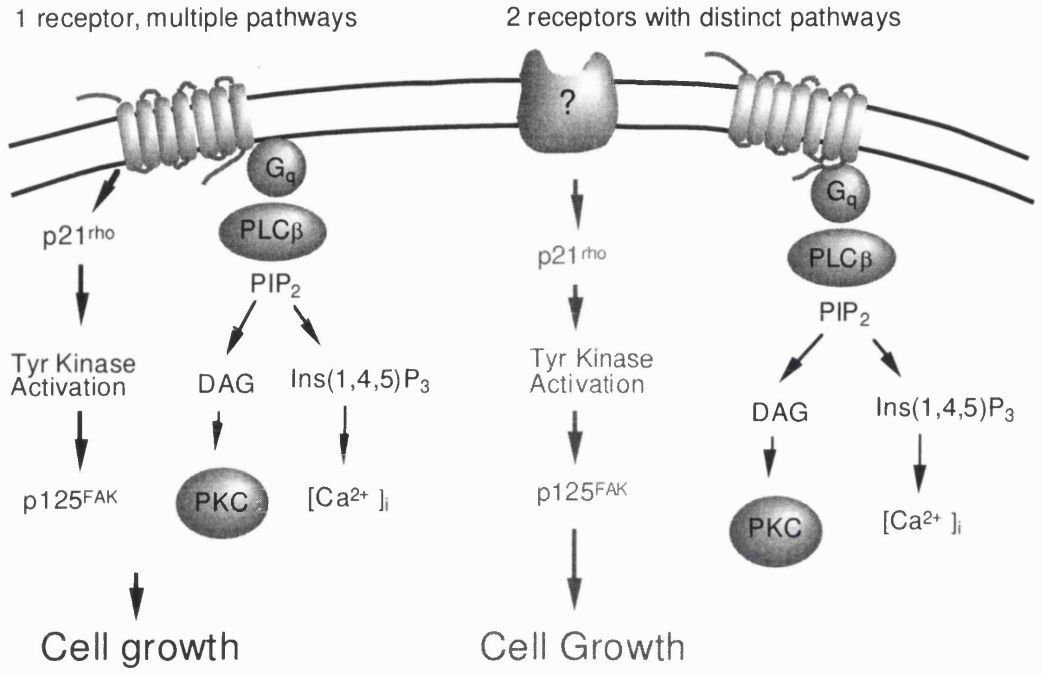

Figure 1.14. Possible mechanisms of multiple coupling for bombesin receptors

Two alternatives are shown. One receptor could couple to all the pathways or there could be two receptors.

Ligands such as thrombin and LPA have also been shown to induce multiple signalling pathways, including protein tyrosine phosphorylation and reinitiation of DNA synthesis. They have in common signals which are transduced by G_q and G_i.

Thrombin was first identified as a mitogen in 1975 when Chen was investigating mitogens present in blood and especially in serum which during its production simulates a wound response. Proteases of the blood clotting cascade were known to be present in serum and they could be obtained in a highly purified form. Addition of purified thrombin to chick embryo fibroblasts caused an increase in [^{3}H]-thymidine incorporation, an increase in cell numbers over 4 days and increased cell motility (Chen and Buchanan, 1975).

LPA is the smallest and simplest phospholipid and has a wide range of biological and biochemical actions (Moolenaar, 1994). It is a platelet-derived serum factor which accumulates rapidly in thrombin-stimulated platelets and can be released from growth factor stimulated fibroblasts.

Cloning of the thrombin receptor revealed a seven transmembrane protein with a novel mechanism of action (reviewed in Grand, 1996). The N-terminal is cleaved to expose a "tethered ligand" which is then thought to bind to the receptor in the same fashion as other peptide ligands and in fact a synthetic peptide to this sequence can elicit similar responses to thrombin. These include pertussis toxin-sensitive and -insensitive activation of PLCβ (Murayama and Ui, 1985; Grand, *et al.*, 1996); pertussis toxin-sensitive inhibition of adenylyl cyclase and Ras activation (Van Corven, *et al.*, 1993; Grand, *et al.*, 1996); partially sensitive Src activation (Chen, *et al.*, 1994), and insensitive Shc (Chen, *et al.*, 1996) and FAK tyrosine phosphorylation and reorganisation of the actin cytoskeleton (Jalink and Moolenaar, 1992; Grand, *et al.*, 1996).

A similar set of signals are elicited upon addition of LPA to cells. These are phospholipid hydrolysis and arachidonic acid release which are pertussis toxin insensitive but potentiated by GTPγS, and [^{3}H]-thymidine incorporation and inhibition of cAMP production which are pertussis toxin sensitive (van Corven, *et al.*, 1989). Later it was shown that LPA stimulates the Ras-MAPK pathway in a pertussis toxin-sensitive manner and it also activates Src (Jalink, *et al.*, 1993). In addition LPA stimulates stress fibre assembly and reorganisation of the actin cytoskeleton, and the tyrosine phosphorylation of FAK which are pertussis toxin insensitive (Ridley and Hall, 1992; Jalink, *et al.*, 1993; Hordijk, *et al.*, 1994).

Thus the action of thrombin and LPA involves two G proteins, a pertussis toxin-sensitive $G_{i/o}$ and -insensitive G_q (judged from pertussis toxin-insensitive phospholipid hydrolysis). The identity of the LPA receptor(s) coupling to these G proteins has remained elusive. Using a ^{32}P-labelled LPA analogue containing a photoreactive acyl chain, a putative receptor of 38 - 40 kDa was identified (Van der Bend, *et al.*, 1992), but unambiguous characterisation awaits the molecular cloning of the receptor. There are discrepancies between the actions of thrombin and thrombin receptor peptide and there does appear to be thrombin specific binding sites on the surface of cells which can elicit responses without the need for proteolytic action (Seiler, *et al.*, 1991; Vouret-Craviari, *et al.*, 1993). This has led to the suggestion that there is more than one thrombin receptor (Grand, *et al.*, 1996). In this context it has not been clearly defined by transfection of the known thrombin receptor which pathways are stimulated by thrombin and the effect on cellular proliferation. One study altered the thrombin

cleavage site to that of enterokinase (Hung, *et al.*, 1992) to show that cleavage of this receptor activates coupling to G_q and G_i but this was not extended to cell proliferation and protein tyrosine phosphorylation. Similarly the exact identity of the bombesin receptor which couples to tyrosine phosphorylation has not been precisely demonstrated.

An approach to investigate how these ligands exert their multiple signalling effects and mitogenic capability is to transfect a defined receptor into an unresponsive cell line and examine the signalling pathways elicited. This approach was taken with the bombesin/GRP receptor and the results are presented in Chapter 3.

1.5. AIMS OF THESIS

This thesis will be into three sections.

1.5.1. Bombesin elicits multiple signal transduction pathways including activation of PLC, tyrosine phosphorylation of FAK and paxillin and reinitiates DNA synthesis. In Swiss 3T3 cells tyrosine phosphorylation does not appear to be downstream of PLC-mediated events. It was proposed recently that a separate bombesin receptor is coupled to tyrosine phosphorylation than the one that is coupled to PLC-mediated hydrolysis of PIP_2. The exact identity of the receptor which mediates tyrosine phosphorylation is an unresolved but fundamental issue. Therefore to address this, the bombesin/GRP receptor was transfected into a bombesin-unresponsive fibroblast cell line, Rat-1. The first set of questions posed are:

Does ligand activation of the bombesin/GRP receptor transfected into Rat-1 cells:

a) Stimulate Ca^{2+} mobilisation and 80K phosphorylation?

b) Stimulate DNA synthesis and cell proliferation?

c) Stimulate the tyrosine phosphorylation of multiple proteins including FAK and paxillin?

1.5.2. MAPK activation is common point of convergence of a variety of mitogenic factors. As described in section 1.3.8.7 receptors that couple to G_i activate MAPK via a Ras-dependent pathway. However, receptors that are coupled to G_q appear to activate MAPK via PKC-dependent and independent routes which could also be via activation of Ras and cellular context could be an important contributing factor. It is known that in

Swiss 3T3 cells bombesin activates the MAPK pathway via PKC-dependent routes and does not cause GTP-loading of Ras. In Rat-1 cells stimulation of the CCK_B receptor causes the activation of Raf-1 independently of PKC (Seufferlein, *et al.*, 1995). This could mean that peptide receptors coupled to G_q in Rat-1 cells could activate MAPK via Ras. Therefore, by transfecting the bombesin/GRP receptor into Rat-1 cells this problem can be addressed. The second set of questions are:

a) Does bombesin stimulate MAPK and Raf-1 activity in Rat-1 cells transfected with the bombesin/GRP receptor?
b) Is the activation of the kinases in this cascade dependent on PKC activity?
c) Does bombesin stimulate the GTP loading of Ras?

1.5.3. Bombesin and other mitogenic neuropeptides mobilise Ca^{2+} to cause a transient increase in $[Ca^{2+}]_{cyt}$ followed by a persistent depletion of intracellular stores. However, the role of Ca^{2+} in initiating mitogenesis is subject to controversy. The Ca^{2+}ATPase inhibitors thapsigargin and DBHQ offer a new approach to investigate the role of Ca^{2+} in DNA synthesis. The third set of questions are:

a) What effect do the Ca^{2+}ATPase inhibitors thapsigargin and DBHQ have on DNA synthesis?
b) How are these effects mediated?

Chapter 2 - MATERIALS AND METHODS

2.1. MATERIALS

The cDNA of the murine bombesin/GRP preferring receptor in pCD2-neo (BNR-pCD2-neo) was kindly provided by Dr. Jim Battey, Laboratory of Biological Chemistry, National Cancer Institute, Bethesda, Maryland, U.S.A).

The bombesin/GRP receptor-specific antagonist D-F_5-Phe^6, D-Ala^{11}-Bombesin(6-13)OMe was a kind gift of Dr. David H. Coy of Peptide Research Laboratories, Tulane University Medical Center, New Orleans, Louisiana, USA.

Anti-Tyr(P) mAb clone Py72 and anti-Ras mAb clone 259 was obtained from the hybridoma development unit, Imperial Cancer Research Fund, London, United Kingdom. Py20 anti-Tyr(P) mAb was from ICN, Buckinghamshire, UK. The 4G10 anti-Tyr(P) mAb was from Tissue Culture Supplies, Buckingham, UK. Anti FAK mAb was obtained from AFFINITI Research Products Ltd, Nottingham, UK. Anti-paxillin mAb 165 was from ICN, High Wycombe, UK. The anti-p74$^{raf\text{-}1}$ affinity purified rabbit polyclonal antibody was obtained from Santa Cruz Biotechnology Ltd., USA. p42^{mapk} antisera were generous gifts from Dr. Johan Van Lint of The Katholieke Universiteit Leuven and Dr. Dominic J Withers of Department of Medicine, Hammersmith Hospital, London, UK.

GST-MEK and GST-p42^{mapk} expression vectors were kind gifts from Professor C. Marshall, Chester Beattie Laboratory, Institute for Cancer Research, London, UK

Thapsigargin, GF 109203X and Fura-2/AME were obtained from Calbiochem. DBHQ was obtained from Aldrich. Dispase, Protein A-agarose conjugate and Protein G-Sepharose was obtained from Boehringer Mannheim, Germany. PDB, insulin, bombesin, econozole, GRP, BSA, litorin, NmB, bombesin fragment amino acids 8-14, endothelin, anti-mouse IgG (whole molecule) - agarose were obtained from Sigma. FBS was obtained from Gibco BRL, Life Technologies.

[*methyl*-^{3}H]thymidine (25 Ci/mmol), *myo*-[2-^{3}H]inositol (16 Ci/mmol), $^{32}P_i$ (10 mCi/ml), ^{125}I-labelled sheep anti-mouse IgG (20 μCi/μg), ^{125}I-labelled Protein A (30 mCi/mg) and $^{45}CaCl_2$ (10 - 40 mCi/mg calcium), (3-[^{125}I]iodotyrosyl^{15})Gastrin releasing peptide (2000 Ci/mmole), and [γ-^{32}P]ATP (5000 Ci/mmol) were obtained from Amersham International, U.K.

All other materials were of the purest grade commercially available.

2.2. METHODS

Throughout this section the principle is described first in *italics* followed by the method in plain text.

2.2.1. TRANSFECTION OF RAT-1 CELLS

A 1.4 kb insert containing the complete cDNA coding region of the murine bombesin/GRP preferring receptor cloned into the mammalian expression vector pCD2-neo (BNR-pCD2-neo) and under the transcriptional control of the SV40 early region promoter, was kindly provided by Dr. Jim Battey. For transfection, Rat-1 cells were plated at a density of 1 x 10^6 cells/100 mm dish. The following day, cultures were washed twice in Dulbecco's Modified Eagle's Medium (DMEM) and 20 μg DNA from BNR-pCD2-neo expression vector, mixed with 40 μg lipofectin reagent, was added to the cultures in 5 ml DMEM at 37°C. After 14 h incubation, 5 ml of 20% foetal bovine serum (FBS) in DMEM were added and incubation extended for a further day. Then, cultures were trypsinised, divided into four and selected in DMEM supplemented with 1 mg/ml neomycin derivative G418. The medium was changed every 3 - 4 days. After 2 - 3 weeks, resistant colonies were cloned by ring isolation and propagated in DMEM supplemented with 5% FBS and 0.5 mg/ml G418.

2.2.2. CELL CULTURE

Only early passage cells were used which were replaced every 6 -8 weeks.

2.2.2.1. RAT-1 CELLS

Stock cultures of Rat-1 cells were maintained as described previously (Higgins, *et al.*, 1992) except that transfected cell lines were propagated in the presence of 0.5 mg/ml G418. For experimental purposes, 5 x 10^4 or 3 x 10^5 cells were subcultured in 33 or 100 mm Nunc Petri dishes respectively with DMEM supplemented with 5% FBS and 0.5 mg/ml G418 as necessary and incubated in a humidified atmosphere of 10% CO_2, 90% air at 37°C until they became 50 - 70% confluent (2 - 3 days), then switched down to 0.5% FBS for 3 - 4 days until they were quiescent.

2.2.2.2. SWISS 3T3 CELLS

Stock cultures of Swiss 3T3 cells were propagated as described previously (Rozengurt and Sinnett-Smith, 1983). For experimental purposes, cells were

subcultured in 33- or 100- mm Nunc Petri dishes with DMEM supplemented with 10% FBS and incubated in a humidified atmosphere of 10% CO_2, 90% air at 37°C until they became confluent and quiescent (6 - 8 days).

2.2.3. [^{125}I]GRP BINDING ASSAY

[^{125}I]GRP is incubated with intact cells at 37°C then binding is terminated by washing rapidly with PBS and solubilising the cells as described previously (Zachary and Rozengurt, 1985). Radioactivity associated with the whole cell is measured.

Confluent, quiescent, cultures of Rat-1 cells in 33 mm dishes were washed twice with DMEM and incubated for the indicated times at 37°C in DMEM with a mixture of [^{125}I]GRP and cold GRP, as described in the figure legends, in the presence of 1 mg/ml bovine serum albumin (BSA). For the competition studies various concentrations of competing peptides (bombesin, GRP, litorin, NmB and bombesin fragment 8-14) were also added to the binding medium. Non-specific binding was determined by adding 1 μM bombesin. After the incubation, binding was terminated by placing cultures on ice where they were rapidly washed 4 times with ice cold phosphate buffered saline (PBS) containing 1 mg/ml BSA. Cultures were dissolved in 1 ml of 0.1 N NaOH/2% $NaCO_3$/1% SDS and the radiation emitted was counted in a gamma counter. Non-specific binding, defined by 1 μM bombesin, was subtracted from total binding to calculate specific binding. Results are expressed as specific binding per 10^6 cells. Specific binding in the transfected cells was 60 - 80% of the total binding. No statistically significant binding was observed in untransfected cells in this assay which is able to detect as little as 200 receptors per cell.

2.2.4. MEASUREMENT OF TOTAL INOSITOL PHOSPHATES

Cells are labelled overnight with [^{3}H]inositol and total inositol phosphates are collected on an anion exchange column then eluted off with ammonium formate as described previously by Murphy and Rozengurt (1992).

Cultures of Swiss 3T3 cells in 33 mm dishes were labelled for 16 - 18 h with *myo*-[2-^{3}H]inositol (15 μCi/ml). Then they were washed twice with DMEM/Waymouth's medium (1:1 [vol/vol]) and incubated in this medium with additions as described in the table legend. LiCl was added to a final concentration of 20 mM for the last 30 min of the incubation to inhibit breakdown of inositol phosphates, see section 1.3.1. Inositol phosphates were extracted by replacing the medium with 1 ml ice-cold 3% $HClO_4$. After 20 min at 4°C the extract was neutralised with 1 M KOH containing 50

mM HEPES, 5 mM EGTA and 0.005% phenol red. Precipitated $KClO_4$ was removed by centrifugation. Samples were diluted to 10 ml with water then loaded onto 1 ml of Dowex AG1-X8 (200 - 400 mesh, $HCOO^-$ form) in Bio-Rad Econo-columns. Columns were washed 3 x 10 ml H_2O and 2 x 10 ml of 60 mM NH_4COOH, 5 mM $Na_2B_4O_7$ and inositol phosphates eluted with 6 ml 1 M NH_4COOH, 0.1 M HCOOH. An aliquot (1 ml) of eluate was counted in 10 ml Ultima Gold.

2.2.5. MEASUREMENT OF Ca^{2+}

Two methods have been used in this thesis. The fluorescent Ca^{2+} indicator, fura-2, allows continuous measurement of the cytoplasmic concentration, whereas the $^{45}Ca^{2+}$ isotope allows measurement of the total cellular Ca^{2+}.

2.2.5.1. USING THE FLUORESCENT Ca^{2+} INDICATOR, FURA-2

Fura-2 is a fluorescent derivative of EGTA (Grynkiewicz, et al., 1985) with four carboxylate groups that make it very membrane-impermeable. This problem has been addressed by masking the carboxyl groups with acetoxymethyl ester (AME) groups. The resulting fura-2/AME is hydrophobic, penetrates the plasma membrane and accumulates in the cytosol. Once inside the cell, the AME are hydrolysed by esterases present in the cytosol and fura-2 is again membrane-impermeable and is trapped inside the cell (Borle and Snowdowne, 1987). $[Ca^{2+}]_{cyt}$ *was measured with the fluorescent* Ca^{2+} *indicator fura-2/AME using procedures previously described (Millar and Rozengurt, 1990b).*

Rat-1 Cells

Confluent, quiescent, cultures of transfected Rat-1 cells in 100 mm dishes were washed twice with DMEM and incubated for 10 min at 37°C in 2.5 mg/ml dispase/DMEM to digest the intercellular matrix. Cells (4 - 5 $x10^6$) in single cell suspension were then incubated at 37°C, 10 min, in 5 ml of DMEM containing 1 μM fura-2/AME freshly prepared from a 1 mM stock maintained in DMSO. Cells were collected by centrifugation and resuspended in 2 ml electrolyte solution (150 mM NaCl, 0.5 mM KCl, 0.9 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM glucose, 16 mM HEPES, the same amino acid composition as DMEM, pH 7.1).

Swiss 3T3 Cells

Cells on 100 mm dishes were washed twice with DMEM and incubated for 10 min in 5 ml of DMEM containing 1 μM fura-2/AME freshly prepared from a 1 mM stock

maintained in DMSO. Cells were washed twice with PBS then gently scraped and resuspended in 2 ml of electrolyte solution (see above).

Both Rat-1 and Swiss 3T3 cells in electrolyte solution were then transferred to a 1 cm^2 quartz cuvette which was kept at 37°C and stirred continuously throughout the experiment. Fluorescence was monitored in a Perkin-Elmer LS-5 luminescence spectrometer with an excitation wavelength of 336 nm and an emission wavelength of 510 nm. $[Ca^{2+}]_{cyt}$ was calculated after sequential addition of Triton X-100 to 0.05% and EGTA to 100 mM to obtain the maximum (F_{max}) and minimum (F_{min}) fluorescence respectively, using the formula: $[Ca^{2+}]_{cyt}$ in nM = $K\ (F - F_{min})/(F_{max} - F)$, where F is the fluorescence at the unknown $[Ca^{2+}]_{cyt}$, and the value of K is 220 nM for fura-2/AME.

2.2.5.2. MEASUREMENT OF TOTAL $^{45}Ca^{2+}$ CONTENT

$^{45}Ca^{2+}$ is equilibrated with total cellular Ca^{2+} overnight. As described in section 1.3.2.1 Ca^{2+} in the intracellular stores can reach nearly mM concentrations whereas the Ca^{2+} in the cytoplasm is sub-micromolar. This method therefore is more a measure of stored Ca^{2+} rather than cytoplasmic Ca^{2+}.

Confluent, quiescent Swiss 3T3 cells in 33 mm dishes were loaded to equilibrium with 4 μCi $^{45}Ca^{2+}$ per ml over 16 h. Cells were then washed twice with DMEM and incubated with factors for 10 min at 37°C. Cells were rapidly washed 2 x 5 ml DMEM with 2 mM EGTA, then 6 x 5 ml DMEM. Cells were dissolved in 1 ml of 2% Na_2CO_3, 0.1 N NaOH, 1% SDS and the total amount of $^{45}Ca^{2+}$ in the cells was measured by liquid scintillation counting in 10 ml Ultima Gold.

2.2.6. MEASURING PKC ACTIVATION BY 80K/MARCKS PHOSPHORYLATION

Cells are labelled overnight with $^{32}P_i$ and 80K/MARCKS is immunoprecipitated. Phosphorylated 80K/MARCKS is visualised by autoradiography.

Confluent, quiescent, cultures of cells in 33 mm dishes were washed twice with DMEM without P_i and labelled overnight in this medium with 50 μCi/ml $^{32}P_i$. Cells were treated with various factors as indicated then lysed in 250 μl of ice cold lysis buffer (10 mM Tris/HCl, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1% Triton X-100 and 1 mM phenylmethylsulphonyl fluoride (PMSF), pH 7.6). Lysates were clarified by centrifugation at 15,000 g for 10 min. 80K/MARCKS was immunoprecipitated for 3 h at 4°C with 80K/MARCKS antiserum (Brooks, *et al.*, 1991) then 1 h with Protein A - agarose. Immunoprecipitates were washed three times with lysis buffer, extracted in 2x sample buffer (200 mM Tris-HCl,

6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol, pH 6.8) and then resolved by one-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (8% acrylamide) (Laemmli, 1970). Gels were fixed in 10% acetic acid and 25% methanol then dried. Phosphorylated 80K/MARCKS was visualised by autoradiography.

2.2.7. MAPK ACTIVATION

This has been measured by 2 approaches. *In vitro* immunecomplex kinase assays measure the enzymatic activity of $p42^{mapk}$ whereas $p42^{mapk}$ bandshift is an indication of the phosphorylation state of the enzyme and hence activation.

2.2.7.1. IMMUNE COMPLEX ASSAY FOR $p42^{mapk}$ ACTIVATION

$p42^{mapk}$ is immunoprecipitated and the immune complex is incubated with an MBP peptide substrate and γ-^{32}P ATP. The peptide is collected on anion exchange paper as described in Withers, et al. (1995).

Quiescent Rat-1 cells in 100 mm dishes were treated with factors and lysed at 4°C in 1 ml of lysis buffer. Lysates were clarified by centrifugation at 15,000 x g for 10 min at 4°C. Immunoprecipitation was performed using a polyclonal anti-$p42^{mapk}$ antibody raised against the C-terminal 9 amino acids of $p42^{mapk}$, and incubating the samples on a rotating wheel for 2 h. Washed protein A-agarose beads (50 µl 1:1 slurry) were added for the second hour. Immune complexes were collected by centrifugation and washed twice in lysis buffer without PMSF and twice in kinase buffer (15 mM Tris-HCl, 15 mM $MgCl_2$). The kinase reaction was performed by resuspending the pellet in 25 µl of kinase assay cocktail containing kinase buffer with 0.5 mM EGTA, 1 mg/ml MBP-peptide (APRTPGGRR), 50 µM ATP, 50 µCi/ml of [γ-^{32}P]ATP. Incubations were performed under linear assay conditions (10 min) at 30°C and terminated by spotting 25 µl of the supernatant onto Whatman P81 cation exchanger chromatography paper. Free [γ-^{32}P]ATP was removed by washing filters four times for 5 min in 0.5% orthophosphoric acid. Filters were immersed in acetone to aid drying before Cerenkov counting. The average radioactivity of two blank samples containing no immune complex was subtracted from the result of each sample. The specific activity of [γ-^{32}P] ATP used was 900 - 1200 cpm/pmol.

2.2.7.2. $p42^{mapk}$ BANDSHIFT

Active MAPK is phosphorylated on threonine and tyrosine residues and this phosphorylated form of MAPK exhibits retarded mobility in SDS-PAGE. Therefore the appearance of a slower migrating isoform, visualised by Western blotting with anti-

MAPK antibodies, is an indication of the activation state of the cellular pool of MAPK (Leevers and Marshall, 1992).

Confluent, quiescent dishes of Swiss 3T3 cells were treated with factors as described in the figure legend for 5 min and lysed in 150 µl of 2x sample buffer. Samples were boiled for 10 min, loaded onto a 10% polyacrylamide gel. Resolved proteins were transferred to Immobilon-P (PVDF) membranes in transfer buffer containing 25 mM Tris base, 0.2 M glycine and 20% methanol for 2.5 h at 100 V (current limited to 0.4 A). Immobilon membranes were blocked in 5% non-fat milk and incubated for 2 h with the polyclonal anti-p42^{mapk} antibody in 3% non-fat milk, 0.02% azide. Bands were visualised by incubating for 1 h with ^{125}I-labelled Protein A followed by autoradiography.

2.2.8. RAF-1 KINASE ASSAY

Raf-1 activity was assayed using a modification of the method described by Alessi, et al. (1994) using a GST-fusion proteins of MEK and p42^{mapk} as substrates for sequential activation, initiated by active Raf-1. This is followed by MAPK phosphorylation of MBP peptide which is then collected on cation exchanger paper as above.

2.2.8.1. PREPARATION OF SUBSTRATES

Overnight cultures of *Escherichia coli* strain BL21 DE3 transformed with GST-p42^{mapk} and GST-MEK expression vectors (pGEX-2T) were diluted 1 in 10 and grown for 1 h. GST-p42^{mapk} was induced with 1 mM IPTG for 4 h at 37°C and GST-MEK induced with 30 µM IPTG at 27°C overnight. The cells were then pelleted, freeze-thawed and lysed. Cell debris was removed by centrifugation and GST-fusion proteins were purified by adding 0.5 ml glutathione-Sepharose beads and rotating for 30 min at 4°C. The GST-p42^{mapk} was cleaved from the GST in thrombin buffer while the GST-MEK was eluted from the glutathione-Sepharose beads with 5 mM glutathione in 50 mM Tris pH 8. Both preparations were then dialysed and concentrated. The purity of each preparation was checked by subjecting the proteins to SDS-PAGE and staining the gels with Coomassie blue. For the p42^{mapk} typical yields were 10 µg/ml of culture, with a purity of >95%. For the GST-MEK typical yields were 5 µg/ml of culture with a purity of >95%.

2.2.8.2. RAF-1 KINASE ASSAY

Quiescent cells were treated as indicated in the figure legends and lysed in lysis buffer as above. Raf-1 immunoprecipitation was performed with an affinity purified

rabbit polyclonal antibody for 2 h with protein A-agarose added for the second hour. Immune complexes were collected by centrifugation and then washed twice in lysis buffer with no PMSF and twice with buffer A (50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 0.5 mM Na_3VO_4 and 0.1% β-mercaptoethanol). Pellets were then resuspended in 30 µl buffer B (30 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 6.5 µg/ml GST-MEK, 100 µg/ml $p42^{mapk}$, 0.03% Brij-35, 10 mM Mg-ATP and 20 mM N-octyl-β-D-glucopyranoside) and incubated at 30°C for 30 min. The reaction was then terminated by diluting the supernatant in 40 µl of buffer A with 1 mg/ml of BSA and, after mixing, 10 µl of the supernatant was removed to a fresh tube. $p42^{mapk}$ activation was then measured using the MBP peptide phosphorylation assay essentially as above.

2.2.9. GTP LOADING OF RAS

Cells are metabolically labelled with $^{32}P_i$, then the cell lysate is partitioned and Ras with bound nucleotide is immunoprecipitated from the detergent phase. The bound nucleotides are eluted, separated by thin layer chromatography and visualised by autoradiography as described in Basu, et al, (1992).

Confluent, quiescent cells in 100 mm dishes were washed twice in DMEM-phosphate and incubated in 5 ml DMEM-phosphate with 20 mM HEPES pH 7.5 and 1 mg/ml tissue culture grade BSA with 1.5 mCi of $^{32}P_i$ and labelled for 16 - 18 h. Cells were then stimulated with factors as described in the figure legend for 2.5 min at 37°C then rinsed with cold PBS and lysed in Ras lysis buffer (50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EGTA, 10 mM benzamidine, 10 µg/ml trypsin inhibitor, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM DTT, 1 mg/ml BSA) with 5 mM $MgCl_2$ and 1% Triton X-114. After removal of cell debris by centrifugation, phases were partitioned in the presence of 0.5 M NaCl for 2 min at 37°C. The detergent gel containing Ras was recovered by spinning down for two minutes at 14,000 g at 20°C and redissolved in Ras lysis buffer containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.05% SDS and 0.5 M NaCl. Ras was immunoprecipitated for 2 h with 25 µl [1:1 slurry] anti-Ras mAb 259 pre-coupled to Protein G - Sepharose (0.1 mg 259 mAb per 1 ml [1:1 slurry] Protein G - Sepharose). All immunoprecipitations were carried out in duplicate along with non-specific controls. Immunoprecipitates were washed with 6 x 1 ml of 50 mM HEPES, pH 7.4, 0.5 M NaCl, 5 mM $MgCl_2$, 0.1% Triton X-100, 0.005% SDS and the nucleotide was eluted with 2 mM EDTA, 2 mM DTT, 0.2% SDS, 0.5 mM GTP, 0.5 mM GDP, at 68°C for 20 min. Eluted nucleotides were separated by thin layer chromatograpy on PEI-cellulose plates (MERCK) run in 1.2 M ammonium formate, 0.8 M HCl. Results were quantitated on a phosphorimager and analysed by ImageQuant (Molecular Dynamics).

2.2.10. ANALYSIS OF TYROSINE PHOSPHORYLATED PROTEINS

The tyrosine phosphorylation of multiple proteins has been analysed by immunoprecipitation of specific proteins and analysis by Western blotting.

2.2.10.1. TYROSINE PHOSPHORYLATION OF MULTIPLE PROTEINS

Proteins are both immunoprecipitated and Western Blotted with anti-phosphotyrosine (Tyr(P)) mAbs.

Confluent, quiescent cultures of Rat-1 cells in 33 mm dishes (2.5 x 10^6 cells total) or Swiss 3T3 cells in 100 mm dishes were washed twice with DMEM, treated with factors in 5 ml of DMEM as indicated and lysed at 4°C in 1 ml lysis buffer as used for 80K/MARCKS immunoprecipitation. Lysates were clarified by centrifugation at 15,000g for 10 min. Supernatants were incubated with anti-Tyr(P) mAb Py72 for 4 h at 4°C then phosphotyrosine immune complexes were precipitated with anti-mouse IgG (whole molecule) - agarose for 1 h at 4°C. Immunoprecipitates were washed three times with lysis buffer, extracted in 2x sample buffer (200 mM Tris-HCl, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol, pH 6.8) and then resolved by SDS-PAGE. Proteins were then transferred to Immobilon transfer membranes in transfer buffer containing 50 mM Tris base, 0.4 mM glycine, 0.1% SDS and 20% methanol for 2 h at 35 V then 2 h at 70 V. Membranes were blocked using 5% non-fat dried milk in PBS, pH 7.2, and incubated for 2 h with anti-Tyr(P) mAb (4G10 1 μg/ml) in PBS containing 3% non-fat dried milk. Immunoreactive bands were visualised by autoradiography using [^{125}I]-labelled sheep anti-mouse IgG (1:1000).

2.2.10.2. TYROSINE PHOSPHORYLATION OF FAK

Tyrosine phosphorylated protein are immunoprecipitated and the presence of FAK in these immune complexes is analysed by Western blotting with anti-FAK mAb.

Cells were stimulated and phosphotyrosyl containing proteins were immunoprecipitated and transferred to Immobilon membranes as above. Membranes were blocked using 5% non-fat dried milk in PBS, pH 7.2, and incubated for 2 h with anti-p125^{FAK} mAb (1:1000) in PBS containing 3% non-fat dried milk. Immunoreactive bands were visualised by autoradiography using [^{125}I]-labelled sheep anti-mouse IgG (1:1000).

2.2.10.3. TYROSINE PHOSPHORYLATION OF PAXILLIN

The tyrosine phosphorylation of paxillin is analysed by immunoprecipitating paxillin and Western blotting with anti-Tyr(P) mAb.

Confluent, quiescent cultures of Rat-1 cells in 33 mm dishes (2.5 x 10^6 cells total) were washed twice with DMEM, treated with factors in 5 ml of DMEM as indicated and lysed at 4°C in 1 ml lysis buffer. Lysates were clarified by centrifugation at 15,000g for 10 min then incubated with anti-paxillin mAb 165 for 2 h at 4°C. Paxillin immune complexes were precipitated with anti-mouse IgG (whole molecule) - agarose for 1 h at 4°C. Immunoprecipitates were washed three times with lysis buffer, extracted in 2x sample buffer and then resolved by SDS-PAGE and transferred to Immobilon transfer membranes as above. Membranes were blocked using 5% non-fat dried milk in PBS, pH 7.2, and incubated for 2 h with anti-Tyr(P) mAb (4G10 1 μg/ml) in PBS containing 3% non-fat dried milk. Immunoreactive bands were visualised by autoradiography using [^{125}I]-labelled sheep anti-mouse IgG (1:1000).

Autoradiograms were scanned with an LKB Ultrascan XL densitometer to quantify tyrosine phosphorylation in terms of peak area.

2.2.11. MEASUREMENTS OF CELLULAR PROLIFERATION

Two basic methods have been used in this thesis. The first involves measuring the incorporation of the DNA-specific base thymidine into stimulated cells as a measure of re-entry into S-phase and DNA synthesis. The second method involves looking for an increase in cell numbers as a measure of cell proliferation.

2.2.11.1. [^{3}H]THYMIDINE INCORPORATION ASSAY

Determinations of DNA synthesis were performed as previously described (Dicker and Rozengurt, 1980). Cells are stimulated in the presence of [^{3}H]thymidine and the amount of radioactivity incorporated into acid-insoluble pools is measured.

Rat-1 Cells

Confluent, quiescent cultures of Rat-1 cells in 33 mm dishes were washed twice with DMEM and incubated at 37°C with DMEM/Waymouth's medium (1:1[vol/vol]) and various additions as described in the figure legends. After times specified [^{3}H]thymidine (0.25 μCi/ml, 1 μM) was added for 3 h.

Swiss 3T3 Cells

Confluent, quiescent cultures of Swiss 3T3 cells in 33 mm dishes were washed twice with DMEM and incubated with DMEM/Waymouth's medium (1:1[vol/vol]) containing [^{3}H]thymidine (0.25 μCi/ml, 1 μM) and various additions as described in the figure legends for 40 h at 37°C.

Cultures were then washed twice with PBS and incubated in 5% trichloroacetic acid at 4°C for 30 min to remove acid-soluble radioactivity. Cultures were washed with ethanol, solubilised in 1 ml of 2% Na_2CO_3, 0.1 M NaOH, 1% SDS and the radioactivity in the acid-insoluble pools determined by scintillation counting in 6 ml Ultima Gold (Packard).

2.2.11.2. AUTORADIOGRAPHY OF LABELLED NUCLEI

Labelled nuclei were determined by autoradiography as described previously (Dicker and Rozengurt, 1980). This method of analysing the incorporation of [^{3}H]thymidine reveals if any differences seen in looking at [^{3}H]thymidine incorporation into acid insoluble pools are reflected in the proportion *of cells that are labelled. This method therefore controls for alterations in the endogenous cellular thymidine pool or in the transport and processing of [^{3}H]thymidine.*

Cultures were washed twice with DMEM and incubated with DMEM/Waymouth's medium (1:1 [vol/vol]) containing [^{3}H]thymidine (2.5 μCi/ml) and various additions as described in the figure legends. After 40 h, the medium was removed and the cultures fixed in formol saline (1:9 [vol/vol]). The cultures were washed twice with Tris-saline pH 7.4 at 4°C, fixed twice (for 5 and 2 min) with 5% trichloroacetic acid at 4°C, and washed three times with 70% ethanol. Autoradiographic film was put on the cultures and developed after a 2 week exposure. After counterstaining with Giemsa, the labelled nuclei were counted.

2.2.11.3. MEASUREMENT OF CELL PROLIFERATION

Confluent, quiescent cultures of Rat-1 cells in 33 mm dishes were washed twice with DMEM and incubated at 37°C in DMEM/Waymouth's with additions as specified in the figure legends. After various times cell number was determined by removing the cells from the dish with a trypsin solution (0.05% trypsin in Ca^{2+} and Mg^{2+} free PBS with EDTA) and counting a portion of the resulting cell suspension in a Coulter counter.

2.2.12. PRETREATMENTS

Pretreatments with thapsigargin and DBHQ were carried out in conditioned medium which is the medium on 6 - 8 day old cells that have attained quiescence and is thus essentially devoid of growth promoting activity.

Stock solutions of thapsigargin were made up at 1 μM in DMSO and stored at -20°C. DBHQ was dissolved fresh each time to 100 μM in DMSO. Dilutions of compounds in DMSO, and DMSO controls, were added to the medium at 1 μl/ml. This concentration of DMSO had no measurable effects on our cells.

RESULTS

and

DISCUSSIONS

Chapter 3 - Signalling Pathways Coupled to The Bombesin/GRP Receptor

At present three mammalian members of the bombesin-like receptor family have been cloned and shown to belong to the G protein-linked receptor superfamily. They are the bombesin/GRP-preferring (Spindel, *et al.*, 1990; Battey, *et al.*, 1991), the neuromedin B- (NmB) preferring (Corjay, *et al.*, 1991; Wada, *et al.*, 1991) and BRS-3 (Gorbulev, *et al.*, 1992; Fathi, *et al.*, 1993), a bombesin-like receptor whose physiological ligand is not yet known. It has been generally assumed that all the early signals and the subsequent mitogenic response elicited by bombesin are mediated by occupancy of the bombesin/GRP receptor. This assumption has recently been questioned. Bold, *et al.* (1994) reported that antagonists that inhibit bombesin-induced Ca^{2+} mobilisation do not prevent bombesin-induced cell growth in human gastric adenocarcinoma cells. However tyrophostin blocked bombesin-induced proliferation of these cells. Bold *et al.* suggested that a bombesin receptor subtype couples to Ca^{2+} mobilisation and a different bombesin receptor subtype, not yet identified, couples to the protein tyrosine phosphorylation pathway and cell growth. However, no direct evidence supporting this hypothesis has been provided.

The experiments presented here were designed to elucidate whether the bombesin/GRP-preferring receptor could couple to both PLC activation as well as to FAK and paxillin tyrosine phosphorylation. Initially, it is shown that expression of the bombesin/GRP preferring receptor in Rat-1 cells, a cell line that can be reversibly arrested in the G_0/G_1 phase of the cell cycle, confers a proliferative response to bombesin in the receptor transfected cells. Using this model system, it is demonstrated that agonist activation of the bombesin/GRP receptor elicits both PLC activation as well as tyrosine phosphorylation of multiple proteins including FAK and paxillin. These results demonstrate, for the first time, that the bombesin/GRP receptor couples to both signalling pathways and to cell proliferation.

3.1. Binding Characteristics of the Expressed Bombesin/GRP Receptor

3.1.1. Expression of the bombesin/GRP receptor in Rat-1 cells.

The mouse bombesin/GRP-preferring receptor cDNA cloned into the mammalian expression vector pCD2, and under the transcriptional control of the SV40 early region promoter, was used for transfection experiments. Rat-1 fibroblasts were

chosen as the recipient cell line because these cells can be reversibly arrested in the G_0/G_1 phase of the cell cycle (Higgins, *et al.*, 1992) and do not express endogenous functional bombesin/GRP receptors (see below). After transfection, 21 G418 resistant colonies were isolated and propagated; six of these cell lines expressed the bombesin/GRP-preferring receptor compared with the control, untransfected Rat-1 cells.

The functional expression of bombesin/GRP receptors in the transfected Rat-1 cells was verified using [^{125}I]-labelled GRP. Figure 3.1 shows that the transfectants have specific binding sites for [^{125}I]GRP. Parental Rat-1 cells did not specifically bind [^{125}I]GRP. The apparent affinity of [^{125}I]GRP for the transfected receptor in BOR 5 and BOR 15 cells (K_d = 1 - 2 nM) was similar to the endogenous receptor expressed in Swiss 3T3 cells (K_d = 0.8 nM). The number of receptors on the transfected cells was 30 - 60% lower than that of Swiss 3T3 cells, about 10^5 per cell (Zachary and Rozengurt, 1985), under identical experimental conditions, see figure 3.2.

3.1.2. TIMECOURSE OF [^{125}I]GRP BINDING TO TRANSFECTED RAT-1 CELLS

When BOR 5 cells were incubated with [^{125}I]GRP, cell associated radioactivity increased rapidly reaching a maximum value after 30 min and decreasing afterwards (figure 3.3). This is consistent with internalisation and degradation of the peptide as reported previously in Swiss 3T3 cells (Zachary and Rozengurt, 1987) and in transformed rat kidney cells (Grady, *et al.*, 1995).

3.1.3. LIGAND RECOGNITION PROFILE OF THE TRANSFECTED BOMBESIN/GRP RECEPTOR

Addition of unlabelled GRP, bombesin, litorin, the structurally related neuromedin B (NmB), or the bombesin C-terminal fragment (amino acids 8-14) inhibited specific [^{125}I]GRP binding to BOR 5 cells in a concentration dependent manner (figure 3.4). The IC_{50} values for bombesin, GRP and litorin were approximately 1 - 3 nM. In contrast NmB inhibited binding with much lower affinity (IC_{50} = 500 nM). The 8-14 bombesin fragment is the C-terminal heptapeptide of bombesin which is the minimum sequence required to show activity, however full activity is seen with the C-terminal nonapeptide (Erspamer, *et al.*, 1988). Consistent with this the 8-14 bombesin peptide did show binding to the transfected bombesin/GRP receptor but with low affinity.

These results show that BOR 5 has specific binding sites with high affinity for bombesin/GRP and low affinity for NmB demonstrating that the transfected receptor in Rat-1 cells showed the characteristic ligand recognition of the bombesin/GRP-preferring receptor (Zachary and Rozengurt, 1985; Sinnett-Smith, *et al.*, 1990).

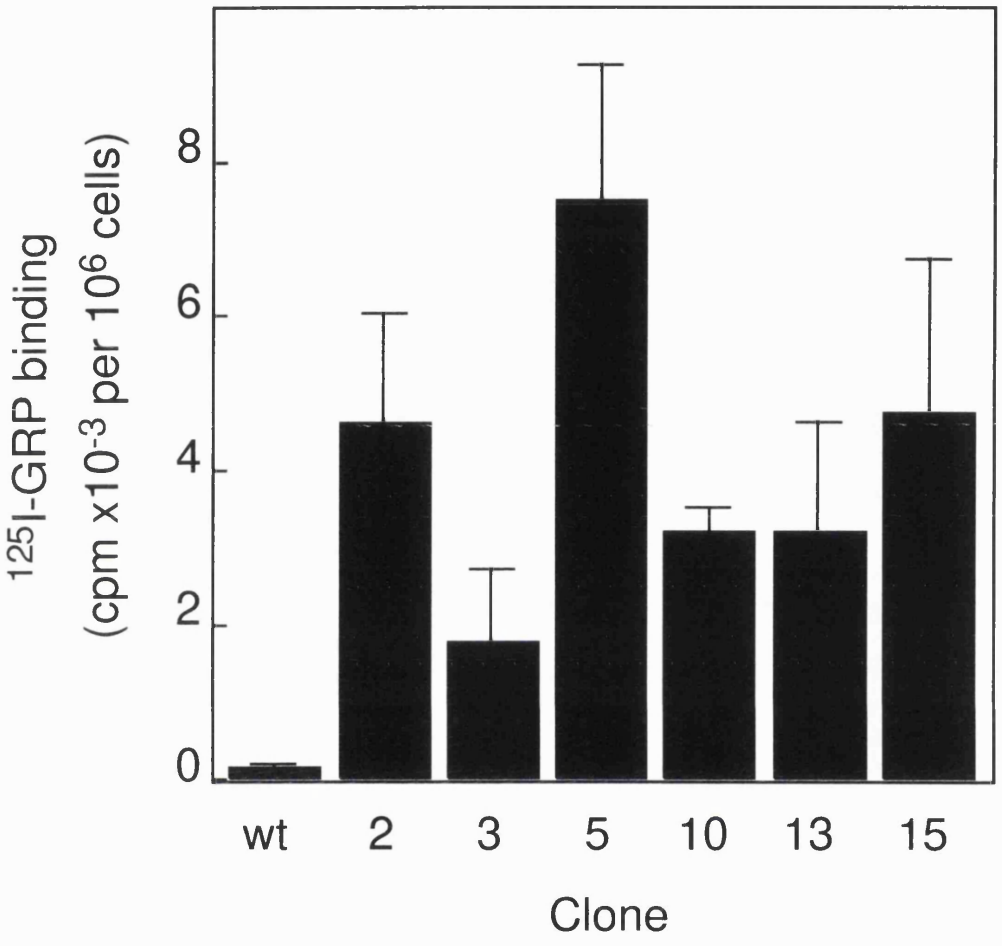

Figure 3.1. Specific [^{125}I]GRP binding to different clones

Confluent, quiescent cultures of transfected and parental (WT) Rat-1 cells were washed twice with DMEM and incubated for 30 min at 37°C in DMEM containing [^{125}I]GRP (0.05 nM, 0.01 μCi and 0.95 nM cold GRP) and 1 mg/ml BSA. Cultures were then placed on ice and rapidly washed 4 x with PBS containing 1 mg/ml BSA. Cultures were dissolved in 0.1 N NaOH/2% $NaCO_3$/1% SDS and counted in a gamma counter. Results are expressed as mean specific binding per 10^6 cells ± SE from 3 independent experiments performed in duplicate.
Non-specific binding was determined in the presence of 1 μM bombesin. Specific binding was 60 - 80% of the total binding.

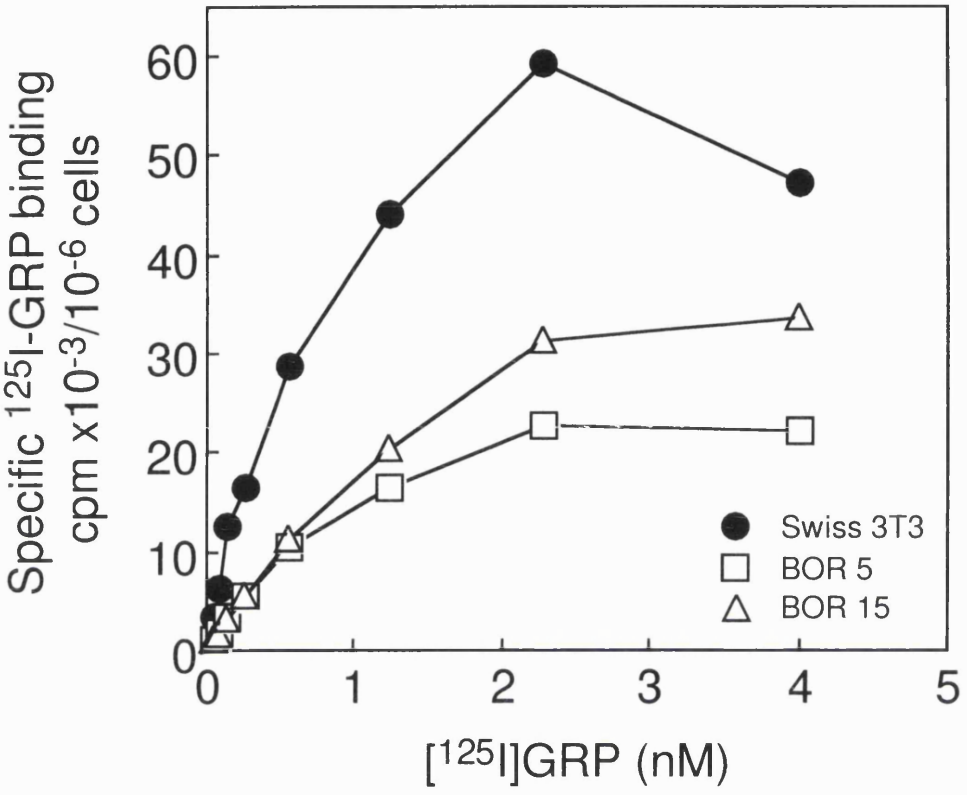

Figure 3.2. Binding of [^{125}I]-GRP as a function of GRP concentration

Confluent, quiescent cultures of transfected Rat-1 cells and Swiss 3T3 cells were washed twice with DMEM and incubated for 30 min at 37°C in DMEM containing various amounts of [^{125}I]GRP and 1 mg/ml BSA. The maximum concentration was 4 nM GRP (0.2 nM, 0.04 μCi and 3.8 nM cold GRP) which was serial diluted 1:1 in DMEM with 1 mg/ml BSA. Cultures were then placed on ice and rapidly washed 4 x with PBS containing 1 mg/ml BSA. Cultures were dissolved in 0.1 N NaOH/2% $NaCO_3$/1% SDS and counted in a gamma counter. Results are expressed as specific binding per 10^6 cells from one experiment performed in duplicate. Non-specific binding was determined in the presence of 1 μM bombesin.

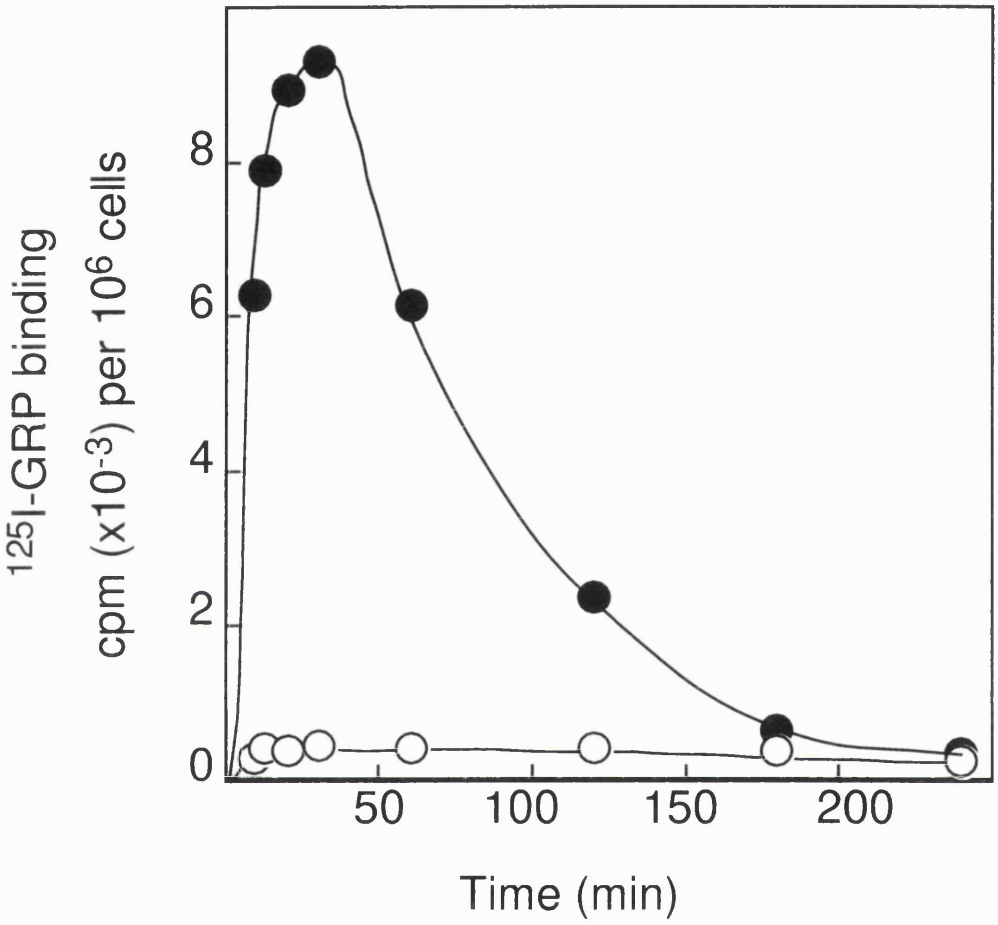

Figure 3.3. Time-course of [^{125}I]GRP binding to BOR 5 cells

Confluent, quiescent cultures of BOR 5 cells were washed twice with DMEM and incubated at 37°C in DMEM with [^{125}I]GRP (as above) in the presence of 1 mg/ml BSA (closed circles). Non-specific binding was determined by adding 1 μM bombesin (open circles). The binding of [^{125}I]GRP to BOR 5 cells was terminated after various times as indicated by rapid washing as in figure 3.1. Cultures were dissolved and counted in a gamma counter. Results are expressed as total binding per 10^6 cells from one experiment performed in duplicate.

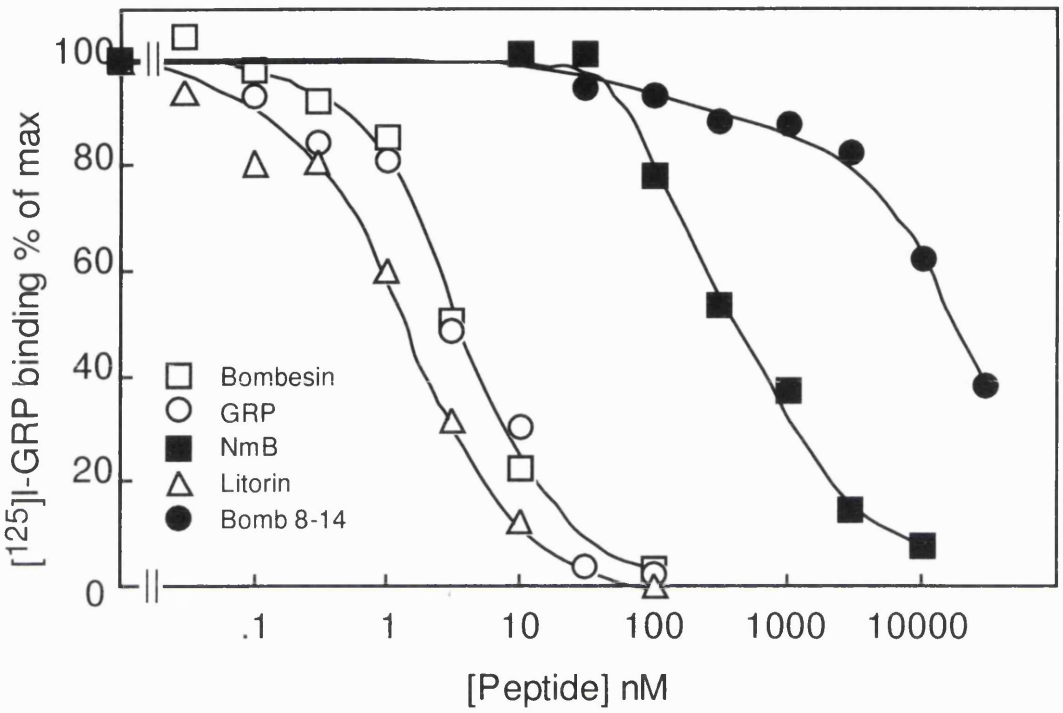

Figure 3.4. Competition Binding of [^{125}I]GRP to BOR 5 cells

Confluent, quiescent cultures of BOR 5 cells were washed twice with DMEM and incubated for 30 min at 37°C in DMEM with [^{125}I]GRP (as above) and various concentrations of competing peptides in the presence of 1 mg/ml BSA. Peptides were bombesin (open squares), GRP (open circles), litorin (open triangles), NmB (closed squares) and bombesin fragment 8-14 (closed circles). Cultures were then placed on ice and washed and counted as in figure 3.1. Results are expressed as a percentage of the total specific binding of [^{125}I]GRP from one experiment performed in duplicate.

3.2. THE TRANSFECTED BOMBESIN/GRP RECEPTOR FUNCTIONALLY COUPLES TO PLC-ACTIVATED SIGNALLING PATHWAYS.

In order to verify that the transfected bombesin/GRP receptor expressed in Rat-1 cells could couple to PLC, the effect of bombesin on the intracellular calcium concentration and on the phosphorylation of the prominent PKC substrate 80K/MARCKS (Rodriguez-Pena and Rozengurt, 1986; Brooks, *et al.*, 1991) was measured.

3.2.1. BOMBESIN STIMULATES INTRACELLULAR Ca^{2+} MOBILISATION

Bombesin increased $[Ca^{2+}]_{cyt}$ in a concentration dependent manner in BOR 5 cells (figure 3.5). The maximum increase, elicited by 100 nM bombesin, was 4.0 fold over the basal level (186 nM ± 5.5, n = 62). In contrast bombesin caused no increase in $[Ca^{2+}]_{cyt}$ in parental Rat-1 cells even up to 1 μM (data not shown). Addition of the specific bombesin/GRP receptor antagonist, D-F_5-Phe^6, D-Ala^{11}-Bombesin(6-13)OMe (Coy, *et al.*, 1992), inhibited bombesin-induced increases in $[Ca^{2+}]_{cyt}$ (figure 3.6) but had no effect on the increase in $[Ca^{2+}]_{cyt}$ induced by endothelin which binds to receptors endogenously expressed in Rat-1 cells. Studies of Ca^{2+} mobilisation with cultures of BOR 15 cells produced exactly the same results.

3.2.2. BOMBESIN STIMULATES 80K/MARCKS PHOSPHORYLATION

Addition of bombesin to cultures of BOR 5 cells prelabelled with $^{32}P_i$ markedly increased the phosphorylation of the prominent PKC substrate, 80K/MARCKS, in a concentration dependent manner (figure 3.7). The level of phosphorylation was comparable to that induced by 200 nM PDB, a direct activator of PKC. In contrast 80K/MARCKS was not phosphorylated in response to bombesin (up to 100 nM) in parental Rat-1 cells.

These results show that the transfected bombesin/GRP receptor is functionally coupled to intracellular signalling pathways initiated by PLC activation.

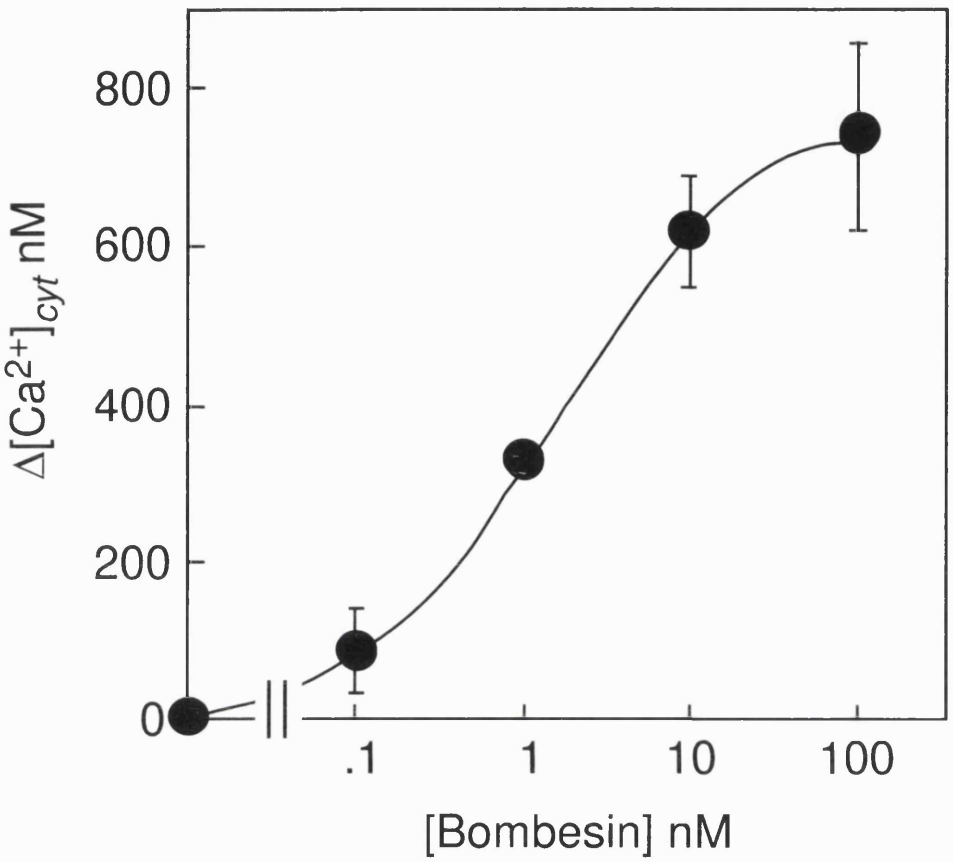

Figure 3.5. Bombesin-induced increase in $[Ca^{2+}]_{cyt}$ in BOR 5 cells

Confluent, quiescent cultures of BOR 5 cells were washed twice in DMEM and detached from the dishes by a 10 min incubation in DMEM with 2.5 μg/ml dispase. Cells were loaded with 1 μM fura-2/AME while in suspension. Loaded cells were pelleted and resuspended in 2 ml electrolyte solution and transferred to a quartz cuvette. Cells were kept at 37°C and stirred continuously while fluorescence was monitored. After a 1 min control period different concentrations of bombesin were added. Results are expressed as the difference in $[Ca^{2+}]_{cyt}$ between unstimulated and stimulated levels from one representative experiment that was performed in duplicate. Similar results were obtained in 3 independent experiments.

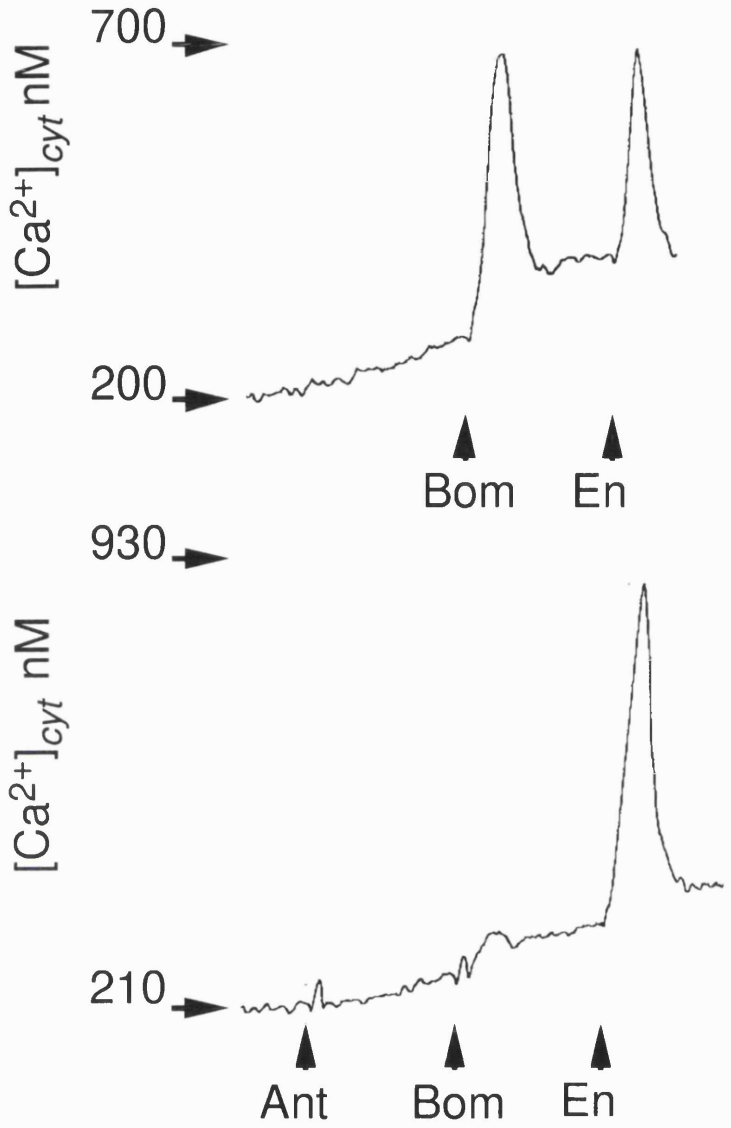

Figure 3.6. Antagonist inhibition of bombesin-induced increase in $[Ca^{2+}]_{cyt}$

Confluent, quiescent cultures of BOR 5 cells were washed twice in DMEM and detached from the dishes by a 10 min incubation in DMEM with 2.5 μg/ml dispase. Cells were loaded with 1 μM fura-2/AME while in suspension. Loaded cells were pelleted and resuspended in 2 ml electrolyte solution and transferred to a quartz cuvette. Cells were kept at 37°C and stirred continuously while fluorescence was monitored. After 3 min of monitoring fluorescence bombesin was added to 1 nM (Bom) and at 5 min endothelin was added to 50 nM (En). At 1 min the bombesin antagonist D-F_5-Phe^6, D-Ala^{11}-Bombesin(6-13)OMe was added to 10 nM as indicated (Ant). Shown are the traces from one representative experiment with $[Ca^{2+}]_{cyt}$ in nM on the right. Similar results were seen in two independent experiments.

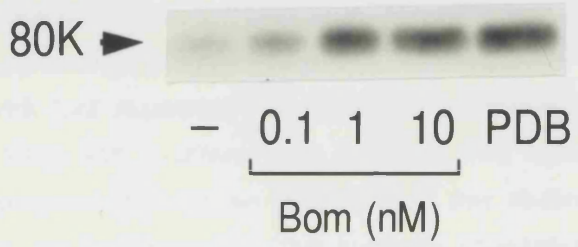

Figure 3.7. Bombesin-induced phosphorylation of 80K/MARCKS

Confluent, quiescent cultures of BOR 5 cells were washed twice in DMEM without phosphate and incubated in this medium with 5 μCi carrier-free $^{32}P_i$ at 37°C for 18 h. Various concentrations of bombesin (0.1 nM, 1 nM, 10 nM), or 200 nM PDB were added for 10 min. Cultures were put on ice and rapidly washed twice with cold DMEM without phosphate and lysed with 250 μl lysis buffer. 80K/MARCKS was immunoprecipitated and resolved on a 8% polyacrylamide gel. ^{32}P-labelled 80K was visualised by autoradiography. Results shown are from one representative experiment that was performed three times with similar results.

3.3. EXPRESSION OF THE BOMBESIN/GRP RECEPTOR CONFERS A GROWTH RESPONSE TO BOMBESIN IN RAT-1 CELLS.

3.3.1. BOMBESIN STIMULATES DNA SYNTHESIS

Next, it was determined whether agonist activation of the transfected bombesin/GRP receptor leads to reinitiation of DNA synthesis in Rat-1 cells. Quiescent cultures of BOR 5 cells were incubated with bombesin and at various times after stimulation the cells were labelled with [^{3}H]thymidine for 3 h. The rate of [^{3}H]thymidine incorporation reached a maximum between 18 h - 24 h after the addition of bombesin (figure 3.8). This is consistent with previous results of bombesin-induced DNA synthesis in Swiss 3T3 cells (Rozengurt and Sinnett-Smith, 1983). DNA synthesis induced by bombesin in cultures of BOR 5 cells was inhibited by the specific bombesin/GRP receptor antagonist, D-F_5-Phe^6, D-Ala^{11}-Bombesin(6-13)OMe, (figure 3.9) with half maximum and maximum inhibition at 1 nM and 30 nM respectively. When BOR 5 cultures were incubated with various concentrations of bombesin, maximum [^{3}H]thymidine incorporation was elicited at 1 nM (data not shown). These results substantiate that bombesin-stimulated reinitiation of DNA synthesis in BOR 5 cells was elicited through a bombesin/GRP preferring receptor.

3.3.2. BOMBESIN INDUCES CELL PROLIFERATION

The mitogenic activity of bombesin in cultures of BOR 5 cells could also be demonstrated by monitoring cell number (rather than [^{3}H]thymidine incorporation into acid precipitable material) over a period of several days (figure 3.10).

The addition of bombesin to BOR 5 in serum-free medium increased cell number compared with control cultures as early as 1 day of incubation and reached a maximum after 3 days of bombesin addition. In other experiments it was also verified that bombesin induced DNA synthesis and cell proliferation in cultures of BOR 15 cells. Thus, the transfected bombesin/GRP receptor is coupled to signal transduction pathways which stimulate DNA synthesis and subsequently cell proliferation.

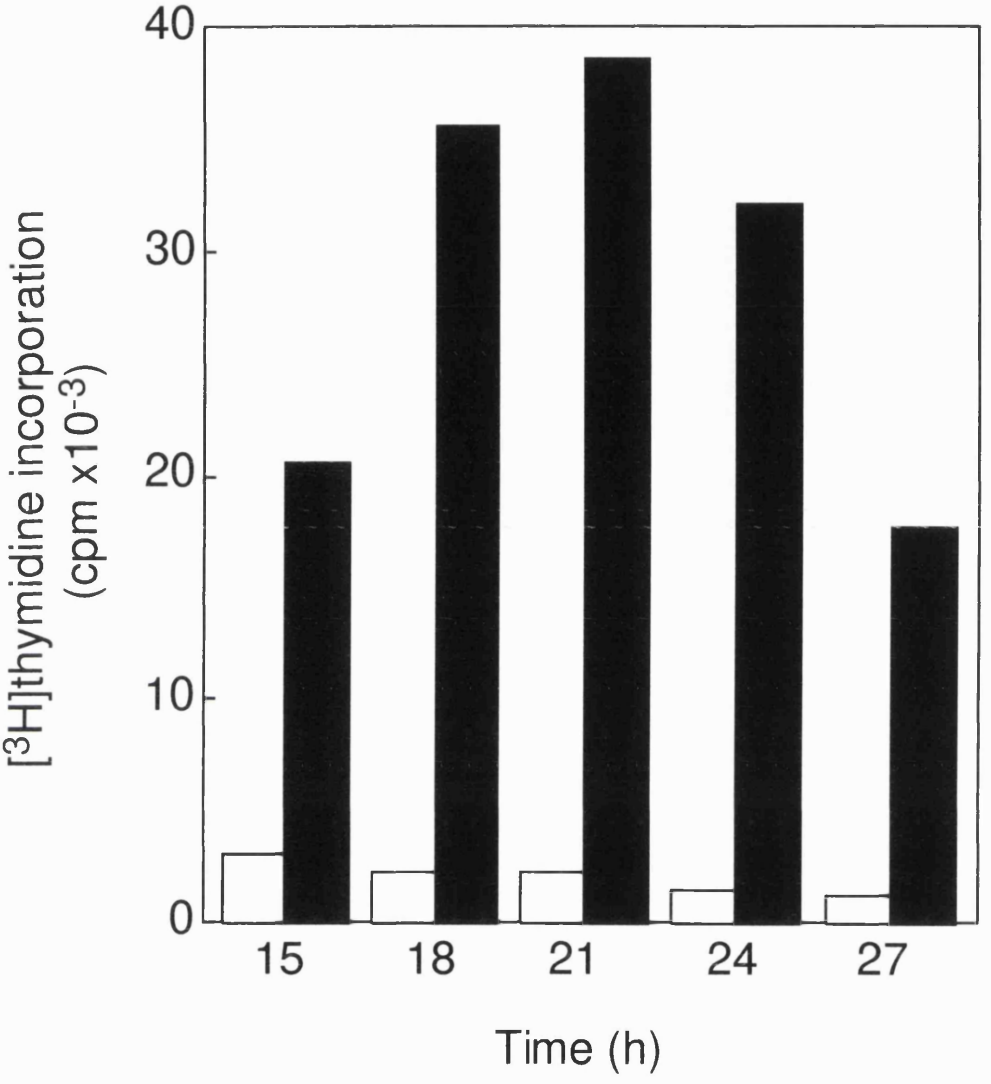

Figure 3.8. Kinetics of [^{3}H]thymidine incorporation induced by bombesin

Confluent, quiescent cultures of BOR 5 cells were washed twice in DMEM and incubated at 37°C in DMEM/Waymouth's [1:1] with (solid bars) or without (open bars) 2 nM bombesin. After the times indicated below each bar 0.25 μCi [^{3}H]thymidine per ml was added for 3 h and the incorporation was stopped as described in Materials and Methods. DNA synthesis was assessed by measuring the level of [^{3}H]thymidine incorporated into half of the acid precipitable material by liquid scintillation counting. Results are expressed as the mean of two determinations from one representative experiment that was performed twice with similar results.

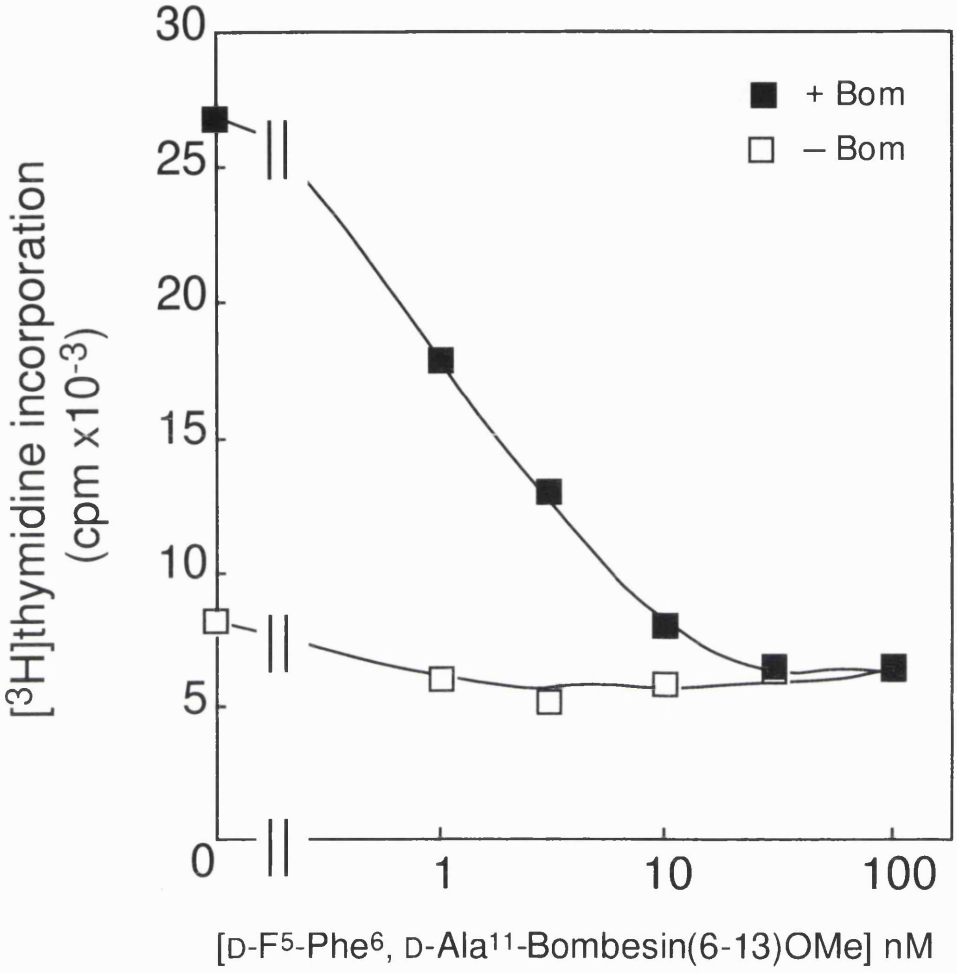

Figure 3.9. Effect of antagonist on bombesin-induced [^{3}H]thymidine incorporation.

Confluent, quiescent cultures of BOR 5 cells were washed twice in DMEM and incubated at 37°C in DMEM/Waymouth's [1:1] with various concentrations of antagonist D-F_5-Phe^6, D-Ala^{11}-Bombesin(6-13)OMe in the presence (closed squares) or absence (open squares) of 1 nM bombesin. [^{3}H]thymidine was added after 18 h and its incorporation was stopped after 6 h of additional incubation as described in Materials and Methods. Results are expressed as the mean cpm from two dishes from a representative experiment that was performed twice with similar results.

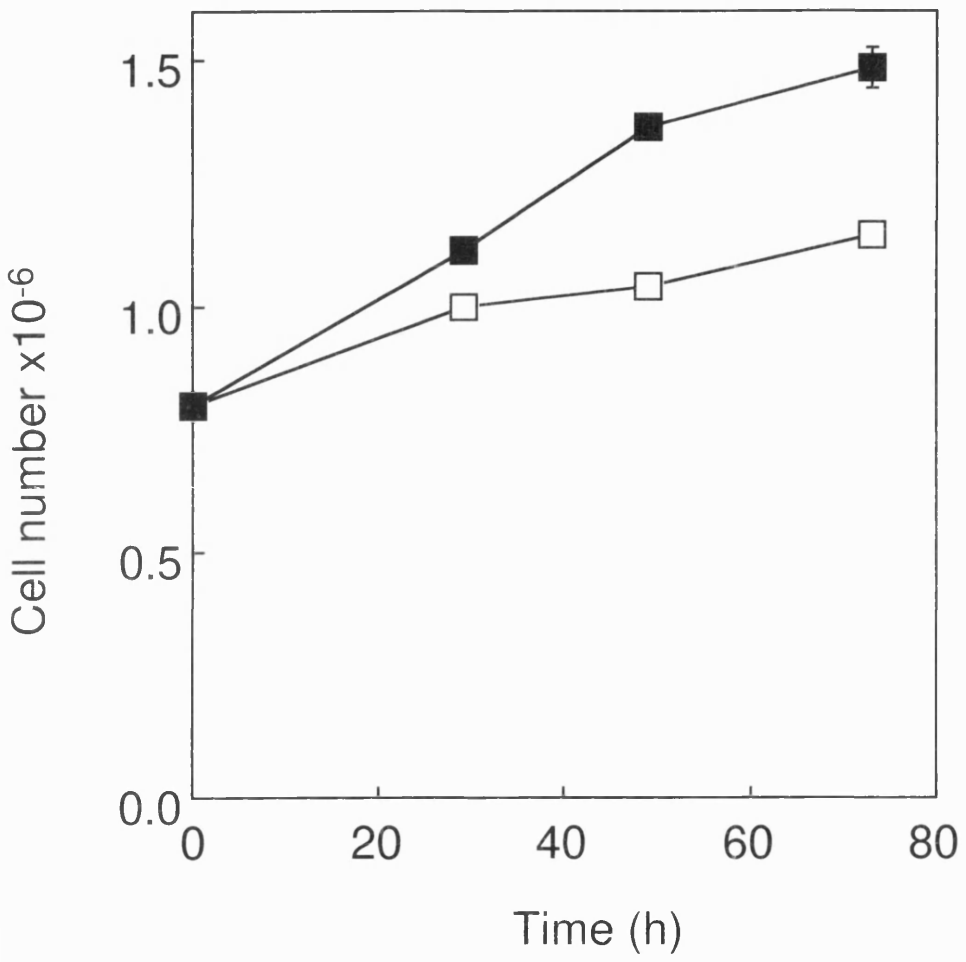

Figure 3.10. Cell proliferation induced by bombesin in BOR 5 cells

Confluent, quiescent cultures of BOR 5 cells were washed twice in DMEM and incubated at 37°C in DMEM/Waymouth's [1:1] with (closed squares) or without (open squares) 1 nM bombesin. Cells were trypsinised off the dishes and counted with a Coulter counter at 0, 24, 48 and 72 hours after stimulation. Each value is the mean ± SE of 5 cultures. Where error bar is not shown, it is smaller than the symbol.

3.4. BOMBESIN STIMULATES TYROSINE PHOSPHORYLATION IN RAT-1 CELLS TRANSFECTED WITH THE BOMBESIN/GRP RECEPTOR

In view of the preceding results that demonstrated that bombesin stimulates Ca^{2+} mobilisation, 80K/MARCKS phosphorylation and cell proliferation, it was next examined whether bombesin can also stimulate tyrosine phosphorylation in Rat-1 cells transfected with the bombesin/GRP receptor. Tyrosine phosphorylation has been implicated in the intracellular signalling of bombesin and other neuropeptides (Rozengurt, 1995). Bombesin stimulates tyrosine phosphorylation of multiple proteins in Swiss 3T3 cells, including broad bands migrating with the apparent molecular masses of 110,000 - 130,000 and 70,000 - 80,000 (Zachary, *et al.*, 1991a; Zachary, *et al.*, 1991b). More recently the focal adhesion associated proteins FAK and paxillin have been identified as prominent tyrosine phosphorylated proteins in Swiss 3T3 cells after stimulation by bombesin (Zachary, *et al.*, 1992; Sinnett-Smith, *et al.*, 1993; Zachary, *et al.*, 1993). FAK is a novel cytosolic tyrosine kinase that lacks Src Homology 2 (SH2) and SH3 domains (Hanks, *et al.*, 1992; Schaller, *et al.*, 1992) but associates with signalling proteins including Src (Cobb, *et al.*, 1994) and paxillin (Hildebrand, *et al.*, 1995). Recent molecular cloning of paxillin revealed a multidomain protein that may function as an adaptor capable of associating with FAK, Crk, Src and vinculin (Turner and Miller, 1994; Salgia, *et al.*, 1995).

3.4.1. BOMBESIN INDUCES TYROSINE PHOSPHORYLATION OF MULTIPLE PROTEINS IN BOR 5 AND BOR 15 CELLS.

Lysates of quiescent cultures of BOR 5 and BOR 15 cells treated with bombesin were immunoprecipitated with the anti-phosphotyrosine (anti-Tyr(P)) monoclonal antibody (mAb) Py72 and the immunoprecipitates were analysed by Western blotting with anti-Tyr(P) mAb 4G10. As shown in figures 3.11 bombesin induced a dose-dependent increase in the phosphotyrosine content of multiple components including bands migrating with an apparent M_r of 110,000 - 130,000 and 70,000 - 80,000. Scanning densitometry of these bands revealed that half maximum and maximum tyrosine phosphorylation was achieved at bombesin concentrations of 0.1 - 0.3 nM and 1 - 3 nM, respectively (figure 3.12). In contrast, parental Rat-1 cells did not show any increase in tyrosine phosphorylation in response to bombesin (100 nM) in these and the following experiments.

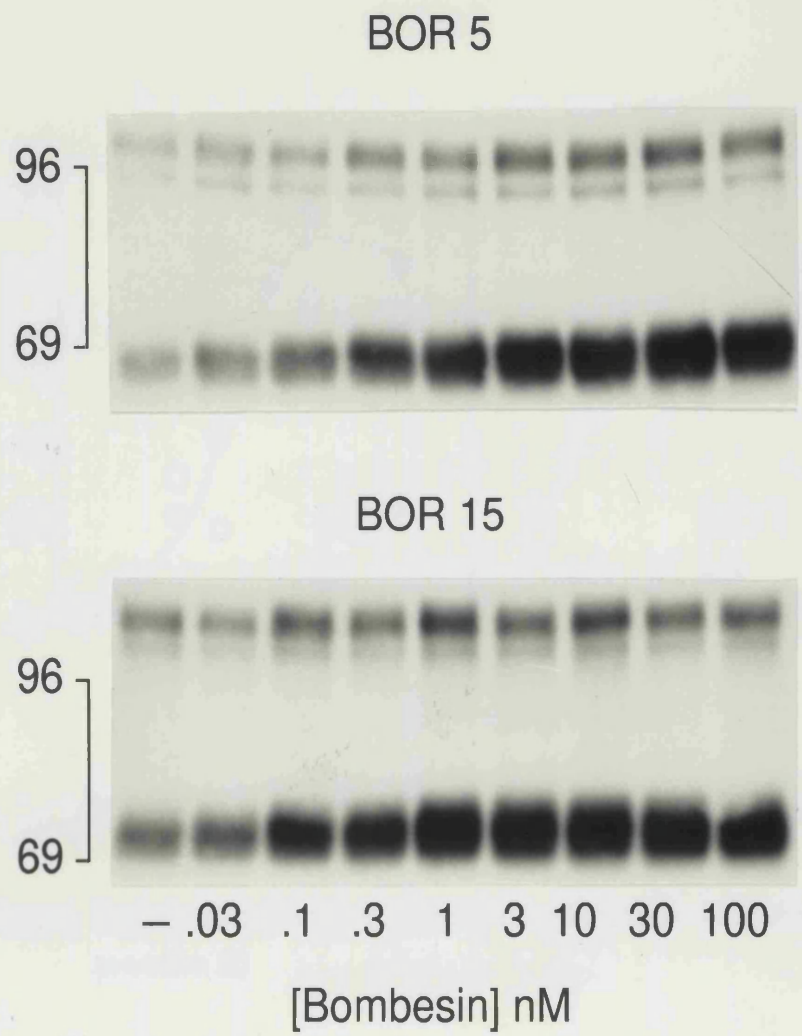

Figure 3.11. Bombesin-induced tyrosine phosphorylation of multiple proteins (I)

Confluent, quiescent cultures of BOR 5 and BOR 15 cells were washed twice in DMEM and incubated at 37°C in DMEM with various concentrations of bombesin for 10 min. Cells were lysed and tyrosine phosphorylated proteins immunoprecipitated with anti-Tyr(P) mAb Py72. Immunoprecipitates were resolved by SDS-PAGE and Western blotted with 4G10 anti-Tyr(P) mAb followed by ^{125}I-labelled anti-mouse IgG. Bands were visualised by autoradiography. Similar results were obtained in at least 3 independent experiments.

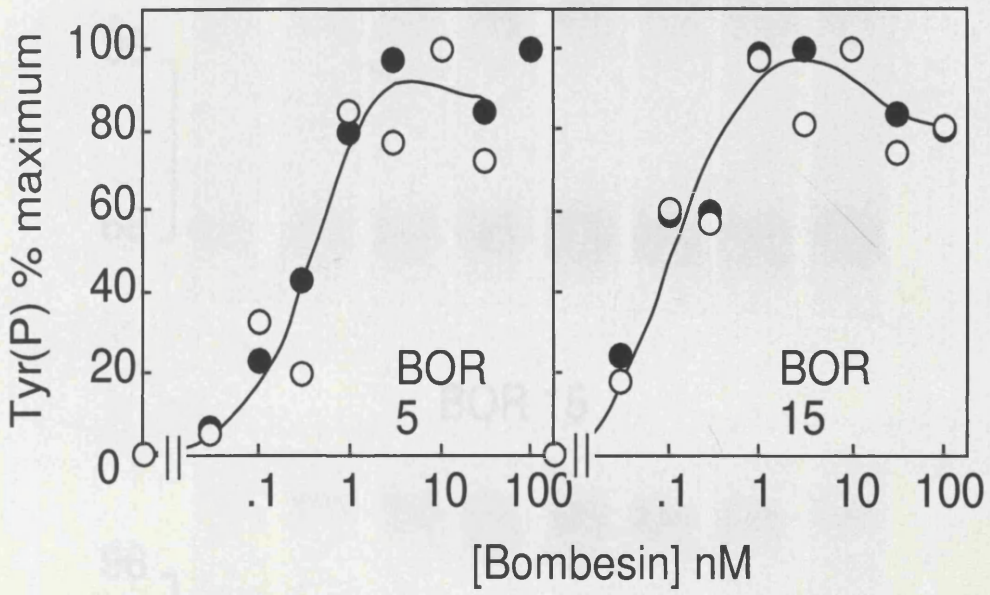

Figure 3.12. Bombesin-induced tyrosine phosphorylation of multiple proteins (II)

Autoradiograms from the previous figure (3.11) were scanned with an LKB Ultrascan XL densitometer to quantify tyrosine phosphorylation in terms of peak area. The 70,000 - 80,000 band is depicted by closed circles and the 110,000 - 130,000 band by open circles. Results shown are the average of three independent experiments. Values are expressed as a percentage of the maximum tyrosine phosphorylation after subtraction of the control.

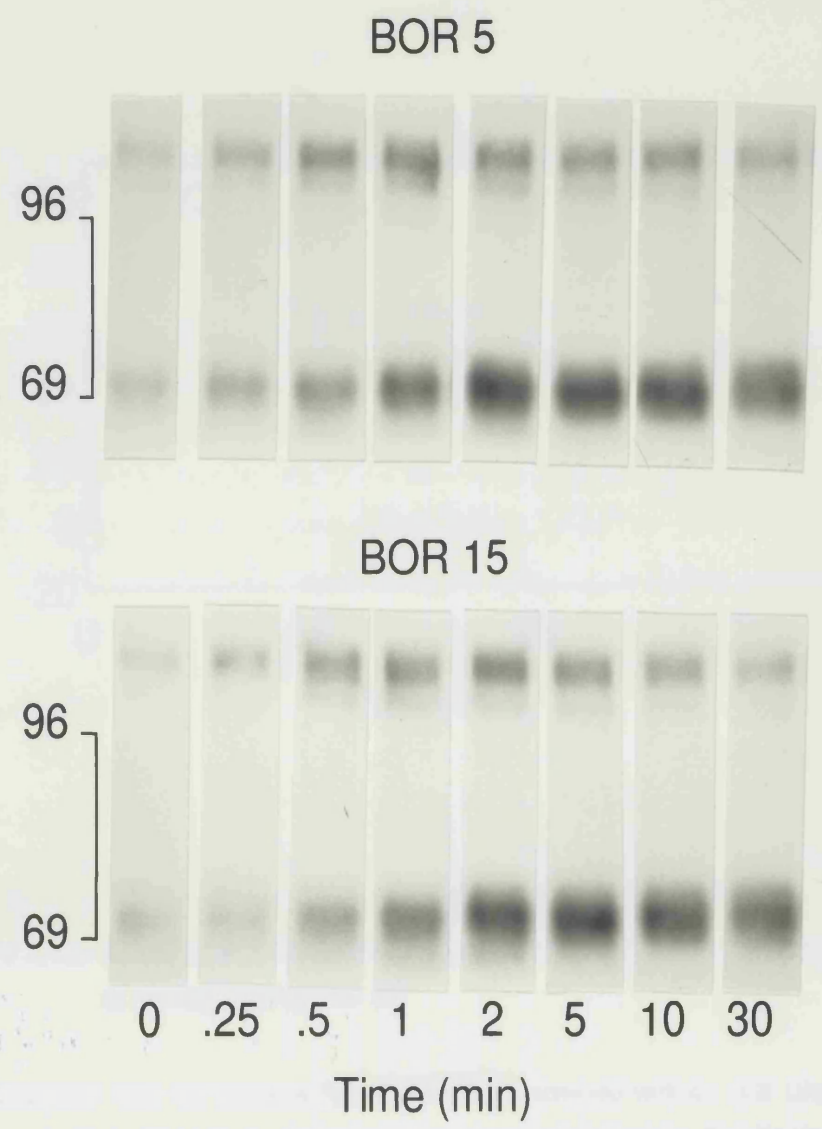

Figure 3.13. Kinetics of bombesin induction of tyrosine phosphorylation of multiple proteins (I)

Confluent, quiescent cultures of BOR 5 and BOR 15 cells were washed twice in DMEM and incubated at 37°C in DMEM with 10 nM bombesin for various times. Cells were lysed and tyrosine phosphorylated proteins immunoprecipitated with Py72 anti-Tyr(P) mAb. Immunoprecipitates were resolved by SDS-PAGE and Western blotted with 4G10 anti-Tyr(P) mAb. Similar results were obtained in at least three independent experiments.

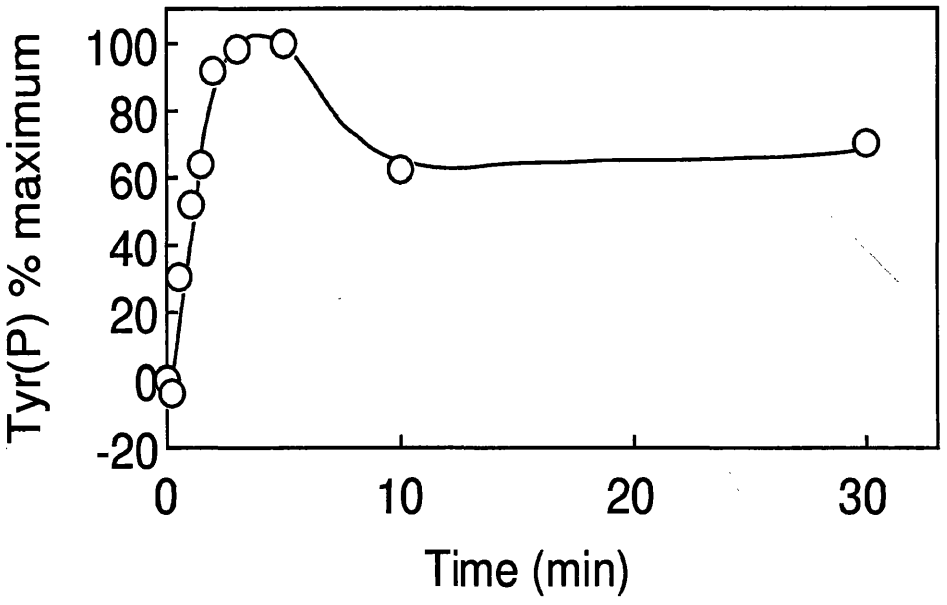

Figure 3.14. Kinetics of bombesin induction of tyrosine phosphorylation of multiple proteins (II)

Autoradiograms from the previous figure (3.13) were scanned with an LKB Ultrascan XL densitometer to quantify tyrosine phosphorylation in terms of peak area. Results shown are of the 70,000 - 80,000 band from a representative experiment of BOR 15 which was repeated at least three times with similar results. Values expressed as a percentage of the maximal tyrosine phosphorylation after subtraction of the control.

The time course of tyrosine phosphorylation induced by bombesin in Rat-1 cells transfected with the bombesin/GRP receptor is shown in figures 3.13 and 3.14. The increase in tyrosine phosphorylation of the M_r 110,000 - 130,000 and the M_r 70,000 - 80,000 bands was detectable within seconds and reached a maximum after 1 min of incubation with 10 nM bombesin in cultures of both BOR 5 and BOR 15 cells. Increased protein tyrosine phosphorylation persisted for at least 3 h.

3.4.2. BOMBESIN-INDUCED TYROSINE PHOSPHORYLATION IS INHIBITED BY THE SPECIFIC BOMBESIN RECEPTOR ANTAGONIST

To further substantiate the involvement of the bombesin/GRP receptor in tyrosine phosphorylation, the bombesin/GRP receptor-specific antagonist D-F_5-Phe^6, D-Ala^{11}-Bombesin(6-13)OMe was used. This antagonist has already been shown to inhibit bombesin-induced Ca^{2+} mobilisation (figure 3.6) and DNA synthesis (figure 3.9). We therefore examined whether it can also inhibit bombesin-induced tyrosine phosphorylation. Cultures of BOR 5 and BOR 15 cells were pretreated with the bombesin/GRP receptor-specific antagonist for 10 min then stimulated by bombesin or endothelin and cell lysates were analysed for tyrosine phosphorylated proteins. Figure 3.15 shows that the antagonist completely blocked bombesin-induced tyrosine phosphorylation of the M_r 110,000 - 130,000 and the M_r 70,000 - 80,000 bands. In contrast, the antagonist did not interfere with the stimulation of tyrosine phosphorylation of similar bands induced by endothelin.

3.4.3. BOMBESIN-INDUCED TYROSINE PHOSPHORYLATION IS DEPENDENT ON THE INTEGRITY OF THE ACTIN CYTOSKELETON

It has been shown that bombesin induces tyrosine phosphorylation in Swiss 3T3 cells through a PKC-independent pathway that requires the integrity of the actin cytoskeleton (Sinnett-Smith, *et al.*, 1993). It was examined whether the stimulation of tyrosine phosphorylation by agonist activation of the transfected bombesin/GRP receptor exhibited similar signalling characteristics. The effect of cytochalasin D, which selectively disrupts the network of actin filaments, on bombesin-induced tyrosine phosphorylation was determined. Figure 3.16 shows that pretreating BOR 5 and BOR 15 cultures with cytochalasin D for 2 h, dramatically inhibited the tyrosine phosphorylation of all bands stimulated by bombesin. In contrast, cytochalasin D did not inhibit EGF-induced tyrosine phosphorylation of its receptor (data not shown) indicating that this drug specifically inhibits the tyrosine phosphorylation of bands M_r 110,000 - 130,000 and 70,000 - 80,000. Thus, bombesin-induced tyrosine phosphorylation in BOR 5 and BOR 15 cells is dependent on the integrity of the actin cytoskeleton.

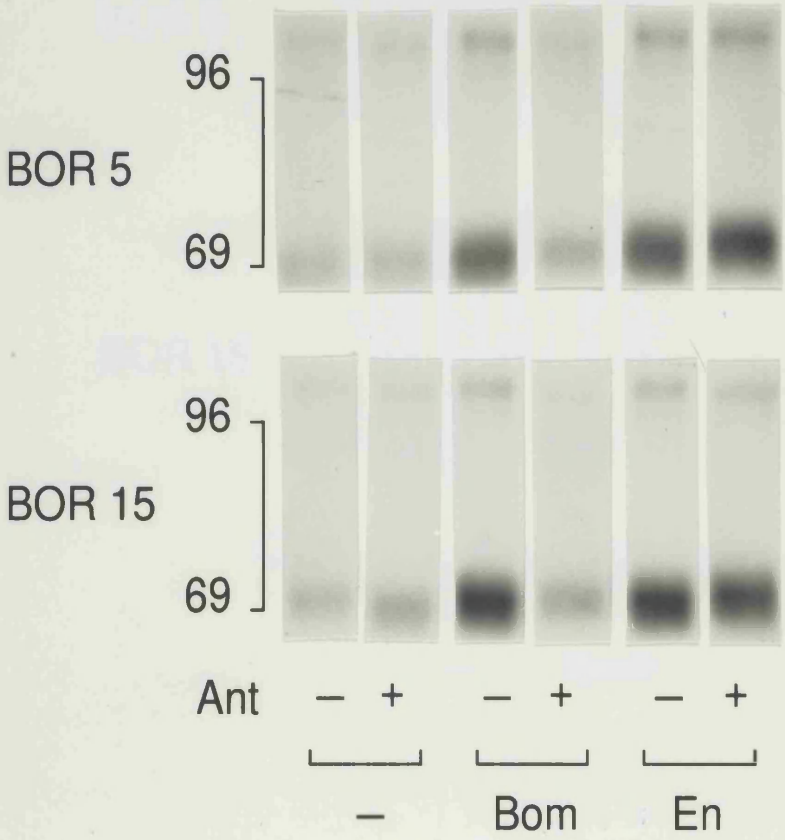

Figure 3.15. Effect of antagonist on bombesin-induced tyrosine phosphorylation of multiple proteins

Confluent, quiescent cultures of BOR 5 and BOR 15 cells were washed twice in DMEM and incubated at 37°C in DMEM with (+) or without (-) 100 nM D-F_5-Phe^6, D-Ala^{11}-Bombesin(6-13)OMe (Ant) for 10 min. Then 10 nM bombesin (Bom) or 50 nM endothelin (En) was added as indicated for 10 min. Cells were lysed and tyrosine phosphorylated proteins immunoprecipitated with Py72 anti-Tyr(P) mAb and Western blotted with 4G10 anti-Tyr(P) mAb. Bands were visualised by autoradiography. Similar results were obtained in two independent experiments.

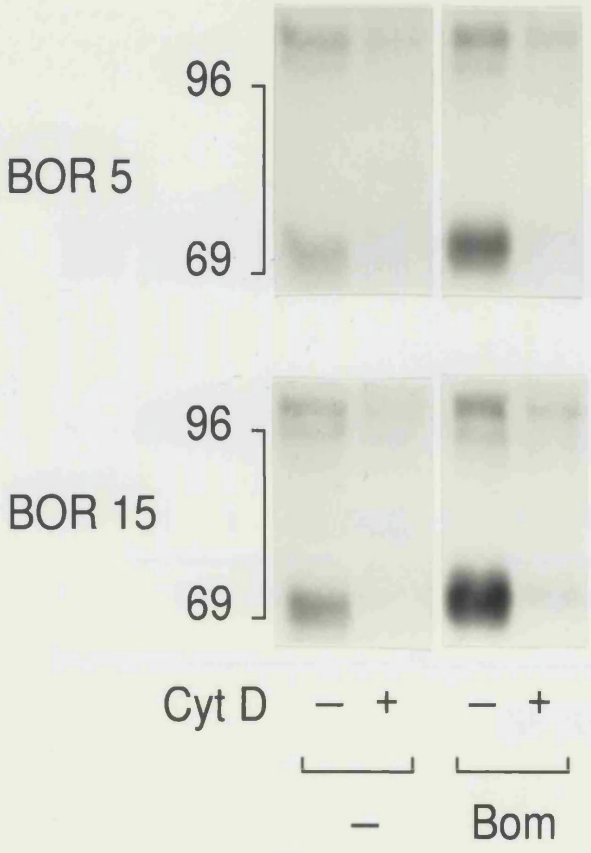

Figure 3.16. Effect of Cytochalasin D on bombesin-induced tyrosine phosphorylation of multiple proteins

Confluent, quiescent cultures of BOR 5 and BOR 15 cells were washed twice in DMEM and incubated at 37°C in DMEM with (+) or without (-) 2 μM cytochalasin D (Cyt D) for 2 h. Then 10 nM bombesin (Bom) was added as indicated for 10 min. Cells were lysed and tyrosine phosphorylated proteins immunoprecipitated with Py72 anti-Tyr(P) mAb and Western blotted with 4G10 anti-Tyr(P) mAb. Bands were visualised by autoradiography. Similar results were obtained in two independent experiments.

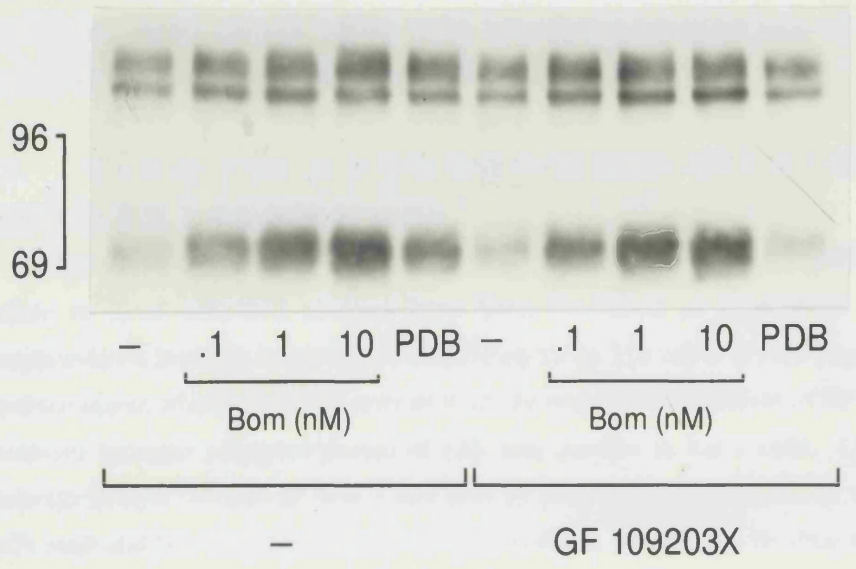

Figure 3.17. Effect of GF 109203X on bombesin-induced tyrosine phosphorylation of multiple proteins

Confluent, quiescent cultures of BOR 5 and BOR 15 cells were washed twice in DMEM and incubated at 37°C in DMEM with or without (-) 3.5 μM GF 109203X for 1 h. Then 0.1, 1 or 10 nM bombesin or 200 nM PDB was added as indicated for 10 min. Cells were lysed and tyrosine phosphorylated proteins immunoprecipitated with Py72 anti-Tyr(P) mAb and Western blotted with 4G10 anti-Tyr(P) mAb. Bands were visualised by autoradiography. Similar results were obtained in three independent experiments.

3.4.4. TYROSINE PHOSPHORYLATION INDUCED BY BOMBESIN IS INDEPENDENT OF PKC

We also verified that protein tyrosine phosphorylation was not downstream of PKC. Although the selective PKC inhibitor bisindolylmaleimide (GF 109203X) (Toullec, *et al.*, 1991) did prevent PDB-induced tyrosine phosphorylation of M_r 110,000 - 130,000 and 70,000 - 80,000 bands, it did not prevent bombesin-induced tyrosine phosphorylation in the transfected Rat-1 cells, figure 3.17.

3.4.5. BOMBESIN STIMULATES TYROSINE PHOSPHORYLATION OF FAK AND PAXILLIN IN BOR 5 AND BOR 15 CELLS

The cytosolic tyrosine kinase FAK and the adaptor protein paxillin which localise to focal adhesion plaques have been identified as prominent tyrosine phosphorylated proteins in bombesin-stimulated Swiss 3T3 cells. It was important to therefore assess whether agonist activation of the transfected bombesin/GRP receptor stimulates tyrosine phosphorylation of FAK and paxillin in Rat-1 cells. Lysates of bombesin-treated cultures of BOR 5 and BOR 15 were immunoprecipitated with anti-Tyr(P) mAb and the immunoprecipitates were analysed by Western blotting with anti-FAK mAb. Figure 3.18 and 3.19 show that bombesin induced tyrosine phosphorylation of FAK in a concentration dependent manner; half maximum and maximum effects were achieved at 0.3 nM and 3 nM respectively.

Parallel cultures of BOR 5 and BOR 15 cells treated with various concentrations of bombesin were lysed and the lysates were immunoprecipitated with anti-paxillin mAb. The immunoprecipitates were analysed by Western blotting with anti-Tyr(P) mAb. Figure 3.18 and 3.19 show that bombesin caused tyrosine phosphorylation of paxillin in a concentration dependent manner. The concentrations of bombesin that induced half-maximum and maximum stimulation were identical to those required to induce tyrosine phosphorylation of FAK (i.e. 0.3 and 3 nM respectively).

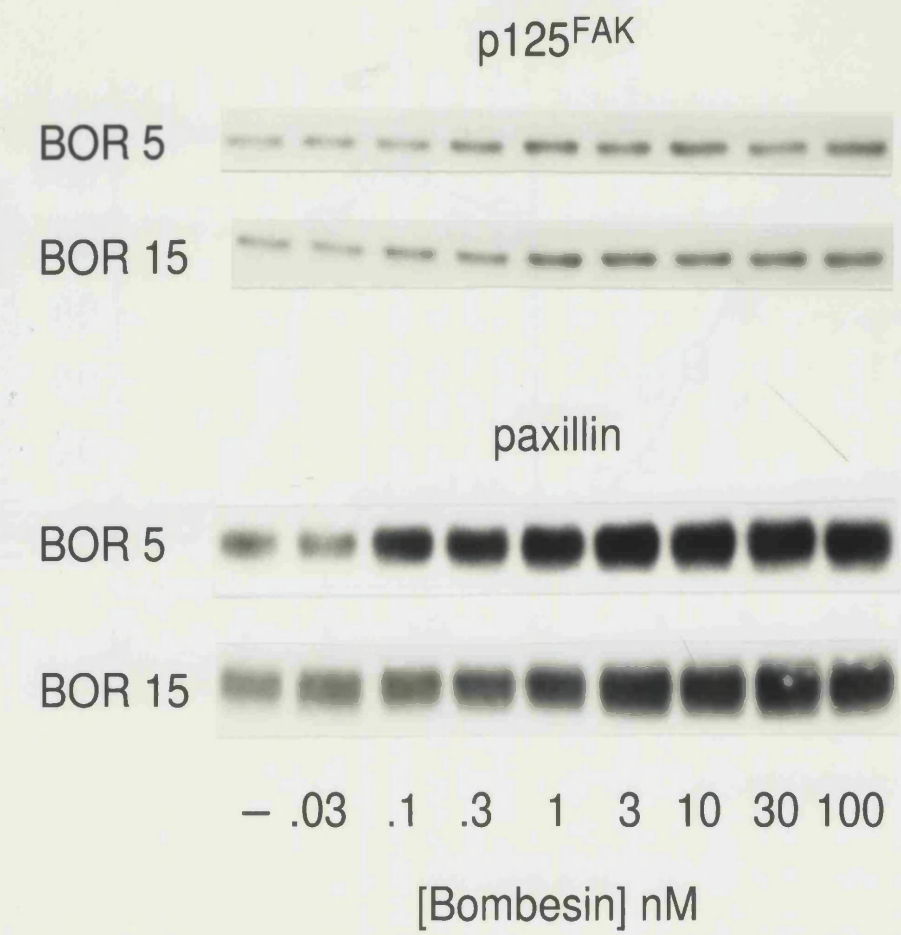

Figure 3.18. Bombesin-induced tyrosine phosphorylation of FAK and Paxillin (I)

FAK. Confluent, quiescent cultures of BOR 5 and BOR 15 cells were washed twice in DMEM and incubated at 37°C in DMEM with various concentrations of bombesin for 10 min. Cells were lysed and tyrosine phosphorylated proteins immunoprecipitated with Py72 mAb. Immunoprecipitates were resolved by SDS-PAGE and Western blotted with anti-p125^{FAK} mAb.
Paxillin. Confluent, quiescent cultures of BOR 5 and BOR 15 cells were washed twice in DMEM and incubated at 37°C in DMEM with various concentrations of bombesin for 10 min. Cells were lysed and paxillin immunoprecipitated with anti-paxillin mAb 165 and Western blotted with 4G10 anti-Tyr(P) mAb.
Similar results were obtained in three independent experiments.

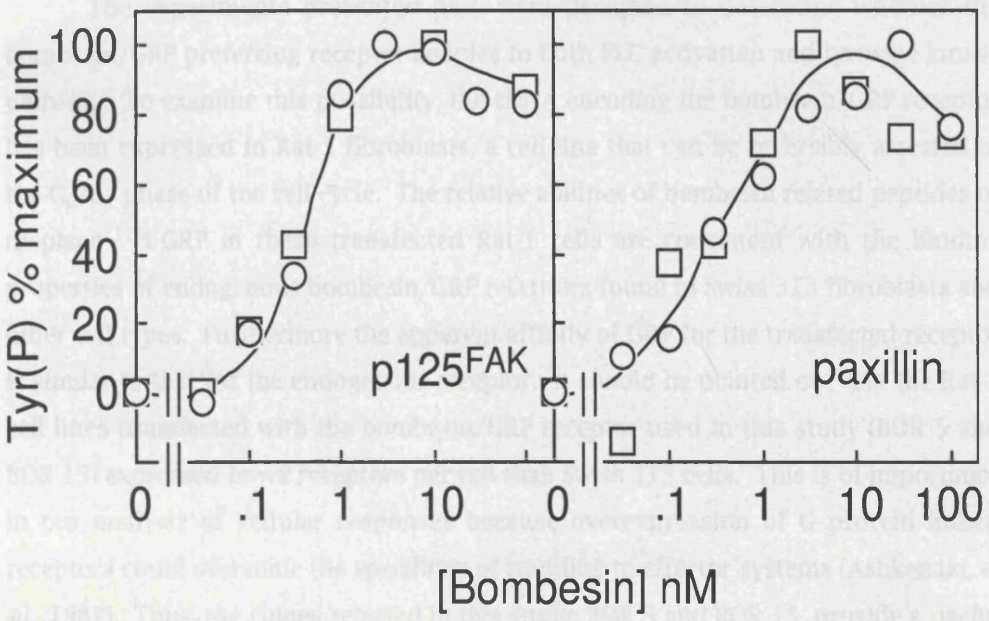

Figure 3.19. Bombesin-induced tyrosine phosphorylation of FAK and Paxillin (II)

Autoradiograms from the previous figure (3.18) were scanned with an LKB Ultrascan XL densitometer to quantify tyrosine phosphorylation in terms of peak area. BOR 5 is represented by squares and BOR 15 by circles. Results shown are from a representative experiment which was repeated three times with similar results. Values expressed as a percentage of the maximum tyrosine phosphorylation after subtraction of the control.

3.5. SUMMARY AND DISCUSSION

3.5.1. MULTIPLE COUPLING FROM ONE RECEPTOR

The experiments presented here were designed to determine whether the bombesin/GRP preferring receptor couples to both PLC activation and tyrosine kinase pathway. To examine this possibility, the cDNA encoding the bombesin/GRP receptor has been expressed in Rat-1 fibroblasts, a cell line that can be reversibly arrested in the G_0/G_1 phase of the cell cycle. The relative abilities of bombesin related peptides to displace ^{125}I-GRP in these transfected Rat-1 cells are consistent with the binding properties of endogenous bombesin/GRP receptors found in Swiss 3T3 fibroblasts and other cell types. Furthermore the apparent affinity of GRP for the transfected receptor is similar to that for the endogenous receptor. It should be pointed out that the Rat-1 cell lines transfected with the bombesin/GRP receptor used in this study (BOR 5 and BOR 15) expressed fewer receptors per cell than Swiss 3T3 cells. This is of importance in our analysis of cellular responses because overexpression of G protein linked receptors could overcome the specificity of coupling to effector systems (Ashkenazi, *et al.*, 1987). Thus, the clones selected in this study, BOR 5 and BOR 15, provide a useful system to examine the signalling pathways activated by the bombesin/GRP receptor.

Our results demonstrate that agonist binding to the transfected bombesin/GRP receptor elicits multiple responses in Rat-1 cells: 1) mobilisation of Ca^{2+} from internal stores leading to a rapid increase in $[Ca^{2+}]_{cyt}$ and increased phosphorylation of the 80K/MARCKS protein mediated by PKC, 2) reinitiation of DNA synthesis and cell proliferation in serum free medium and 3) tyrosine phosphorylation of multiple proteins including broad bands of M_r 110,000 - 130,000 and 70,000 - 90,000. We identified two major substrates in the transfected cells as FAK and paxillin. None of these responses were elicited by bombesin in the parental Rat-1 cells.

The characteristics of protein tyrosine phosphorylation induced by agonist binding to the bombesin/GRP receptor transfected into Rat-1 cells were examined in detail in two clones, BOR 5 and BOR 15. An increase in the tyrosine phosphorylation of multiple proteins including FAK and paxillin occurred at low concentrations of bombesin, the half maximum being 0.3 nM. This is similar to that observed in Swiss 3T3 cells (half maximum 0.08 - 0.3 nM) (Zachary, *et al.*, 1992; Sinnett-Smith, *et al.*, 1993; Zachary, *et al.*, 1993). Both Swiss 3T3 cells (Zachary, *et al.*, 1991a) and transfected Rat-1 cells show similar time dependence of tyrosine phosphorylation, increases are detected within seconds of bombesin addition, reach a maximum at 1 min and remain elevated for hours. Bombesin stimulation of tyrosine phosphorylation in BOR 5 and BOR 15 cells, as in Swiss 3T3 cells, is not prevented by the specific PKC inhibitor GF 109203X showing that this pathway is not downstream of PKC. FAK and

paxillin are localised in the focal adhesions which form at the termini of actin stress fibres and disruption of the actin cytoskeleton by cytochalasin D prevents tyrosine phosphorylation of these proteins in Swiss 3T3 cells (Sinnett-Smith, *et al.*, 1993; Zachary, *et al.*, 1993). In BOR 5 and BOR 15 cells bombesin-induced tyrosine phosphorylation of multiple proteins was also prevented by cytochalasin D. Therefore the protein tyrosine phosphorylation pathway activated by the transfected bombesin/GRP receptor in Rat-1 cells shows the same characteristics as that induced by bombesin stimulation of Swiss 3T3 cells.

In the transfected Rat-1 cells the increases in Ca^{2+} mobilisation, protein tyrosine phosphorylation and DNA synthesis were inhibited by the specific bombesin/GRP receptor antagonist, D-F_5-Phe^6, D-Ala^{11}-Bombesin(6-13)OMe (Coy, *et al.*, 1992), further substantiating that all these responses emanate from the same receptor. This is the first report demonstrating that ligand activation of a transfected bombesin/GRP receptor elicits multiple signalling pathways and leads to cell proliferation. This is also the first time that a G protein linked receptor retains the endogenous ability to activate different pathways and cell proliferation.

The results presented here raise important questions regarding the mechanism(s) by which a single seven transmembrane receptor subtype can couple to multiple signalling pathways. While the mechanism by which bombesin/GRP receptor and other seven-transmembrane domain receptors couple to PLC through heterotrimeric G proteins of the G_q subfamily is increasingly understood (Lee and Rhee, 1995), little is known about the pathways coupling the bombesin/GRP receptor to tyrosine phosphorylation of FAK and paxillin.

Recent work has implicated activation of the monomeric G protein of the p21^{rho} subfamily as one step in the signalling pathway leading to tyrosine phosphorylation of these proteins (Rankin, *et al.*, 1994; Seckl, *et al.*, 1995). Thus, our results suggest that the bombesin/GRP receptor couples to both heterotrimeric (G_q) and monomeric (p21^{rho}) G proteins.

3.5.2. MULTIPLE COUPLING VIA βγ SUBUNITS

The demonstration that certain seven transmembrane domain receptors couple to p21^{ras} via βγ subunits of the heterotrimeric G protein of the G_i subfamily (see section 1.3.8.7) suggests a possible coupling mechanism between the bombesin/GRP receptor and p21^{rho}. It is thought that βγ from G_i activate Ras by stimulating the phosphorylation of the adaptor protein Shc which causes the formation of the Shc/Grb2/mSos complex. Sos then exchanges GDP for GTP on ras. Other GEFs (C3G, Vav) can associate with other adaptors (Crk, Crk-l) (Smit, *et al.*, 1996) and p21^{rho} is also regulated by GEFs and GAPs (Takai, *et al.*, 1995). Thus by analogy, the βγ subunits

generated by activation of G_q/G_{11} could be preferentially coupled to activation of $p21^{rho}$ and thereby to the tyrosine phosphorylation pathway, whereas the α subunit of G_q stimulates PLC (Lee and Rhee, 1995). This would imply specificity of $\beta\gamma$ complexes coupling to different $G\alpha$ and activating different effectors.

Section 1.1.6.4 presents some evidence for $\beta\gamma$ specificity but this is not well documented at the functional level. In the context of activation of $p21^{rho}$, a limited selection of combinations were tested which did not induce stress fibre formation, see table 1.IV. Intriguingly, specificity in localisation is demonstrated for γ_5, which has been shown by immunostaining to localise to focal adhesions and stress fibres. In contrast γ_3 had a more diffuse cellular distribution (Hansen, *et al.*, 1994). It is unclear if this of functional importance.

3.5.3. MULTIPLE COUPLING VIA A DISTINCT Gα SUBUNIT

Alternatively bombesin-induced tyrosine phosphorylation of FAK and paxillin could be mediated by a $G\alpha$ subunit. There is evidence which suggests the involvement of α_q. For example, agonists which cause tyrosine phosphorylation of FAK including bombesin, vasopressin, bradykinin, endothelin, angiotensin, carbachol (M_1, M_3 receptor subtypes) and norepinephrine (α_1 receptor subtypes) (Huckle, *et al.*, 1990; Force, *et al.*, 1991; Leeb-Lundberg and Song, 1991; Tsuda, *et al.*, 1991; Zachary, *et al.*, 1991a; Gutkind and Robbins, 1992), bind to receptors which have been shown to couple to G_q (Gutowski, *et al.*, 1991; Berstein, *et al.*, 1992; Offermanns, *et al.*, 1994a). Although thrombin and LPA couple to the Ras/MAPK through a pertussis toxin-sensitive G_i signalling pathway (Van Corven, *et al.*, 1993; Hordijk, *et al.*, 1994) they can activate PLCβ via pertussis toxin-insensitive pathways (Murayama and Ui, 1985; van Corven, *et al.*, 1989; Grand, *et al.*, 1996). Receptors which couple solely to G_i, for example M_2-Ach and M_4-Ach receptors, are not associated with tyrosine phosphorylation of FAK (Gutkind and Robbins, 1992) and adrenomedullin, which elevates cAMP, also does not stimulate protein tyrosine phosphorylation (Withers, *et al.*, 1996). Thus tyrosine phosphorylation is associated with receptors coupled to G_q. Also it has been recognised for some time that Ca^{2+} and PIP_2 have important roles to play in cell cytoskeletal arrangements (Janmey, 1994). 80K/MARCKS crosslinks actin and this is inhibited by Ca^{2+}/CaM and PKC-dependent phosphorylation. Aderem (1992), proposed that when cells are stimulated with mitogenic peptides the ability of 80K/MARCKS to crosslink actin is reduced and actin around the cell membrane becomes destabilised. This could allow cellular shape changes required during the mitotic cycle to occur. Interestingly $G\alpha_q$ and $G\alpha_{11}$ have been shown to associate with actin although it is not known if this is of any functional consequence (Ibarrondo, *et al.*, 1995).

It is established that the bombesin/GRP receptor couples to G_q (Offermanns, *et al.*, 1994a). However, it has been convincingly shown that bombesin-induced tyrosine

phosphorylation of FAK is PKC- and Ca^{2+}-independent (see section 1.3.7.8). Another explanation could be that one domain of the bombesin/GRP receptor may couple to G_q and thereby to PLC, whereas a separate domain could lead to activation of $p21^{rho}$ and to the tyrosine phosphorylation of FAK and paxillin through interaction with a different heterotrimeric G-protein.

The concept of a single receptor subtype coupling to multiple G proteins is not without president. Multiple coupling can be observed when components are ectopically expressed and these results must be interpreted with caution. In one study the α_2-AR was shown to couple to G_s, G_i and G_q (Chabre, *et al.*, 1994), however, both receptors and G proteins were transfected, potentially compromising specificity. Also coupling of the M_2 receptor to PLC was shown to be dependent on the number of receptors expressed (Ashkenazi, *et al.*, 1987). It has already been stressed that the bombesin/GRP receptor in the present study was not over-expressed. Table 3.I shows receptors for which multiple G protein coupling has been demonstrated. These are single receptor subtypes defined by transfection. Several methods have been used to show *bona fida* coupling to more than one G protein and not, for example, $\beta\gamma$ signalling. These include comparisons with other G protein-coupled receptor which couple specifically to only one pathway in the same cell system; the use of pertussis toxin if the G_i family was involved, and more recently photolabelling with [α-^{32}P]GTP azidoanilide and specific immunoprecipitation of α subunits.

Table 3.I. Seven transmembrane receptors that couple to multiple G proteins

Receptor	Gα	comment	ref.
parathyroid	G_s, G_q	compared to other GPCR	a
thrombin	G_i, G_q	PTX sensitivities	b
LH	G_s, G_q	compare to other GPCR	c
Calcitonin	G_s, G_q	compare to other GPCR	d
α_2-AR	G_i, G_s	PTX, CTX, immunoprecipitation	e
TSH	$G_{s(s,l)}$, $G_{(q,11)}$	photolabel	f
A_3 adenosine	G_i, G_q	photolabel	g
TSH	G_i, $G_{12,13}$, G_q	photolabel	h

Refs.: **a** (Abou-Samra, *et al.*, 1992); **b** (Hung, *et al.*, 1992); **c** (Gudermann, *et al.*, 1992); **d** (Chabre, *et al.*, 1992); **e** (Eason, *et al.*, 1992); **f** (Allgeier, *et al.*, 1994); **g** (Palmer, *et al.*, 1995); **h** (Laugwitz, *et al.*, 1996). GPCR, G protein-coupled receptor; PTX, pertussis toxin; CTX, cholera toxin.

Where it has been measured, the EC_{50} of the receptor agonist for the activation of the Gα effector is shown in table 3.II. Interestingly different pathways are activated at different concentrations of agonist with $G\alpha_q$ mediated events occurring at higher ligand concentration.

Table 3.II. Comparison of receptor-ligand EC_{50} values between different Gα mediated signals

Receptor	EC_{50} of receptor agonist for G protein activation		
LH	G_s = 0.8 nM	G_q = 2.4 nM	
Calcitonin	G_s = 0.16 nM	G_q = 3.7 nM	
α_2-AR	G_i = 0.16 μM	G_s = 17 μM	
TSH	G_i = 3 nM	$G_{12,13}$ = 10 nM	G_q = 30 nM

Refs. as in previous table.

If the effects of bombesin/GRP on cellular events measured in this laboratory in Swiss 3T3 cells are examined more carefully an interesting pattern emerges (see table 3.III). Effects which are dependent on G_q mediated effects such as PKC and Ca^{2+} have an $EC_{50} \approx 1$ nM, whereas effects which are PKC-independent occur at $EC_{50} \approx 0.3$ nM. This difference in EC_{50} values could reflect coupling to a different Gα subunit. Thus the bombesin/GRP receptor could couple to rho and tyrosine phosphorylation of FAK and paxillin via different heterotrimeric G-proteins than those which couple it to PLC.

It has already been documented that G protein-coupled receptors are designed to interact with more than one G protein. Mutational studies within the third intracellular loop show that different Gα require different stretches of amino acids on the receptor to bind. For example in the α_{2A}-AR-C10 amino acids 218-228 are required for G_s coupling but they do not affect G_i coupling (Eason and Liggett, 1995). Also in the α_1-AR distinct areas are required for coupling to different members of the G_q family (Wu, *et al.*, 1995b) see figure 3.20.

Table 3.III. Comparison of EC_{50} values of cellular effects induced by bombesin

Effector	EC_{50} [Bom] nM	PKC/Ca^{2+}	ref.
PKC activation	0.4 - 0.6	+	a
Ca^{2+} mobilisation	1	+	b
inositol accumulation	>2.5	+	c
AA release	4	N/D	d
cAMP enhancement	1	+	e
fos induction	1	+	f
DNA synthesis	1	+	g
DNA synthesis + Ins	0.3	-	h
Na^+/H^+ exchange	0.4	±	i
Na^+/K^+ exchange	0.4	±	j
general Tyr(P)	0.3	-	k
Tyr kinase activity	0.1	-	l
FAK-Tyr(P)	0.08 - 0.3	-	m
paxillin-Tyr(P)	0.09	-	n

Refs.: **a** (Zachary, *et al.*, 1986); **b** (Mendoza, *et al.*, 1986; Lopez-Rivas, *et al.*, 1987); **c** see table 5.II, this thesis; **d** (Millar and Rozengurt, 1990a); **e** (Millar and Rozengurt, 1988); **f** (Rozengurt and Sinnett-Smith, 1987; Mehmet, *et al.*, 1990); **g** and **h** (Rozengurt and Sinnett-Smith, 1983; Rozengurt and Sinnett-Smith, 1987); **i** (Mendoza, *et al.*, 1986); **j** (Mendoza, *et al.*, 1986); **k** (Zachary, *et al.*, 1991a); **l** (Zachary, *et al.*, 1991b); **m** (Zachary, *et al.*, 1992; Sinnett-Smith, *et al.*, 1993); **n** (Zachary, *et al.*, 1993). N/D, not done.

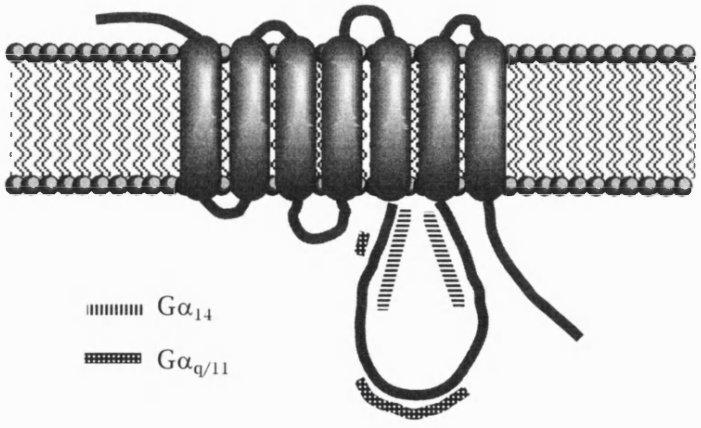

Figure 3.20. Different areas in the 3rd cytoplasmic loop of α_{2A}-AR-c10 are required for coupling to $G\alpha_{14}$ vs $G\alpha_{q/11}$

If the bombesin/GRP receptor is coupling to a second Gα then the recent demonstration that microinjected $G\alpha_{12}$ and $G\alpha_{13}$, but not $G\alpha_q$, induce Rho-dependent stress fibre assembly is a potential clue (Buhl, *et al.*, 1995). G_{12} and G_{13} were originally identified by homology cloning (Strathmann and Simon, 1991). They comprise a fourth class of α subunit and, like G_q, are unable to be modified by pertussis toxin. Expression of activated mutants of G_q, G_{12} and G_{13} increases Na^+/H^+ exchange causing cellular alkalisation (Dhanasekaran, *et al.*, 1994; Voyno-Yasenetskaya, *et al.*, 1994a). The effect of α_q and α_{12} on Na^+/H^+ exchange is PKC-dependent but that of α_{13} is not (Dhanasekaran, *et al.*, 1994). Interestingly, table 3.III shows that in Swiss 3T3 cells bombesin stimulates tyrosine phosphorylation and Na^+/H^+ exchange with a similar low (sub-nanomolar) EC_{50}. Also bombesin-induced tyrosine phosphorylation is PKC-independent and Na^+/H^+ exchange is partially PKC-independent. These observations are consistent with bombesin coupling to a $G_{12/13}$-like G protein.

Agonists which have already been shown to activate G_{12} and G_{13} by [α-^{32}P]GTP azidoanilide photolabelling are thromboxane A_2, thrombin and TSH. In each case they also cause GTP labelling of G_q (Offermanns, *et al.*, 1994b; Laugwitz, *et al.*, 1996). However it was not shown categorically that there was only one receptor involved. It is assumed that there is only one TSH receptor because the transfected receptor couples to both cAMP and PLC regulation (van Sande, *et al.*, 1990), but Offermanns, *et al.* (1994b) points out that in their study multiple receptors for thromboxane A_2 and thrombin cannot be excluded. Indeed the possibility of multiple thrombin receptors is debated (reviewed in Grand, *et al.*, 1996). This controversy stems from the differing intracellular events induced by thrombin and the thrombin receptor agonist peptide (Seiler, *et al.*, 1991; Vouret-Craviari, *et al.*, 1993), and see section 1.4. In addition recent experiments with cells derived from a thrombin receptor knockout mouse support the existence of additional thrombin receptors (Connolly, *et al.*, 1996).

In summary, this is the first time that a seven transmembrane receptor, defined by transfection, has been shown to couple to multiple signal transduction pathways and induce cell growth.

Chapter 4 - The MAPK Pathway Stimulated By Bombesin in Rat-1 Cells

MAPK is a common point of convergence for most mitogenic signalling pathways but there are exceptions, see Withers, *et al.* (1995). The mechanism by which G protein-coupled receptors activate the MAPK kinase pathway is beginning to be unravelled. It is clear that receptors coupled to G_i such as M_2-Ach, thrombin, LPA and α_2-AR activate Ras via $\beta\gamma$ subunits, but the situation for receptors linked to G_q is less clear. Some reports document G_q-coupled receptors activating MAPK via PKC dependent routes, however there are reports of these receptors utilising ras sometimes via $\beta\gamma$ subunits similar to G_i-coupled receptors. Section 1.3.8.9 described how controversial the literature is regarding G_q-coupled receptors especially regarding Ras versus PKC routes to MAPK and some of this can be explained by cellular context.

In Swiss 3T3 cells bombesin-induced MAPK is completely dependent on PKC (Pang, *et al.*, 1993) and does not increase Ras.GTP loading (Satoh, *et al.*, 1990b; Mitchell, *et al.*, 1995) Neither does it activate Raf-1 (Mitchell, *et al.*, 1995; Seufferlein, *et al.*, 1996). Thus bombesin activates MAPK via PKC and not Ras. Consistent with this is the observation that in COS 7 cells bombesin-stimulated MAPK is not dependent on $\beta\gamma$ subunits (Faure, *et al.*, 1994). It remains to be demonstrated that bombesin can activate ras.

In Rat-1 cells stimulation of the CCK_B receptor causes the activation of Raf-1 via a PKC-independent mechanism (Seufferlein, *et al.*, 1995). This could mean that peptide receptors coupled to G_q in Rat-1 cells could activate MAPK via Ras. The transfected bombesin/GRP receptor confers a mitogenic response to bombesin in Rat-1 cells, so the effect of bombesin on the activation of MAPK was examined. The results in this chapter show that bombesin can activate MAPK and Raf-1 activity in a PKC-independent manner. In addition it is shown that bombesin activates Ras.GTP loading in Rat-1 cells transfected with the bombesin/GRP receptor.

4.1. Bombesin Stimulation of MAPK Activity in Rat-1 Cells Transfected with the Bombesin/GRP Receptor

4.1.1. Bombesin Stimulates MAPK Activity

First it was established that bombesin did stimulate MAPK in Rat-1 cells transfected with the bombesin/GRP receptor. Quiescent BOR 15 cells were treated with various factors for 5 min then the were cells lysed. $p42^{mapk}$ was

immunoprecipitated and subjected to an *in vitro* immunecomplex kinase assay, using MBP peptide as a substrate. The results in figure 4.1 show that bombesin activated MAPK to the same level as EGF, which was approximately 15-fold over background levels. In contrast PDB activated MAPK to a level that was 30% of that induced by bombesin and EGF.

4.1.2. DOSE RESPONSE OF BOMBESIN-INDUCED MAPK ACTIVITY IN BOR 15 CELLS

Bombesin stimulation of MAPK was then examined more closely. BOR 15 cells were treated with increasing concentrations of bombesin for 5 min then MAPK was immunoprecipitated and kinase activity measured as above. Figure 4.2 shows that bombesin induced a concentration dependent increase in MAPK activity the half maximum and maximum concentration being 0.3 and 10 nM respectively. Thus bombesin stimulated a robust increase in MAPK activation in the transfected Rat-1 cells.

4.1.3. BOMBESIN-STIMULATED MAPK ACTIVITY IS INDEPENDENT OF PKC IN BOR 15

In view of the role of PKC in G_q-mediated MAPK activation in some cell types, it was important to assess the contribution of PKC to the MAPK response induced by bombesin in bombesin/GRP receptor transfected Rat-cells. This was examined both by down regulation of phorbol ester-sensitive isoforms of PKC by prolonged treatment with 800 nM PDB, and by use of the specific PKC inhibitor GF 109203X. The specific PKC inhibitor, GF 109203X, is a bisindolylmaleimide which selectively inhibits PKC (assayed on isoforms α, βI, βII and γ) by competing with ATP for the ATP binding site (Toullec, *et al.*, 1991). Subsequently it has also been shown to inhibit other DAG-regulated PKC isoforms δ and ε (Yeo and Exton, 1995). The results presented in figure 4.3 show that phorbol ester-mediated MAPK activation was significantly attenuated by GF 109203X (66% inhibition) and completely abolished by chronic pretreatment with PDB. In contrast EGF, which does not activate PKC in Rat-1 cells was not significantly inhibited by PKC inhibition and only slightly by PKC down regulation. Similarly bombesin-induced MAPK was not significantly inhibited by GF 109203X or pretreatment with PDB.

Thus the results in figures 4.1 - 4.3 demonstrate that bombesin induces a strong stimulation of MAPK activity in Rat-1 cells transfected with the bombesin GRP receptor and this is a PKC-independent event.

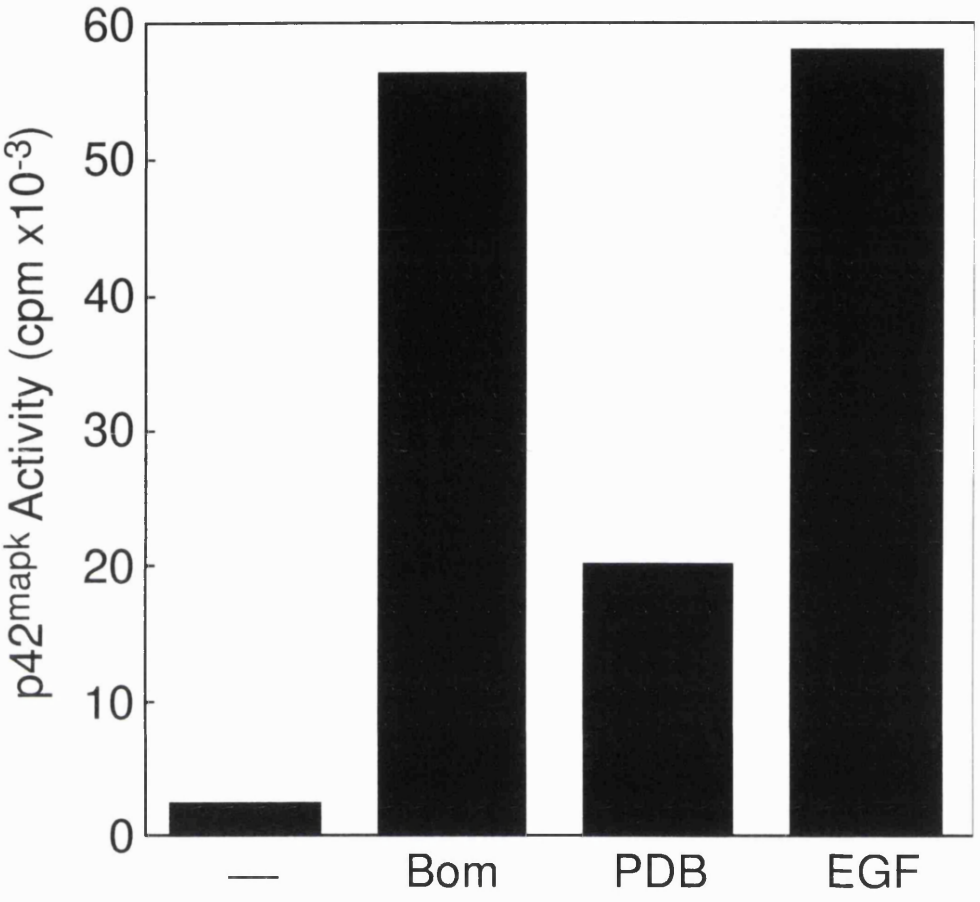

Figure 4.1. p42^{mapk} activation induced by various factors

BOR 15 cells in 100 mm dishes were washed twice with DMEM and incubated in 5 ml DMEM without (–) or with 1 nM Bombesin (Bom), 200 nM PDB or 5 ng/ml EGF for 5 min at 37°C. Cells were lysed and p42^{mapk} was immunoprecipitated with polyclonal anti-p42^{mapk} antibodies. MAPK activity was measured by an *in vitro* kinase assay using MBP peptide as a substrate as described in materials and methods. Results shown are from one experiment performed in duplicate. Similar results were obtained in two independent experiments.

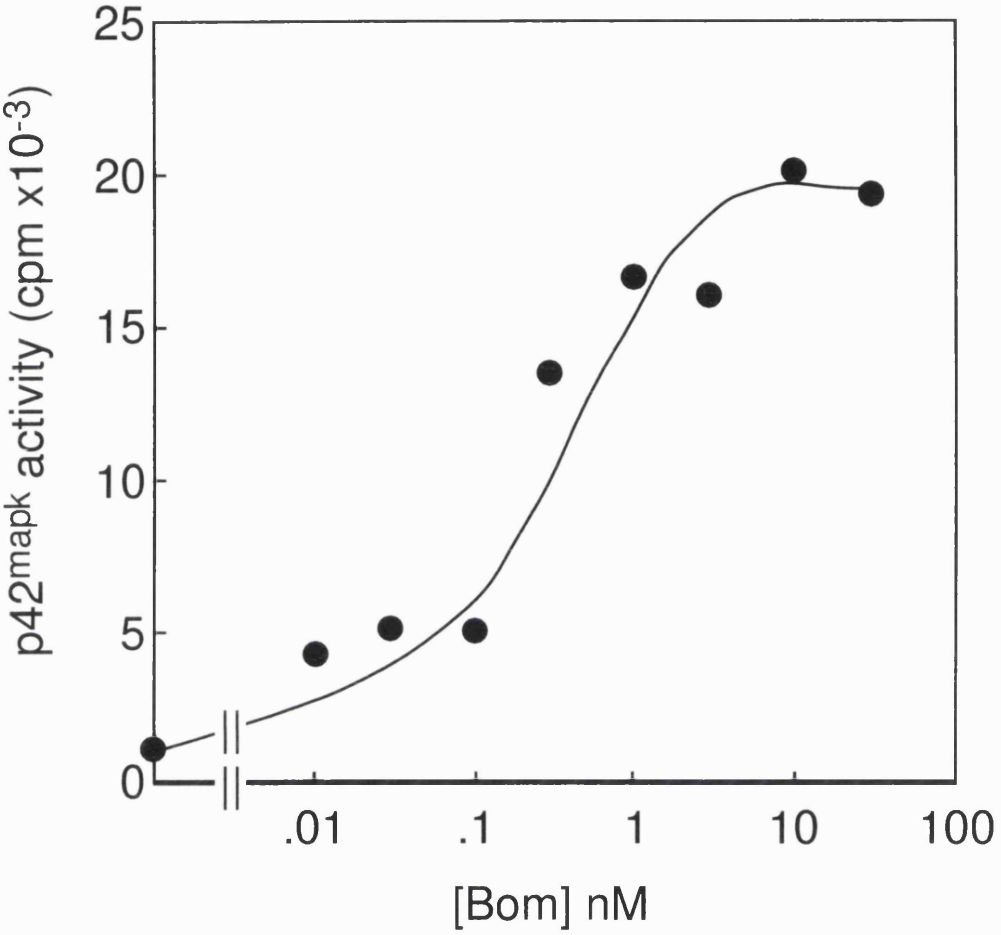

Figure 4.2. Bombesin-induced increase in p42^{mapk} activity

BOR 15 cells in 100 mm dishes were washed twice in DMEM and incubated in 5 ml DMEM with increasing concentrations of bombesin for 5 min at 37°C. p42^{mapk} was immunoprecipitated followed by an *in vitro* kinase reaction as above. Results shown are the mean of two independent experiments that were performed in duplicate.

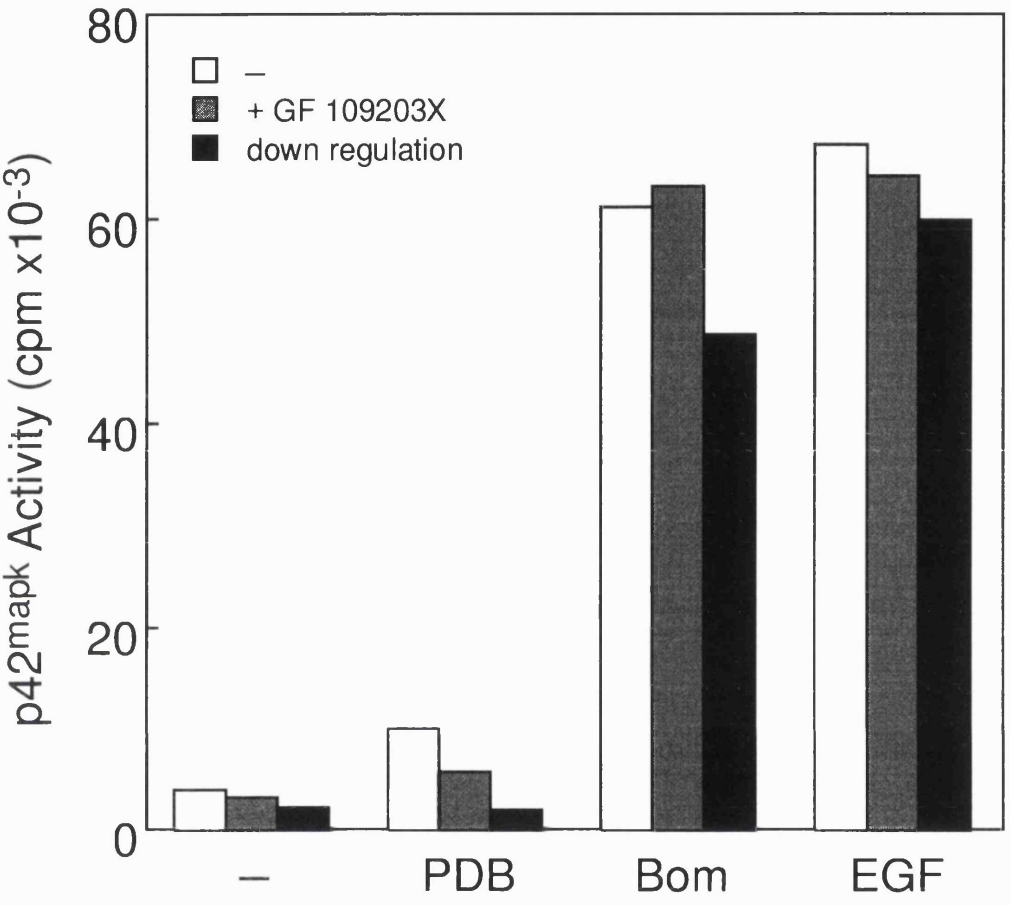

Figure 4.3. Effect of PKC inhibition and down regulation on bombesin-stimulated MAPK activity

5 day-old cells were pretreated with (black bars) or without 800 nM PDB for 48 h. Then all the cells were washed twice in DMEM and incubated in 5 ml DMEM for 1 h with (grey bars) or without 3.5 μM GF 109203X. The cells were stimulated without (–) or with 100 nM PDB, 1 nM bombesin (Bom) or 5 ng/ml EGF for 5 min at 37°C. The cells were lysed and $p42^{mapk}$ was immunoprecipitated and kinase activity assayed as above. Results shown are from one representative experiment that was performed in duplicate. Similar results were obtained in two independent experiments.

4.2. BOMBESIN STIMULATION OF RAF-1 ACTIVITY IN TRANSFECTED RAT-1 CELLS

Next, the upstream signals were investigated. The prototypical MEK activator is Raf-1, although other Raf and non-Raf MEK activators exist. In Rat-1 cells the G_q-coupled M_1-Ach receptor does not activate Raf-1 (Russell, *et al.*, 1994) and in Swiss 3T3 cells bombesin does not activate Raf-1 (Mitchell, *et al.*, 1995). This would suggest that bombesin/GRP receptor transfected into Rat-1 cells would not activate Raf-1. However receptors that are coupled to G_q have been shown to activate Raf-1 (Seufferlein, *et al.*, 1995) or require functional Raf-1 to activate MAPK (Hawes, *et al.*, 1995). The ability of bombesin to stimulate Raf-1 activity in transfected Rat-1 cells was determined.

4.2.1. BOMBESIN STIMULATES RAF-1 ACTIVATION IN BOR 15 CELLS

Quiescent BOR 15 cells were stimulated with 1 nM bombesin for increasing periods of time. The cells were lysed and Raf-1 was immunoprecipitated. Raf-1 immunocomplexes were incubated with GST-MEK and recombinant ERK2 proteins in the presence of ATP to reconstitute the MAPK cascade *in vitro*. Recombinant ERK2 was then collected and used to phosphorylate MBP peptide in the presence of γ-^{32}P-ATP as above. The results are shown in figure 4.4. Bombesin induced a rapid and transient activation of Raf-1 kinase activity. The maximum activation occurred 3 min after bombesin stimulation and was nearly all over by 10 min.

4.2.2. BOMBESIN-INDUCED RAF-1 ACTIVATION IS NOT DEPENDENT ON PKC

Receptors coupled to G_q activate PKC and PKC has been proposed to directly activate Raf-1. In addition phorbol esters can cause activation of Raf-1 in at least some cell types. However, in Rat-1 cells the CCK_B receptor activated Raf-1 in a PKC-independent manner (Seufferlein, *et al.*, 1995). Therefore the effect of GF 109203X was examined on bombesin stimulation of Raf-1 activity. Quiescent BOR 15 cells were pretreated with 3.5 μM GF 109203X for 1 h prior to stimulation by various factors for 3 min. The results are shown in figure 4.5. In Rat-1 cells PDB activates MAPK 3-7 fold above unstimulated levels (figures 4.1 and 4.3). However figure 4.5 shows that PDB does not stimulate Raf-1 activity in Rat-1 cells. Raf-1 activity stimulated by EGF, which does not stimulate PKC in Rat-1 cells, was not inhibited by the specific PKC inhibitor GF 109203X. In fact, a small stimulation was noticed in every case suggesting that GF 109203X eliminates a constitutive feed-back loop in Rat-1 cells. Similarly, bombesin-induced Raf-1 activity was not affected by the PKC inhibitor demonstrating that bombesin activates Raf-1 via a PKC-independent route.

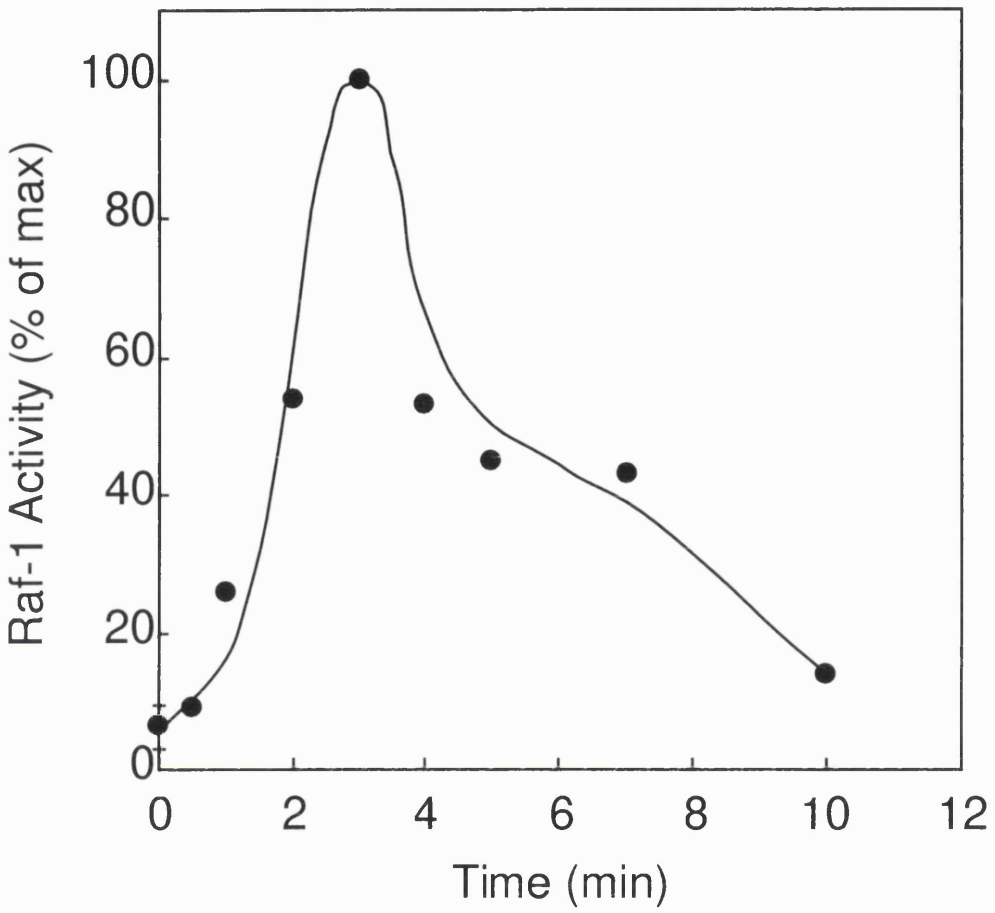

Figure 4.4. Time course of bombesin-induced Raf-1 activity

BOR 15 cells in 100 mm dishes were washed twice in DMEM and incubated in 5 ml DMEM with 1 nM bombesin for increasing times at 37°C. Cells were lysed and Raf-1 was immunoprecipitated with polyclonal C12 anti-Raf-1 antibody. Raf-1 activity was measured by a two stage *in vitro* immunecomplex kinase assay as described in materials and methods. Results shown are the mean of two independent experiments performed in duplicate.

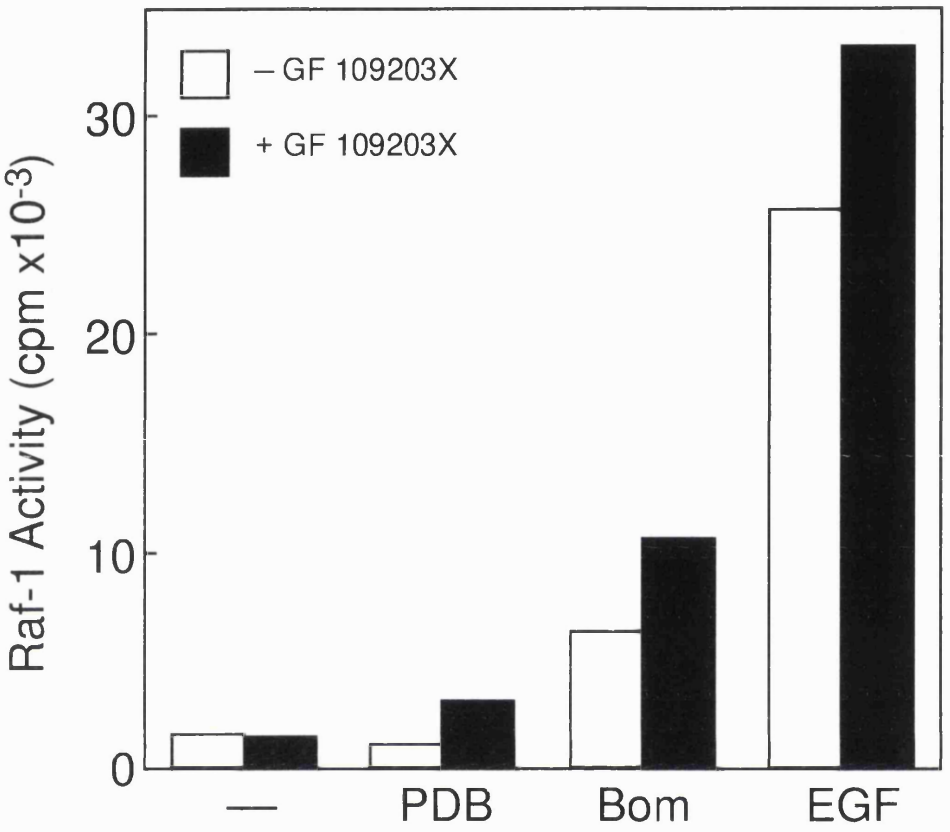

Figure 4.5. Effect of PKC inhibition on Raf-1 activity induced by various mitogens.

BOR 15 cells in 100 mm dishes were washed twice with DMEM and incubated in 5 ml DMEM for 1 h with (closed bars) or without (open bars) 3.5 μM GF 109203X. Cells were then stimulated for 3 min at 37°C without (–) or with 1 nM bombesin (Bom), 200 nM PDB or 5 ng/ml EGF. Raf-1 was immunoprecipitated and kinase activity measured as above. Results shown are from one representative experiment performed in duplicate. Similar results were obtained in two independent experiments.

4.2.3. PERTUSSIS TOXIN DOES NOT INHIBIT BOMBESIN-INDUCED RAF-1 ACTIVITY

G protein-coupled receptors that do not activate MAPK via PKC-dependent mechanisms are often coupled to G_i and in the previous chapter evidence of coupling to multiple G-proteins was presented. Coupling to G_i can be identified with the use of pertussis toxin which will ADP-ribosylate a specific arginine residue in $G\alpha_i$ thus preventing its activation, see section 1.1.6.5. This also means that the α_i does not dissociate from the receptor or its $\beta\gamma$ subunit, and hence pertussis toxin can block both α_i and $\beta\gamma$ signalling. To assess whether bombesin-induced activation of Raf-1 was mediated by a G protein from the G_i family, BOR 15 cells were pretreated for 3 h with 30 ng/ml pertussis toxin and then stimulated with bombesin, LPA or EGF for 3 min. LPA stimulates the MAPK pathway and Raf-1 activity in a pertussis toxin-sensitive fashion (Hordijk, *et al.*, 1994; Seufferlein, *et al.*, 1995). In agreement with this, treatment of BOR 15 cells with pertussis toxin inhibited LPA-induced Raf-1 activity (figure 4.6). In contrast EGF, which signals through a receptor tyrosine kinase that does not use G_i proteins, was not affected by pertussis toxin pretreatment. Similarly bombesin-induced Raf-1 activation was not inhibited by pretreatment with pertussis toxin.

The results in figures 4.4 - 4.6 demonstrate that ligand activation of the bombesin/GRP receptor transfected into Rat-1 fibroblasts induces Raf-1 kinase activity which is pertussis toxin-insensitive and PKC-independent.

4.3. BOMBESIN STIMULATION OF RAS.GTP LOADING IN TRANSFECTED RAT-1 CELLS

Because bombesin-induced stimulation of Raf-1 activity was PKC-independent in Rat-1 transfected with the bombesin/GRP receptor cells the possibility that it could activate Ras was investigated. Quiescent BOR 15 cells were labelled overnight with ^{32}P-orthophosphate and then stimulated for 2.5 min with various factors. Ras was immunoprecipitated and the bound guanine nucleotides were eluted and separated by thin layer chromatography. The results are shown in figures 4.7 and 4.8. Bombesin increased the proportion of Ras in the GTP bound form from 28% to 43% (a 50% increase). This was half the level of EGF-induced stimulation which increased Ras.GTP from 28% to 61% (a 115% increase). Thus activation of the transfected bombesin/GRP receptor stimulated the GTP loading of Ras.

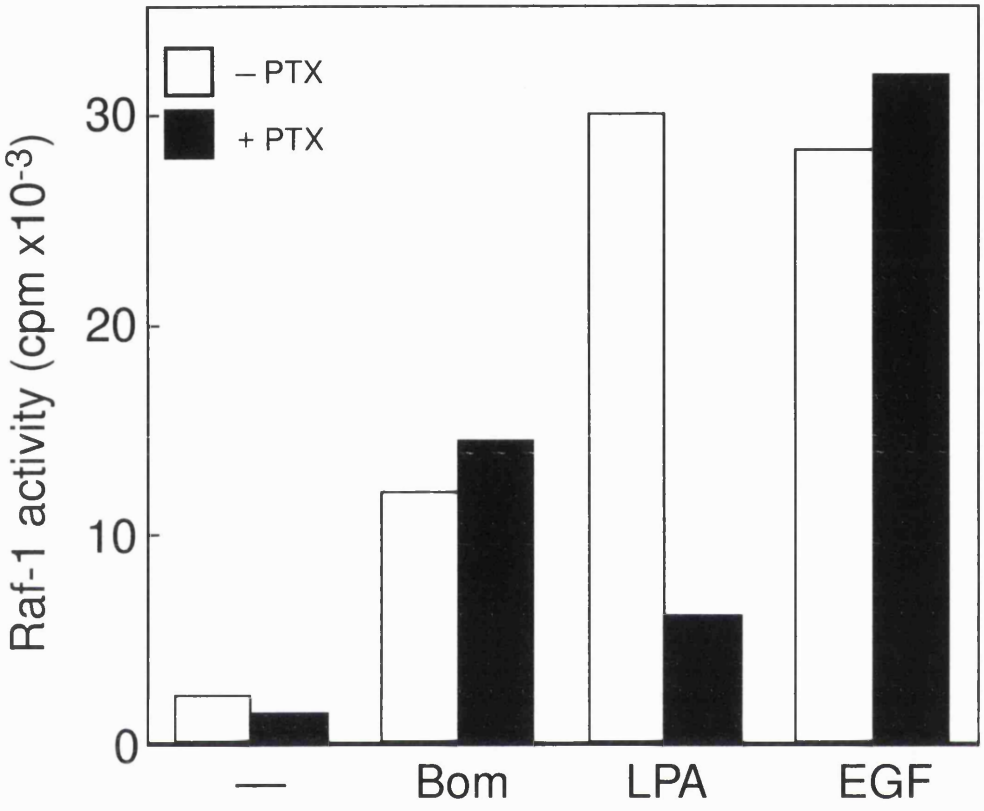

Figure 4.6. Effect of pertussis toxin on Raf-1 activity induced by various mitogens.

BOR 15 cells in 100 mm dishes were washed twice in DMEM and incubated in 5 ml DMEM with (closed bars) or without (open bars) 30 ng/ml pertussis toxin for 4 h. Cells were then stimulated for 3 min at 37°C without (–) or with 1 nM bombesin (Bom), 2 μM LPA or 5 ng/ml EGF. Raf-1 was immunoprecipitated and kinase activity measured as above. Results shown are from one experiment that was performed in duplicate.

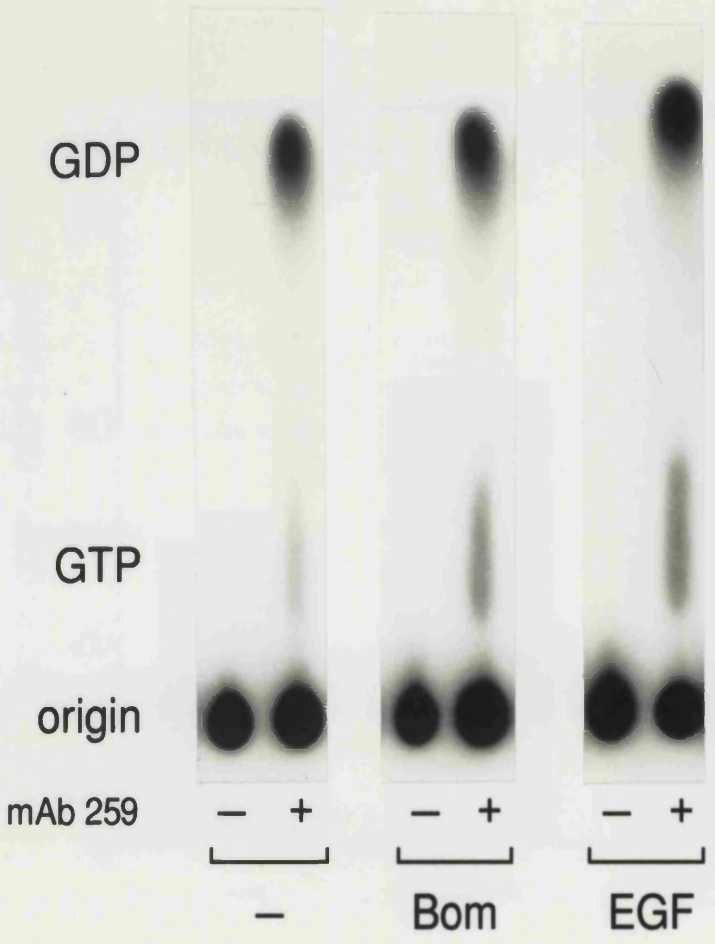

Figure 4.7. Bombesin stimulation of loading (I)

BOR 15 cells in 100 mm dishes were washed twice in DMEM without phosphate and incubated overnight in 5 ml DMEM without phosphate supplemented with 1 mg/ml BSA, 20 mM HEPES and 1.6 mCi orthophosphate. Cells were stimulated without (–) or with 1 nM bombesin (Bom) or 5 ng/ml EGF for 2.5 min at 37°C. The cells were lysed and ras was immunoprecipitated with mAb 259 in duplicate (+) with non-specific controls (–) from the detergent phase as described in materials and methods. The guanine nucleotides were eluted from ras and resolved by thin layer chromatography on PEI cellulose plates as described in materials and methods and visualised by autoradiography. Results shown are from one representative experiment that was performed with duplicate immunoprecipitations from duplicate cultures. Similar results were obtained in 2 independent experiments.

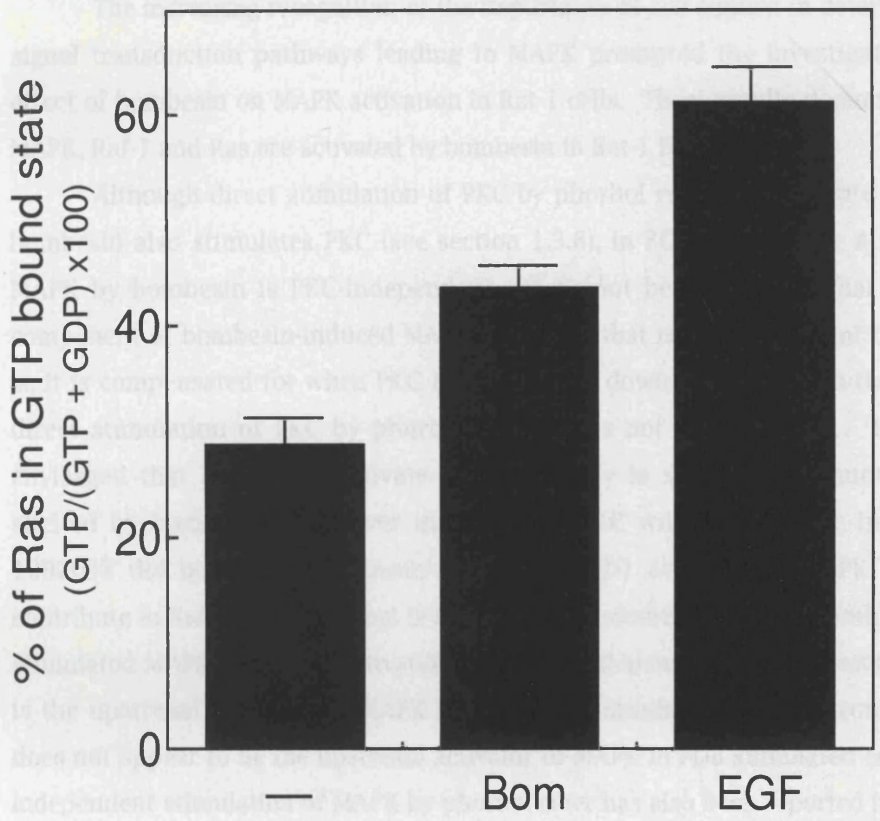

Figure 4.8. Bombesin stimulation of Ras.GTP loading (II)

Labelled nucleotides from the previous figure (4.7.) were quantified in terms of total volume with a Phosphorimager using ImageQuant from Molecular Dynamics. The results are presented as the mean percent of Ras in GTP bound form (GTP/(GTP + GDP) x100) ± SE, of duplicate immunoprecipitations from duplicate cultures.

4.4. SUMMARY AND DISCUSSION

4.4.1. MULTIPLE MEK ACTIVATORS

The increasing recognition of the importance of cell context in determining the signal transduction pathways leading to MAPK prompted the investigation of the effect of bombesin on MAPK activation in Rat-1 cells. These results demonstrate that MAPK, Raf-1 and Ras are activated by bombesin in Rat-1 fibroblasts.

Although direct stimulation of PKC by phorbol esters can activate MAPK and bombesin also stimulates PKC (see section 1.3.8), in BOR 15 cells the activation of MAPK by bombesin is PKC-independent. It cannot be discounted that there is a component of bombesin-induced MAPK activation that is PKC-dependent but if there is, it is compensated for when PKC is inhibited or down regulated. On the contrary, direct stimulation of PKC by phorbol esters does not activate Raf-1. It could be envisaged that PKC could activate Raf-1 but only in synergy with another signal elicited by bombesin. However inhibition of PKC with the specific inhibitor GF 109203X did not affect bombesin-stimulated Raf-1 activity. Thus PKC does not contribute to Raf-1 activation that is stimulated by bombesin. Because both bombesin-stimulated MAPK and Raf-1 activation were PKC-independent this suggests that Raf-1 is the upstream activator of MAPK in bombesin-stimulated cells. In contrast Raf-1 does not appear to be the upstream activator of MAPK in PDB stimulated cells. Raf-1-independent stimulation of MAPK by phorbol ester has also been reported by (Chao, *et al.*, 1994).

Bombesin activates MAPK kinase to the same level as EGF but is much poorer at stimulating Raf-1. This is not a new observation. Faure and Bourne (1995) show that compared to EGF, LPA stimulates little Raf-1 and MEK activity but a comparable amount of MAPK activity in Rat-1 and Swiss 3T3 cells. But they do point out that low levels of Raf-1 activity may still saturate MAPK activation. Thus in BOR 15 cells the smaller Raf-1 activation stimulated by bombesin could saturate MAPK activation and a larger stimulation of Raf-1 by EGF is not translated to further stimulation of MAPK.

PDB could activate MAPK via a different MEK activator. For example in PC12 cells TPA transiently activates Raf-1 which is partially PKC-dependent, but gives a prolonged activation of A-Raf which is completely PKC-dependent (Bogoyevitch, *et al.*, 1995; Erhardt, *et al.*, 1995). Also it is well documented that there are Raf-1-independent mechanisms of ERK activation (Chao, *et al.*, 1994; Vaillancourt, *et al.*, 1994; Zheng, *et al.*, 1994; Erhardt, *et al.*, 1995; Faure and Bourne, 1995) and also uncharacterised MEK activators that non-Raf isoforms (Pang, *et al.*, 1995; Reuter, *et al.*, 1995).

4.4.2. BOMBESIN ACTIVATION OF RAS. MEDIATION BY Gβγ?

How does bombesin activate Ras in BOR 15 cells? In Swiss 3T3 cells the bombesin receptor couples to G_q and is completely dependent on PKC for MAPK activation. So when PKC is removed by inhibition or down regulation why is MAPK activation not compensated for by βγ-mediated Ras activation? It could be argued that βγ from G_q are different, however, the evidence presented in section 1.1.6.4 suggests that there is little specificity between βγ subunit combinations when activating effectors although it is realised that expression levels could be an important part of specificity.

Another explanation could be that the levels of expression of $G\alpha_q$ may not be high enough to support the release of enough βγ upon activation of the receptor. For example in βγ-mediated activation of adenylyl cyclase type II, Taussig and Gilman (1995) describes that this is not sensitive to βγ combinations, but rather to the concentration. $G\alpha_s$ activates adenylyl cyclase type II at picomolar concentrations whereas Gβγ activates at nanomolar concentrations. Therefore βγ stimulation of adenylyl cyclase type II occurs from a much more abundant receptor-G-protein complex. Similarly $G\alpha_q$ activates PLC EC_{50} = 0.6 nM whereas βγ activates EC_{50} = 25 nM (Park, *et al.*, 1993). Exton (1994) notes that G proteins that are the source of βγ that stimulate PLC are more abundant than G_q or G_{11}, i.e. they may be released from G_i or G_o subtypes. If the level of G protein expression is important this could explain why the more abundant G_i utilises a signalling pathway that is well agreed upon. If G_q is less abundant then thresholds for activation of βγ effectors which lead to the activation of Ras may or may not be reached. Also if the receptor couples to multiple G proteins (see Chapter 3) then the sum of the multiple Gα could be important. Alternatively other components of the pathway downstream of βγ (discussed in section 1.3.8.10) could be expressed at higher levels in Rat-1 than in Swiss 3T3.

4.4.3. CELLULAR CONTEXT

To conclude this chapter it has been shown that in Rat-1 cells bombesin stimulates the MAPK pathway via a different mechanism than has been reported previously in Swiss 3T3 cells, which involves the activation of Ras. The regulation of the MAPK pathway is different in some specialised cells such as neurons where voltage dependent Ca^{2+} influx will activate Src and Ras (Rosen, *et al.*, 1994; Farnsworth, *et al.*, 1995; Rusanescu, *et al.*, 1995), and in B cells where leukocyte specific tyrosine kinases such as Lyn and Syk are necessary for G protein-coupled receptor MAPK activation (Wan, *et al.*, 1996). However, among fibroblasts there are differences and these have been described in earlier sections. In the context of bombesin, in Swiss 3T3 cells bombesin does not activate Ras (Satoh, *et al.*, 1990b and unpublished observations from this laboratory) and the activation of MAPK is completely PKC-dependent (Pang,

et al., 1993; Seufferlein, *et al.*, 1996). However the results presented here have shown that in Rat-1 cells bombesin-induced MAPK activation is independent of PKC and most likely is dependent on the activation of Ras. This has important implications. Raf-1 is not the only downstream effector of Ras. PI3K has been suggested to be downstream of Ras (Rodriguez-Viciana, *et al.*, 1994) and a number of other molecules have been shown to bind to Ras in a GTP-dependent manner potentially stimulating diverse signalling pathways (Marshall, 1996). Also if $\beta\gamma$ are stimulating protein tyrosine kinases (EGF receptor, Src, Tsk, Btk), serine kinases homologous to the yeast Ste20p, or lipid kinases such as PI3K (see section 1.3.8.10) then more signalling pathways could branch out from these points.

In conclusion, G_q coupled receptors have the potential to activate MAPK via multiple pathways such as a PKC route and a Ras route, the latter of which is probably mediated by $\beta\gamma$ subunits. The exact route to MAPK activation in a given cell may depend on the exact repertoire and expression levels of the individual components of the multiple pathways.

Chapter 5 - The Calcium Signal

As discussed in section 1.3.2.5, the possibility that Ca^{2+} fluxes could play a role in the transduction of mitogenic signals has attracted intense interest. Neuropeptides such as bombesin, vasopressin, endothelin and bradykinin, which are potent mitogens for Swiss 3T3 cells (Rozengurt, 1986), induce a rapid and transient increase in the cytoplasmic concentration of Ca^{2+} followed by a persistent depletion of Ca^{2+} from stores in the ER (Lopez-Rivas and Rozengurt, 1984; Mendoza, *et al.*, 1986; Lopez-Rivas, *et al.*, 1987; Nanberg and Rozengurt, 1988). This effect is mediated by the second messenger $Ins(1,4,5)P_3$ (Berridge, 1993) which is generated in response to receptor-mediated activation of G_q, the GTP-binding protein that couples neuropeptide receptors to PLCβ (Berstein, *et al.*, 1992; Lee, *et al.*, 1992). However, hydrolysis of PIP_2 also generates DAG which activates PKC, a well known pathway leading to gene expression and cell proliferation (Rozengurt, *et al.*, 1984; Nishizuka, 1986; Nishizuka, 1988; Rozengurt and Sinnett-Smith, 1988a). Furthermore, insulin and EGF can stimulate DNA synthesis in Swiss 3T3 cells without inducing Ca^{2+} mobilisation (Rozengurt, *et al.*, 1983b; Vara and Rozengurt, 1985). Similarly, mutant receptors for platelet-derived growth factor and fibroblast growth factor defective in PLCγ activation have been shown to retain mitogenic activity upon ligand activation (Mohammadi, *et al.*, 1992; Peters, *et al.*, 1992; Valius, *et al.*, 1993; Valius and Kazlauskas, 1993). Thus, the contribution of Ca^{2+} to mitogenic signalling remains unclear.

An approach for assessing the role of Ca^{2+} in the transition from G_0 into DNA synthesis, is to use of specific inhibitors of the Ca^{2+} ATPase pump of the ER, which is responsible for the accumulation of Ca^{2+} into these stores. The tumour promoter thapsigargin and the structurally distinct compound DBHQ have been identified as inhibitors of Ca^{2+} accumulation in the ER (Moore, *et al.*, 1987; Takemura, *et al.*, 1989; Thastrup, *et al.*, 1989; Thastrup, *et al.*, 1990; Bian, *et al.*, 1991; Sagara and Inesi, 1991). Addition of these compounds to intact cells induces mobilisation of Ca^{2+} from internal stores, bypassing PLC-mediated formation of $Ins(1,4,5)P_3$. Previous studies have shown that the addition of either thapsigargin or DBHQ, at micromolar concentrations, profoundly inhibited cell proliferation or induced cytotoxic effects (Ghosh, *et al.*, 1991; Short, *et al.*, 1993). These findings are difficult to reconcile with the fact that mitogenic neuropeptides and other growth promoting factors also cause persistent depletion of Ca^{2+} from the ER and thapsigargin has been shown to be a tumour promoter.

The experiments presented here were designed to assess the effect of gradual depletion of Ca^{2+} from the ER on the ability of quiescent cells to exit from G_0 and enter DNA synthesis.

5.1. EFFECT OF THAPSIGARGIN AND DBHQ ON PDB-STIMULATED DNA SYNTHESIS

Initial experiments confirmed that thapsigargin (2 - 10 nM) inhibited DNA synthesis induced by a variety of growth promoting factors in Swiss 3T3 cells. However, when the dose-dependency of this effect was carefully examined, a stimulatory action was discovered at low concentrations of thapsigargin. This prompted a detailed analysis of the effects of Ca^{2+}ATPase pump inhibitors on cellular DNA synthesis.

5.1.1. THAPSIGARGIN AND DBHQ STIMULATE [^{3}H]THYMIDINE INCORPORATION IN SYNERGY WITH PDB

Quiescent cultures of Swiss 3T3 cells were preincubated with a range of concentrations of DBHQ or thapsigargin for 6 h. The cultures were then exposed to fresh medium containing PDB, the same concentrations of thapsigargin or DBHQ and [^{3}H]thymidine. Cumulative [^{3}H]thymidine incorporation was measured after 40 h of incubation. Figure 5.1 shows that both DBHQ and thapsigargin caused a striking stimulation of DNA synthesis in the presence of PDB. The optimum effects were observed at 7.5 μM DBHQ and 0.5 nM thapsigargin where they reached 63% and 62%, respectively, of the maximum response induced by FBS. The stimulation of [^{3}H]thymidine incorporation by these agents was induced within a narrow concentration range. At higher concentrations of DBHQ or thapsigargin cell toxicity (detachment) was clearly evident after 40 h of incubation. DBHQ and thapsigargin added alone did not induce any [^{3}H]thymidine incorporation in Swiss 3T3 cells.

To examine the extent to which the pretreatment with 0.5 nM thapsigargin and 7.5 μM DBHQ affected the resulting stimulation of [^{3}H]thymidine incorporation, confluent quiescent cultures of Swiss 3T3 cells were preincubated with these agents for various times. Cultures were then washed, thapsigargin or DBHQ was replaced and PDB added. Cumulative [^{3}H]thymidine incorporation was measured after 40 h. The results in figure 5.2 reveal that DBHQ or thapsigargin synergised with PDB in stimulating [^{3}H]thymidine incorporation without preincubation but the effect was enhanced after a pretreatment of 6 h. Similar enhancement of [^{3}H]thymidine incorporation was seen when the cells were preincubated with thapsigargin or DBHQ for 8 h instead of 6 h.

To further substantiate the observed synergy between PDB and DBHQ or thapsigargin, the effect of a range of concentrations of PDB in the presence of a fixed and optimum concentration of either DBHQ (7.5 μM) or thapsigargin (0.5 nM) was examined.

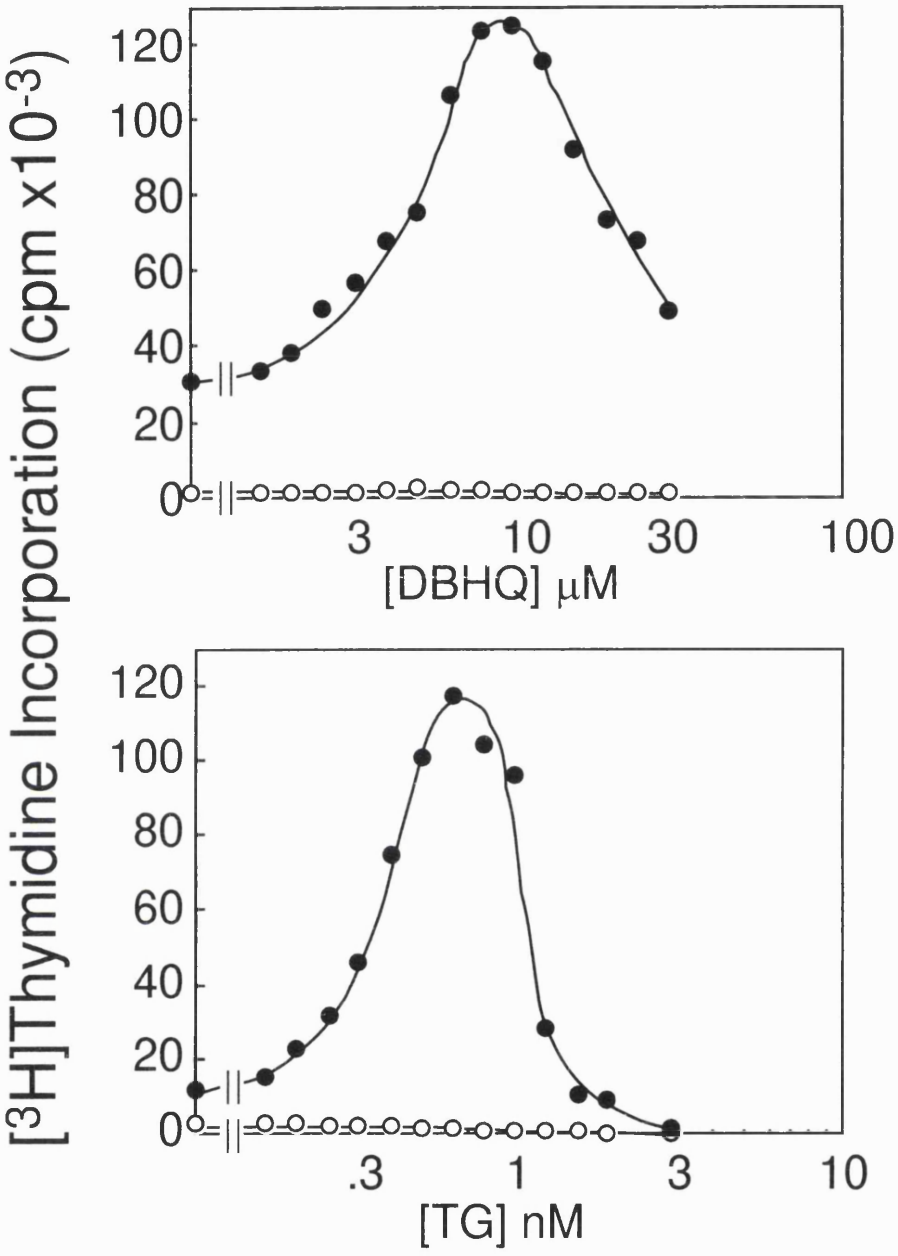

Figure 5.1. Dose response curve for the stimulation of DNA synthesis by DBHQ or thapsigargin

Confluent, quiescent cultures of Swiss 3T3 cells were pretreated with various concentrations of DBHQ (upper), or thapsigargin (lower) for 6 h. The cells were washed twice with DMEM and incubated at 37°C in 2 ml of 1:1 (vol/vol) DMEM/Waymouth's medium containing 0.25 μCi [^{3}H]thymidine per ml and various concentrations of DBHQ or thapsigargin in presence (closed circles) or absence (open circles) of 80 nM PDB. After 40 h, DNA synthesis was assessed by measuring the level of [^{3}H]thymidine incorporated into half of the acid precipitable material by liquid scintillation counting. Each point is the mean of two determinations from one representative experiment. 10% FBS gave an incorporation of 197 x 10^3 cpm (upper) or 189 x 10^3 cpm (lower). Similar results were obtained in 17 - 22 independent experiments.

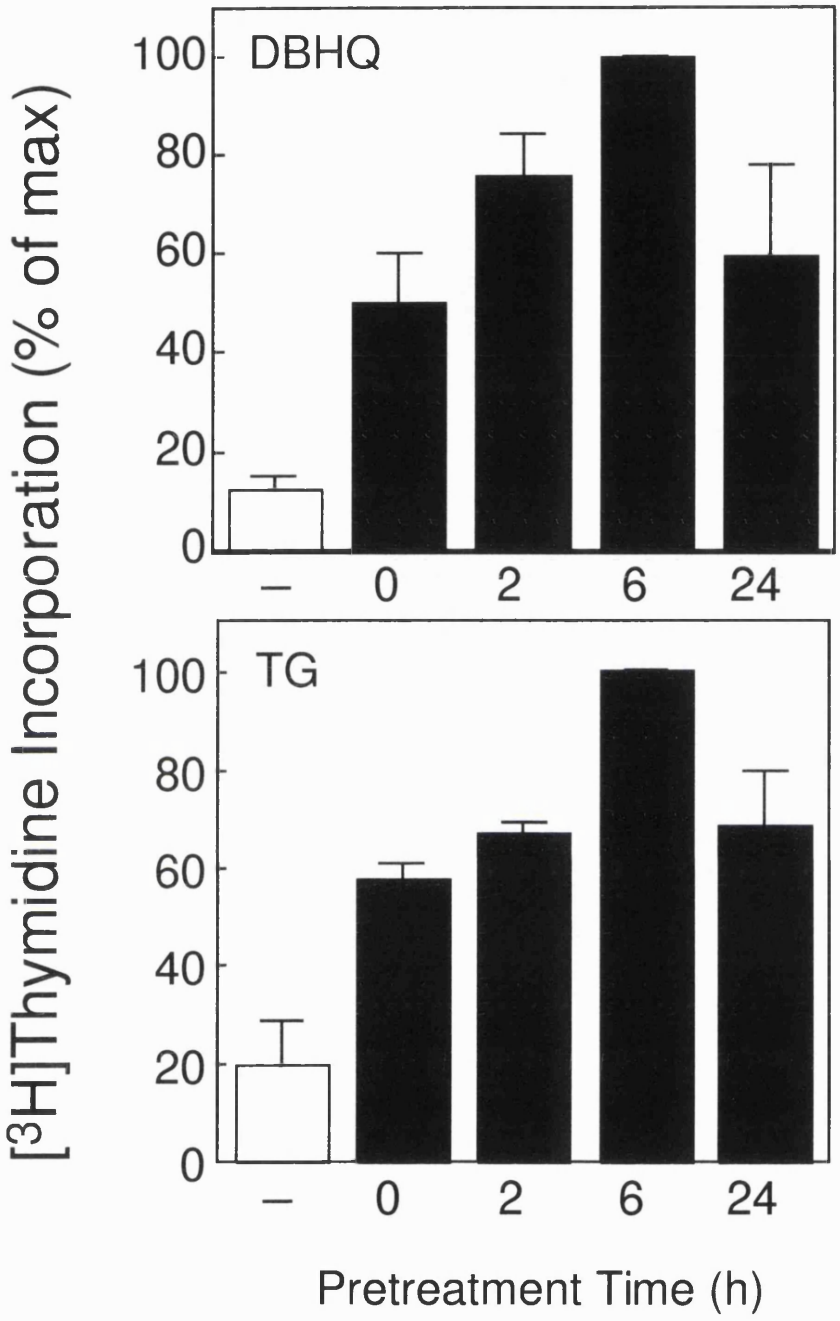

Figure 5.2. Effect of the length of preincubation with DBHQ and thapsigargin on the subsequent stimulation of DNA synthesis by these compounds in synergy with PDB

Confluent, quiescent cultures of Swiss 3T3 cells were pretreated without (-, open bars) or with (closed bars) 7.5 μM DBHQ (upper) or 0.5 nM thapsigargin (lower) for 0, 2, 6, or 24 h. Then (as above), the cells were washed twice with DMEM and incubated for 40 h in DMEM/Waymouth's medium with [^{3}H]thymidine, 7.5 μM DBHQ (upper) or 0.5 nM thapsigargin (lower) and 20 nM (upper) or 80 nM PDB (lower). DNA synthesis is expressed as percent of maximum. Each bar is the mean of 3 - 8 determinations. Error bars are standard error of the mean (SEM).

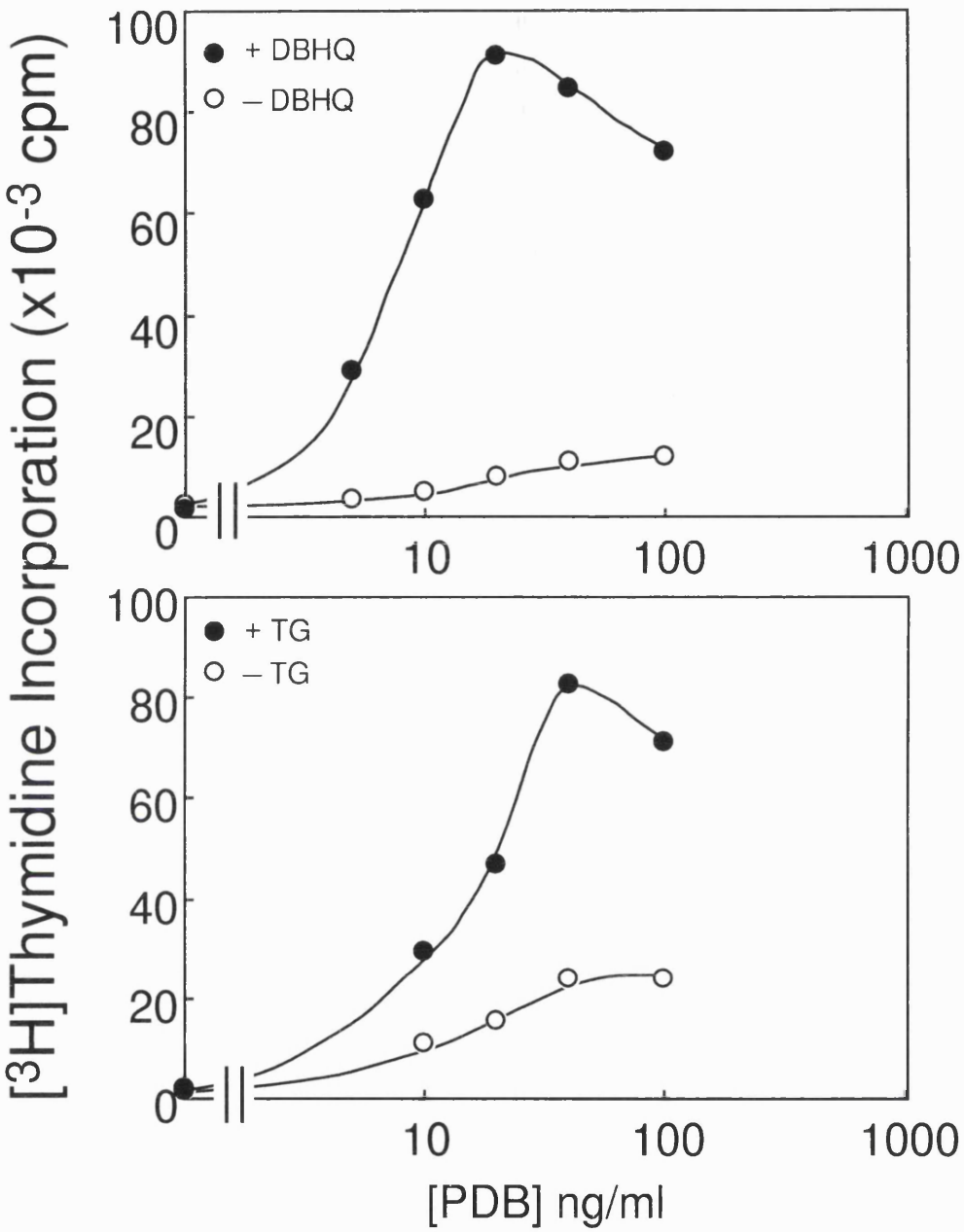

Figure 5.3. Dose response curve for the stimulation of DNA synthesis by PDB

Confluent, quiescent cultures of Swiss 3T3 cells were pretreated for 6 - 8 h without (open circles) or with (closed circles) 7.5 μM DBHQ (upper) or 0.5 nM thapsigargin (lower). Then, as above, the cells were washed twice with DMEM and incubated for 40 h in DMEM/Waymouth's medium with [^{3}H]thymidine and various concentrations of PDB, in the absence (open circles) or presence (closed circles) of 7.5 μM DBHQ (upper) or 0.5 nM thapsigargin (lower). 10% FBS gave 164 x 10^3 cpm (upper) and 168 x 10^3 cpm (lower). Similar results were obtained in 3 independent experiments.

The results show striking synergistic stimulation of [^{3}H]thymidine incorporation by PDB in the presence of either DBHQ or thapsigargin (figure 5.3). Maximum effects were produced at a concentration of 80 nM PDB. PDB alone did not induce any significant [^{3}H]thymidine incorporation in Swiss 3T3 cells, in agreement with previous results (Dicker and Rozengurt, 1978; Dicker and Rozengurt, 1980).

5.1.2. KINETICS OF THAPSIGARGIN AND DBHQ STIMULATED [3H]THYMIDINE INCORPORATION IS CONSISTENT WITH ENTRY TO S-PHASE

To verify that the increase in [^{3}H]thymidine incorporation was due to DNA synthesis, the incorporation of [^{3}H]thymidine by the combination of PDB with either DBHQ or thapsigargin was also assessed as a function of time (figure 5.4). These compounds stimulated [^{3}H]thymidine incorporation after a lag period of 20 - 22 h. This is consistent with the duration of the transition from G_0/G_1 to DNA synthesis stimulated by neuropeptide growth factors (e.g. bombesin, vasopressin) in Swiss 3T3 cells (Dicker and Rozengurt, 1980; Rozengurt and Sinnett-Smith, 1983).

5.1.3. THAPSIGARGIN AND DBHQ INCREASE THE PROPORTION OF CELLS THAT REINITIATE DNA SYNTHESIS

To assess whether PDB and thapsigargin or DBHQ interact synergistically in eliciting initiation of DNA synthesis rather than in changing the specific activity of the [^{3}H]-thymidine precursor pool, quiescent cultures of Swiss 3T3 cells were treated with PDB and thapsigargin or DBHQ and incorporation of [^{3}H]thymidine into DNA was quantified by autoradiography of labelled nuclei. As shown in figure 5.5 (left panel), thapsigargin and DBHQ caused a marked enhancement of the labelling index (from 7% in cultures treated with PDB alone to 44% and 54% in cultures treated also with 0.5 nM thapsigargin and 7.5 μM DBHQ respectively). Thus, PDB in combination with either thapsigargin or DBHQ synergistically enhances the proportion of cells that enter into DNA synthesis. This was in very good agreement with the cumulative [^{3}H]thymidine incorporation into parallel cultures expressed as a percentage of the response induced by 10% FBS (figure 5.5, right panel).

The results shown in figures 5.1 - 5.5 demonstrate that DBHQ and thapsigargin, at low concentrations, promote synergistic stimulation of DNA synthesis in combination with PDB.

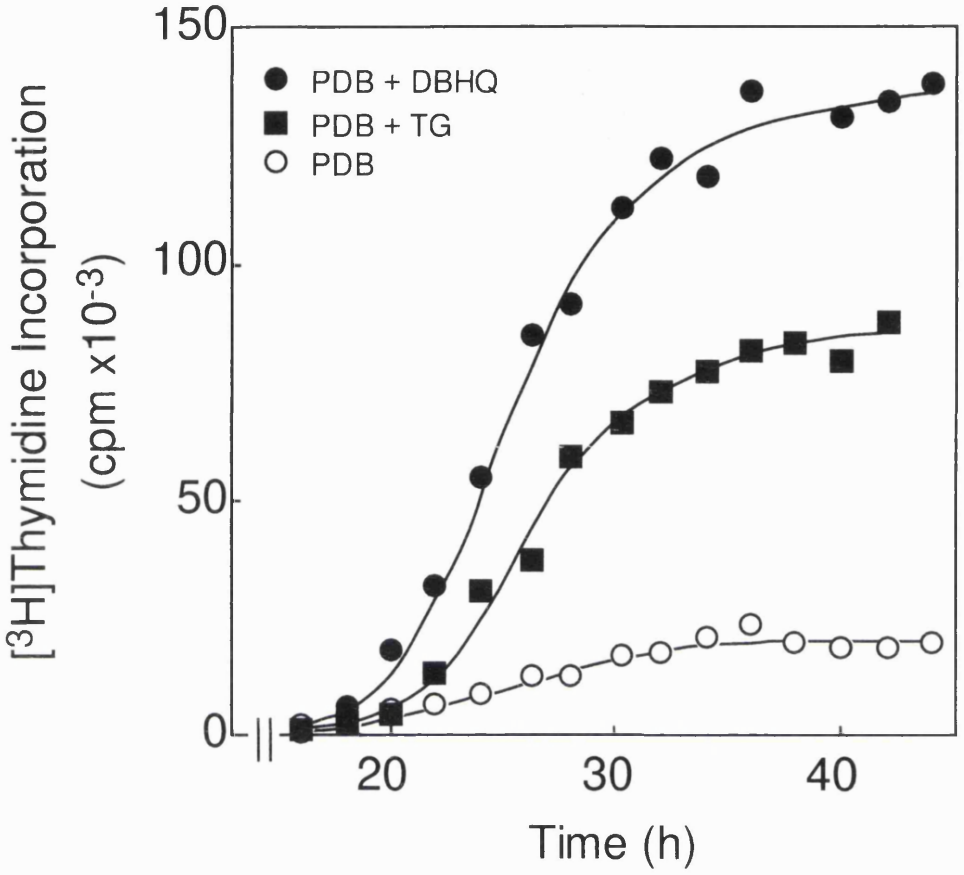

Figure 5.4. Time course for incorporation of [^{3}H]thymidine in the presence of thapsigargin and DBHQ

Confluent, quiescent cultures of Swiss 3T3 cells were pretreated for 6 - 8 h without (open circles) or with 0.5 nM thapsigargin (squares) or 7.5 μM DBHQ (closed circles). Then the cells were washed twice with DMEM and incubated for various times (from 16 h to 44 h) in DMEM/Waymouth's medium with [^{3}H]thymidine, 80 nM PDB and in the absence (open circles) or presence of 0.5 nM thapsigargin (squares) or 7.5 μM DBHQ (closed circles). A similar lag period prior to DNA synthesis was obtained in 3 independent experiments.

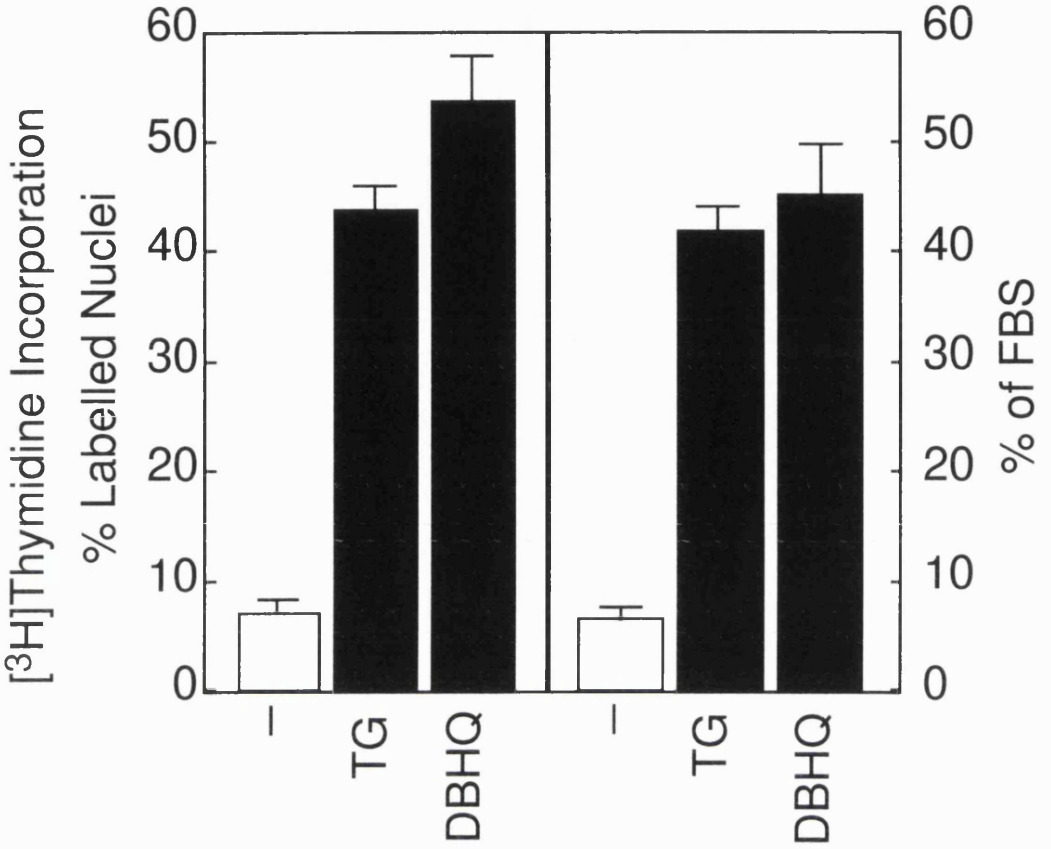

Figure 5.5. Effect of thapsigargin and DBHQ on DNA synthesis assessed by autoradiography and liquid scintillation counting of [^{3}H]thymidine incorporation

Confluent, quiescent cultures of Swiss 3T3 cells were pretreated for 6 - 8 h without (open bars) or with (closed bars) 0.5 nM thapsigargin or 7.5 μM DBHQ. Then, the cells were washed twice with DMEM and incubated for 40 h in DMEM/Waymouth's medium with 2.5 μCi [^{3}H]thymidine per ml (left panel) or 0.25 μCi/ml (right panel), and with 20 nM PDB either in the absence (-, open bars) or presence (closed bars) of 0.5 nM thapsigargin (TG) or 7.5 μM DBHQ. For autoradiography the experiment was stopped and cells fixed by replacing medium with formol saline. Labelled nuclei were visualised by *in situ* autoradiography using Kodak autoradiographic stripping plate AR10. Films were then developed *in situ* . Values on the left are the percent of nuclei which were labelled, counted from eight microscopic fields (total of 65 - 80 cells per field) per dish. 10% FBS labelled 93% of nuclei. Values on the right are expressed as percentage of the values obtained with 10% FBS and are presented for comparison with the autoradiography results. For both left and right panels values are the mean of 2 dishes with the error bars showing the range.

5.2. EFFECT OF DBHQ WITH OTHER MITOGENS

Next, it was examined whether DBHQ could act synergistically with signalling pathways other than those mediated by PKC. As shown in table 5.I, the effect of DBHQ on DNA synthesis in the presence of various growth promoting factors was determined.

Table 5.I. Effect of DBHQ on DNA synthesis induced by various mitogens

MITOGEN(S)	[^{3}H]Thymidine Incorporation (% of FBS)	
	—	+ DBHQ
—	1.3 ± 0.9	0.8 ± 0.5
INS	10.4 ± 3.3	17.3 ± 3.8
EGF	6.5 ± 1.3	8.0 ± 2.5
FOR + IBMX	4.2 ± 2.3	4.5 ± 1.0
BK	4.1 ± 1.3	8.8 ± 4.3
BOM	9.1 ± 3.5	36.7 ± 6.5
PDB	7.1 ± 0.7	43.7 ± 3.5

Confluent, quiescent cultures of Swiss 3T3 cells were pretreated for 8 h without (-) or with (+) 7.5 μM DBHQ. Then, as in Fig. 5.1, the cells were washed twice with DMEM and incubated for 40 h in DMEM/Waymouth's medium with [^{3}H]thymidine and various mitogens: 1 μg/ml insulin (INS), 5 ng/ml EGF, 50 μM forskolin (FOR), 25 μM IBMX, 50 nM bradykinin (BK), 2.5 nM bombesin (BOM) and 20 nM PDB, in the presence (+) or absence (-) of 7.5 μM DBHQ. Values are expressed as percent of [^{3}H]thymidine incorporation obtained with 10% FBS and are the mean, with the error shown as SE, of 4 independent experiments performed in duplicate, or 27 experiments for PDB.

The polypeptides EGF and insulin bind to receptors endowed with intrinsic, ligand dependent, tyrosine kinase activity which are not linked to PLCγ and hence to PKC activation in Swiss 3T3 cells (Rozengurt, *et al.*, 1983b; Vara and Rozengurt, 1985). These polypeptides failed to act synergistically with DBHQ. Similarly, the cAMP elevating agents forskolin and IBMX which are potent mitogens for Swiss 3T3 cultures in the presence of insulin (Rozengurt, *et al.*, 1981b), did not increase DNA synthesis in the presence of DBHQ. Interestingly, bradykinin which causes a rapid but transient activation of PKC (Issandou and Rozengurt, 1990), also failed to act synergistically with DBHQ.

5.2.1. SUB-MAXIMAL CONCENTRATIONS OF BOMBESIN SYNERGISE WITH THAPSIGARGIN AND DBHQ

Bombesin, a potent mitogen for Swiss 3T3 cells, induces persistent stimulation of PKC even at concentrations sub-maximal for inducing DNA synthesis. As shown in table 5.I and figure 5.6 (right panel), 2.5 nM bombesin (a concentration which was sub-maximal) acted synergistically with DBHQ in promoting DNA synthesis in Swiss 3T3 cells. This synergistic effect was also demonstrated when DNA synthesis was determined by autoradiography of [^{3}H]thymidine labelled nuclei (figure 5.6, left panel). Bombesin (2.5 nM) was almost as effective as PDB in promoting DNA synthesis in the presence of DBHQ. This synergy was not evident when saturating doses of bombesin (10 nM) were used (data not shown). These results strongly suggest that DBHQ-mediated Ca^{2+} mobilisation acts synergistically with agents that induce persistent activation of PKC. Similar results were obtained when thapsigargin was added instead of DBHQ.

5.3. EXAMINATION OF THE SYNERGISM BETWEEN PKC AND THAPSIGARGIN OR DBHQ

5.3.1. THE SYNERGISTIC STIMULATION OF DNA SYNTHESIS REQUIRES FUNCTIONAL PKC

The preceding results prompted a verification that the synergistic effects between DBHQ and either PDB or bombesin on stimulation of DNA synthesis are dependent on PKC. It is known that treatment with saturating doses of PDB (800 nM) causes down regulation of all phorbol ester-sensitive isoforms of PKC present in Swiss 3T3 cells (Rodriguez-Pena and Rozengurt, 1984), i.e. PKCs α, δ and ε (Olivier and Parker, 1992). As shown in figure 5.7, down regulation of phorbol ester sensitive PKCs strikingly decreased the [^{3}H]thymidine incorporation induced by DBHQ and PDB or DBHQ and bombesin.

In order to substantiate the requirement of PKC activity for synergistic stimulation of DNA synthesis by DBHQ and PDB or bombesin, we used the inhibitor GF 109203X which selectively inhibits PKC isoforms α, βI, βII, γ, δ and ε in Swiss 3T3 cells (Toullec, *et al.*, 1991; Yeo and Exton, 1995).. Pretreatment of Swiss 3T3 cells with 3.5 μM GF 109203X for 1 h before addition of PDB or bombesin, conditions which have been previously shown to effectively inhibit PKC activity in intact Swiss 3T3 cells (Sinnett-Smith, *et al.*, 1993), also strikingly decreased the [^{3}H]thymidine incorporation induced by DBHQ and PDB or DBHQ and bombesin (figure 5.8). The results shown in figures 5.7 and 5.8 demonstrate that PKC is necessary for the synergistic stimulation of DNA synthesis induced by DBHQ and either PDB or bombesin.

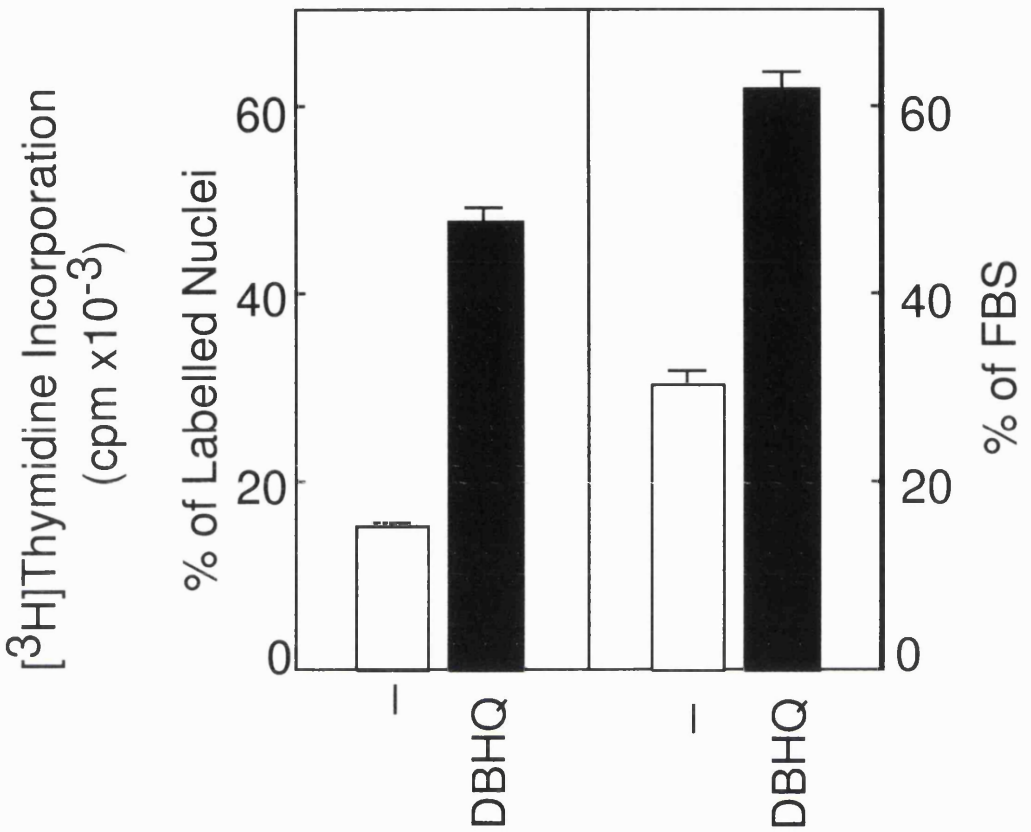

Figure 5.6. Effect of DBHQ on DNA synthesis induced by bombesin assessed both by autoradiography and liquid scintillation counting of [^{3}H]thymidine incorporation as comparison

Confluent, quiescent cultures of Swiss 3T3 cells were pretreated for 6 - 8 h without (open bars) or with 7.5 μM DBHQ (closed bars). Cells were then washed twice with DMEM and incubated for 40 h in DMEM/Waymouth's medium with 2.5 μCi [^{3}H]thymidine per ml (left) or 0.25 μCi/ml (right), and with 2.5 nM bombesin, in the absence (open bars) or presence of 7.5 μM DBHQ (closed bars). Values in left panel are the percent of nuclei which were labelled, counted from eight microscopic fields (total of 65 - 80 cells per field) per dish. 10% FBS labelled 93% of nuclei. Values in right panel are expressed as mean percentage of the values obtained with 10% FBS, with error as SEM.

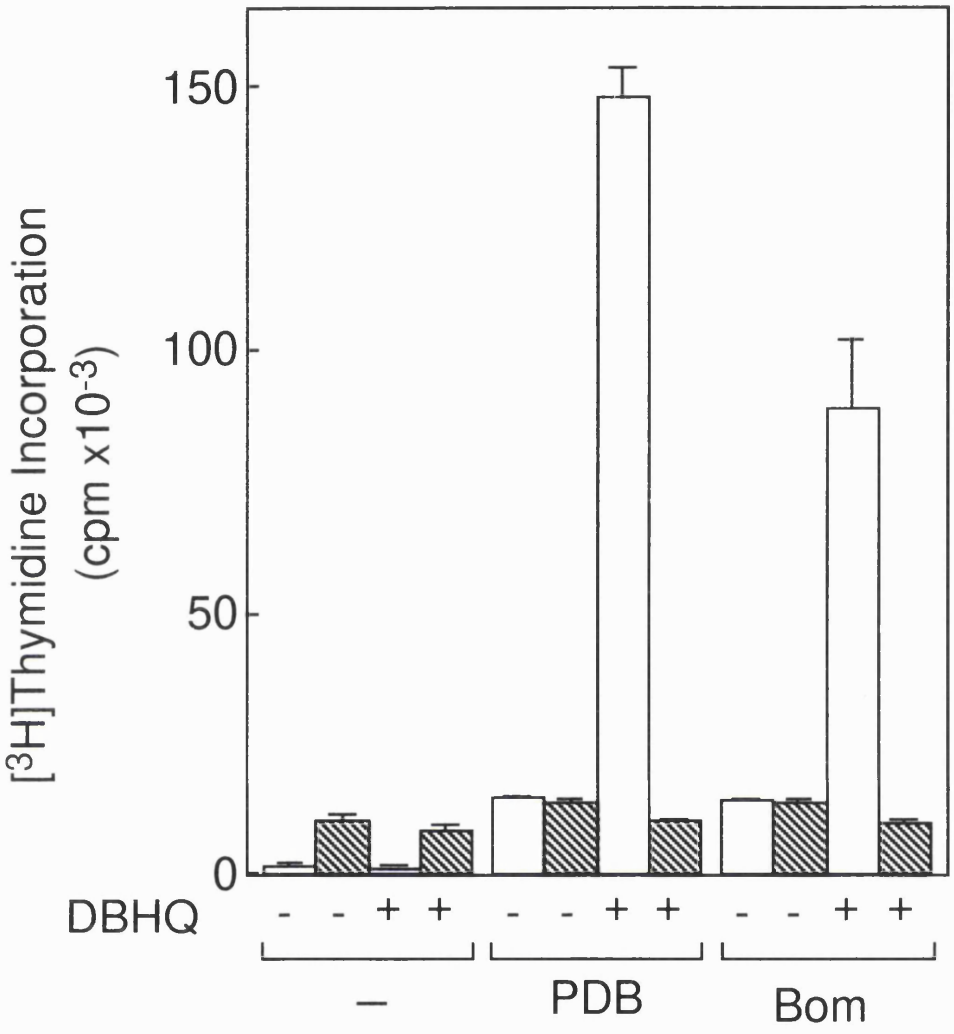

Figure 5.7. Effect of down regulating PKC on [^{3}H]thymidine incorporation induced by DBHQ and either PDB or bombesin

Confluent, quiescent cultures of Swiss 3T3 cells were treated in the absence (open bars) or presence (striped bars) 800 nM PDB for 48 h to down regulate PKC. Then, the cells were pretreated without (-) or with 7.5 μM DBHQ (+). After 6 h, the cells were washed twice with DMEM and incubated in DMEM/Waymouth's medium with [^{3}H]thymidine in the absence (-) or presence of 80 nM PDB or 1 nM bombesin (Bom), and without (-) or with (+) 7.5 μM DBHQ. Results shown are the mean of two determinations with the error bar showing the range. Similar results were obtained in two independent experiments.

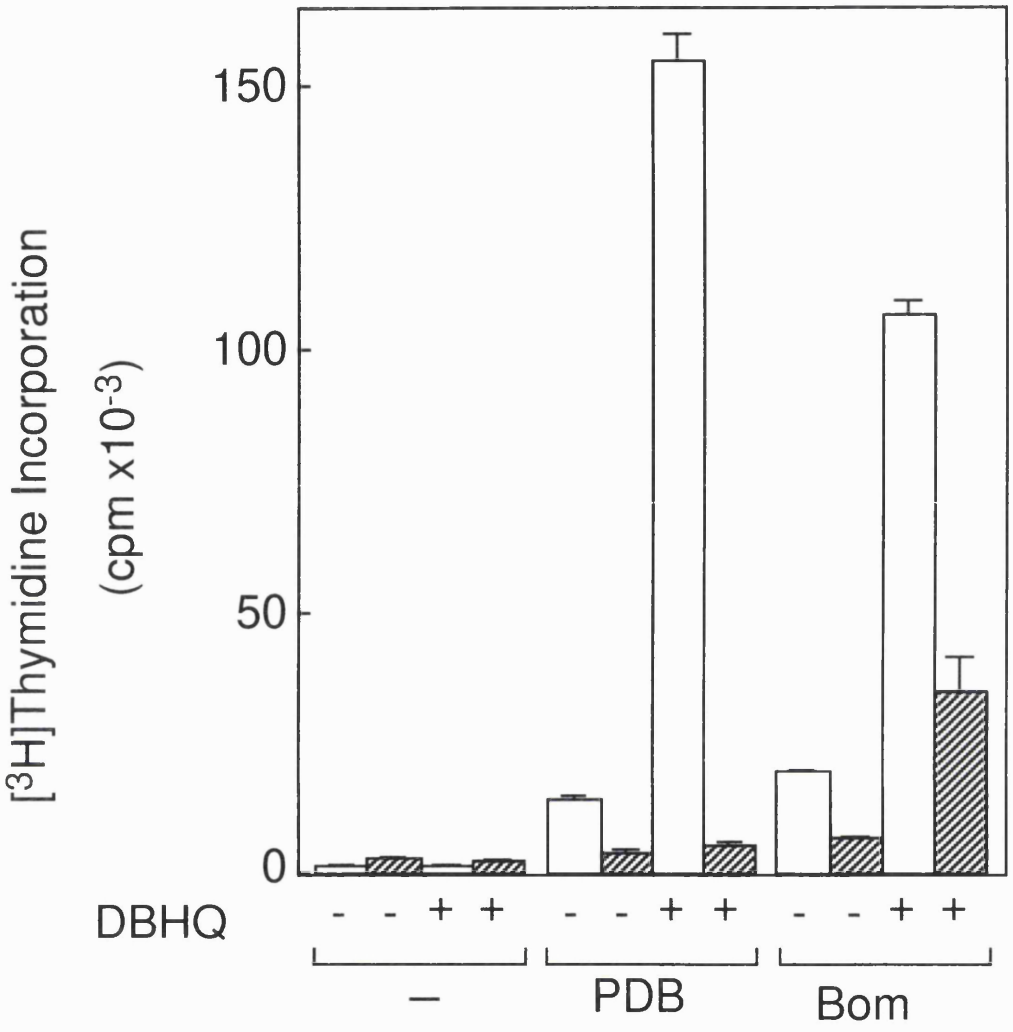

Figure 5.8. Effect of inhibiting PKC on [^{3}H]thymidine incorporation induced by DBHQ and either PDB or bombesin

Confluent, quiescent cultures of Swiss 3T3 cells were pretreated without (-) or with (+) 7.5 μM DBHQ. After 6 h, the cells were washed twice with DMEM, incubated in DMEM/Waymouth's medium with [^{3}H]thymidine and DBHQ was replaced. Then, cells were treated without (open bars) or with 3.5 μM GF 109203X (striped bars) for 1 h before the addition of nothing (-), 80 nM PDB or 1 nM bombesin (Bom). Results shown are the mean of two determinations with the error bar showing the range. Similar results were obtained in two independent experiments.

5.3.2. THAPSIGARGIN AND DBHQ DO NOT POTENTIATE PDB-MEDIATED SIGNAL TRANSDUCTION

Some PKC isoforms are Ca^{2+}-dependent see section 1.3.3.1 and of these Swiss 3T3 cells contain PKCα (Olivier and Parker, 1992; Neri, *et al.*, 1994). Therefore treatment with thapsigargin or DBHQ could enhance PDB-mediated signals, or alternatively, converge with them at a downstream locus further into G_1. To distinguish between these possibilities we determined whether prior exposure of Swiss 3T3 cells to 0.5 nM thapsigargin or 7.5 μM DBHQ for 6 h had any effect on PDB-mediated events.

80K/MARCKS

Cells that were metabolically labelled with $^{32}P_i$ overnight were stimulated and 80K/MARCKS was immunoprecipitated. Figure 5.9 (upper panel) shows that preincubation with thapsigargin or DBHQ did not affect the ability of PDB to stimulate the phosphorylation of 80K/MARCKS, a prominent and specific substrate of PKC in intact cells (Rozengurt, *et al.*, 1983b).

MAPK activation

Phorbol esters activate MAPK, see section 1.3.8 resulting in phosphorylation on tyrosine and threonine residues and a lowered electrophoretic mobility on SDS-PAGE (Leevers and Marshall, 1992). To assess whether thapsigargin or DBHQ increased PDB-stimulated MAPK activity, whole cell lysates were resolved by SDS-PAGE and MAPK was visualised by Western blotting with anti-$p42^{mapk}$ polyclonal antibody. Figure 5.9 (lower panel) demonstrates that thapsigargin or DBHQ did not affect the activation of MAPK by exposure to PDB. The activation of MAPK by bombesin shows a biphasic profile, there is a large transient activation that peaks at 5 - 10 min followed by a lower sustained plateau that lasts for several hours (Withers, *et al.*, 1995). It was verified that pretreating cells with thapsigargin or DBHQ did not alter the timecourse of PDB-induced MAPK activation by incubating cells for 5 or 60 min (instead of 20 min) with PDB. There was no difference between pretreated cells and control cells.

Tyrosine phosphorylation

Recently, it has been shown that PDB stimulates the tyrosine phosphorylation of multiple substrates including bands of M_r 110,000 - 130,000 and M_r 70,000 - 80,000 (Sinnett-Smith, *et al.*, 1993). The novel cytosolic tyrosine kinase FAK has been identified as a prominent tyrosine phosphorylated protein of the M_r 110,000 - 130,000 band whereas paxillin corresponds to the M_r 70,000 - 80,000 heterogeneous band (Sinnett-Smith, *et al.*, 1993; Zachary, *et al.*, 1993).

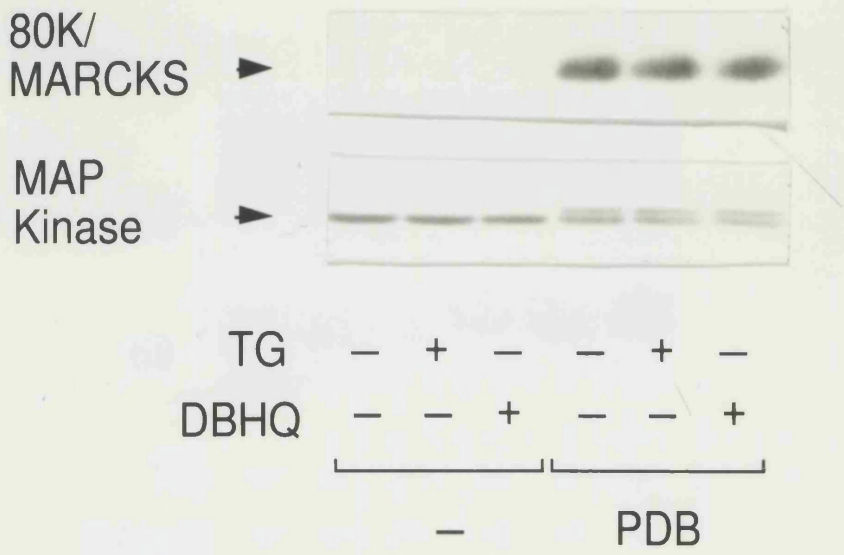

Figure 5.9. Effect of thapsigargin and DBHQ on PDB-mediated signal transduction (I)

Upper panel. 80K/MARCKS phosphorylation

Confluent, quiescent cultures of Swiss 3T3 cells were loaded with $^{32}P_i$ overnight then pretreated for 6 h without or with 0.5 nM thapsigargin or 7.5 μM DBHQ as indicated. Cells were incubated 10 min, 37°C, with or without 80 nM PDB as indicated. Cells were lysed and immunoprecipitated with 80K/MARCKS antiserum and resolved by SDS-PAGE. ^{32}P-labelled bands were visualised by autoradiography. Similar results were obtained in two independent experiments.

Lower panel. MAP kinase activation

Confluent, quiescent cultures of Swiss 3T3 cells were pretreated for 6 h with or without 0.5 nM thapsigargin or 7.5 μM DBHQ as indicated. Cells were then washed twice with DMEM and incubated in DMEM at 37°C, without (-) or with 0.5 nM thapsigargin (TG) or 7.5 μM DBHQ in the presence or absence of 80 nM PDB as indicated for 5, 20 or 60 min. Cells were lysed with 2x sample buffer and proteins separated by SDS-PAGE. Proteins were transferred to Immobilon membranes and Western blotted with MAP kinase antiserum. Bands were visualised by autoradiography. Results shown are after 20 min of stimulation. Similar results were obtained when PDB was added for 5 or 60 min instead of 20 min (results not shown). Similar results were obtained in 3 independent experiments.

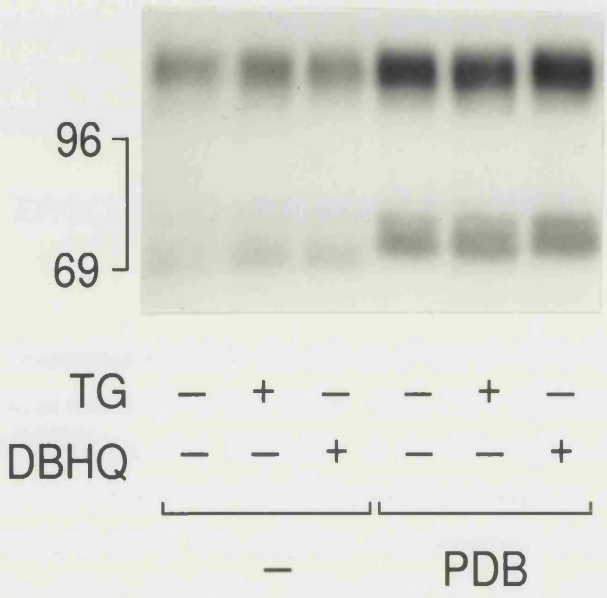

Figure 5.10. Effect of thapsigargin and DBHQ on PDB-mediated signal transduction (II)

Tyrosine phosphorylation of proteins M_r 110,000 - 130,000 and M_r 70,000 - 80,000

Confluent, quiescent cultures of Swiss 3T3 cells were pretreated for 6 h with or without 0.5 nM thapsigargin or 7.5 μM DBHQ as indicated. Then, cells were washed twice with DMEM and incubated in DMEM for 10 min, 37°C, with or without (-) 0.5 nM thapsigargin (TG) or 7.5 μM DBHQ and in the presence or absence of 80 nM PDB as indicated. Cells were lysed and immunoprecipitated with agarose-linked anti-Tyr(P) mAb Py72 and resolved by SDS-PAGE. Phosphotyrosyl proteins were transferred to Immobilon membranes and Western blotted with 4G10 and Py20 anti-Tyr(P) mAbs. Bands were visualised by autoradiography. Similar results were obtained in two independent experiments.

As shown in figure 5.10 (lower panel), pretreatment with thapsigargin or DBHQ did not change the basal level of tyrosine phosphorylation or affect the PDB induced increase in tyrosine phosphorylation of the M_r 110,000 - 130,000 or M_r 70,000 - 80,000 bands. In accordance with all these results, treatment of Swiss 3T3 cells with either DBHQ or thapsigargin did not alter the extent to which [^{3}H]PDB bound to intact cells (data not shown). These results indicate that thapsigargin and DBHQ do not directly potentiate PKC-mediated signals.

5.4. EFFECT OF THAPSIGARGIN AND DBHQ ON INTRACELLULAR CALCIUM STORES

Thapsigargin inhibits the activity of the ER Ca^{2+}ATPase leading to a depletion of the intracellular Ca^{2+} stores (Moore, *et al.*, 1987; Takemura, *et al.*, 1989; Thastrup, *et al.*, 1989; Thastrup, *et al.*, 1990; Bian, *et al.*, 1991; Sagara and Inesi, 1991). However, most previous studies on the effects of thapsigargin on cell proliferation and Ca^{2+} sequestering in the ER have used high concentrations of this compound (0.1 - 10 μM). Here, we found synergistic stimulation of DNA synthesis at sub-nanomolar concentrations. Consequently, it was important to examine the effects of low concentrations of thapsigargin and DBHQ on intracellular Ca^{2+}.

5.4.1. THAPSIGARGIN AND DBHQ INHIBIT BOMBESIN-INDUCED Ca^{2+} MOBILISATION

Specifically, if treatment with mitogenic concentrations of thapsigargin or DBHQ inhibits the accumulation of Ca^{2+} into the Ins(1,4,5)P_3-sensitive pool, it should prevent the increase in $[Ca^{2+}]_{cyt}$ induced by subsequent addition of Ca^{2+}-mobilising agents. Figure 5.11 shows that treatment with 0.5 nM thapsigargin or 7.5 μM DBHQ markedly reduced the ability of 2.5 nM bombesin to induce a rapid and transient increase in the $[Ca^{2+}]_{cyt}$ from Swiss 3T3 cells as measured by the fluorescent Ca^{2+} indicator fura-2. Preincubation with 0.5 nM thapsigargin or 7.5 μM DBHQ for 6 - 8 h inhibited bombesin-induced increase in $[Ca^{2+}]_{cyt}$ by 79 ± 2% (n = 7) and 90 ± 1% (n = 9), respectively (Figure 5.12).

5.4.2. KINETICS OF THAPSIGARGIN AND DBHQ INHIBITION OF BOMBESIN-INDUCED Ca^{2+} MOBILISATION

The reduction in bombesin-induced Ca^{2+} mobilisation by thapsigargin or DBHQ as a function of length of pretreatment time was examined. Figure 5.13 demonstrates that maximum inhibition of the increase in $[Ca^{2+}]_{cyt}$ was reached within 1 h of exposure to thapsigargin or DBHQ which was maintained over a period of several hours.

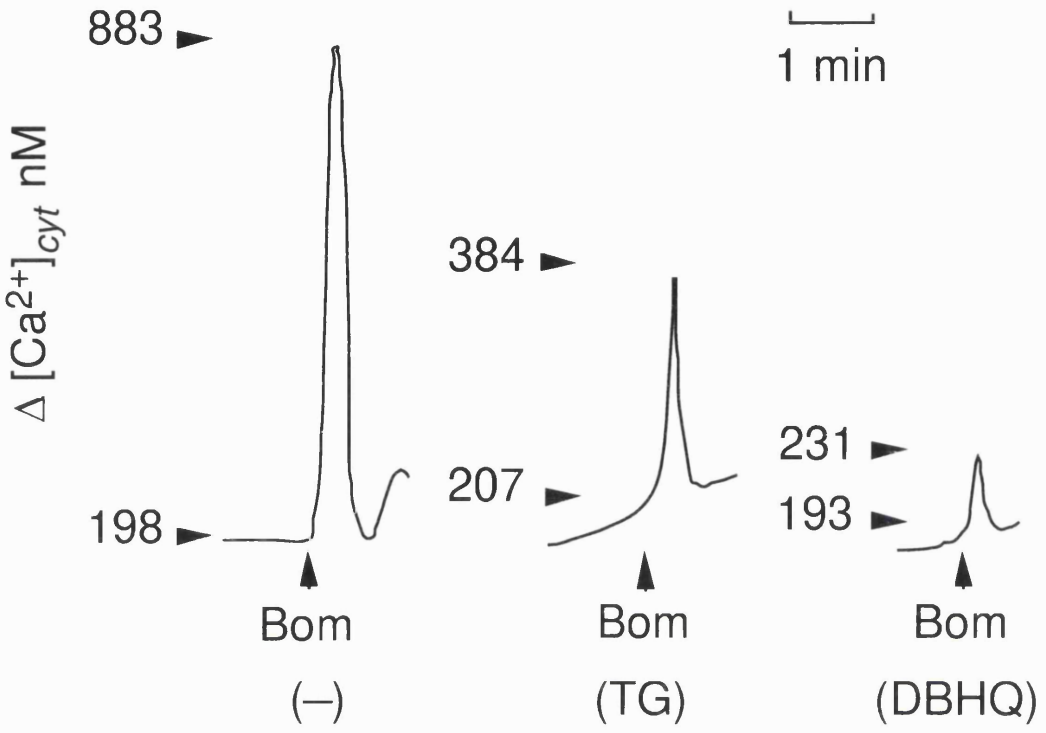

Figure 5.11. Reduction of bombesin induced Ca^{2+} mobilisation by thapsigargin and DBHQ

Confluent, quiescent Swiss 3T3 cells in 100 mm Nunc dishes were incubated at 37°C for 8 h without (-) or with 0.5 nM thapsigargin (TG) or 7.5 μM DBHQ. The cells were then washed twice with DMEM and incubated with DMEM containing 1 μM Fura-2/AME in the absence (-) or presence of 0.5 nM thapsigargin (TG) or 7.5 μM DBHQ for 10 min at 37°C. The cells were washed twice with PBS and gently scraped into 2 ml electrolyte solution containing 0.5 nM thapsigargin (TG) or 7.5 μM DBHQ as necessary and transferred to a quartz cuvette which was kept at 37°C and stirred continuously throughout the experiment. Fluorescence was monitored. After 1 min control time bombesin was added to 2.5 nM (shown by arrow). Values on the left hand side of each tracing (A) are $[Ca^{2+}]_{cyt}$ in nM.

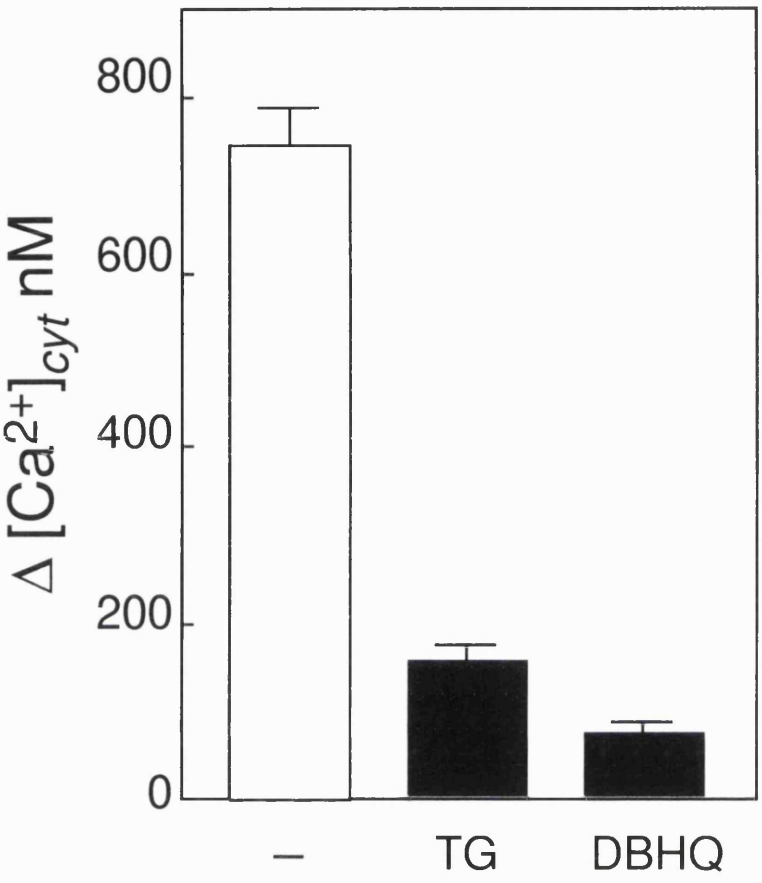

Figure 5.12. The mean increase in $[Ca^{2+}]_{cyt}$ induced by bombesin after pretreatment with thapsigargin or DBHQ

Confluent, quiescent Swiss 3T3 cells in 100 mm Nunc dishes were incubated at 37°C for 8 h without (-) or with 0.5 nM thapsigargin (TG) or 7.5 μM DBHQ. The cells were then washed twice with DMEM and incubated with DMEM containing 1 μM Fura-2/AME and fluorescence was monitored as in the previous figure. After 1 min control time bombesin was added to 2.5 nM (shown by arrow). Values on the left hand side of each tracing (A) are $[Ca^{2+}]_{cyt}$ in nM.
This figure shows the mean increase in $[Ca^{2+}]_{cyt}$ induced by 2.5 nM bombesin in control cells (-, open bars) or in cells pretreated (closed bars) with thapsigargin (TG) or DBHQ. Errors are presented as SEM. The number of samples are n = 17 (-), n = 7 (TG), n = 9 (DBHQ).

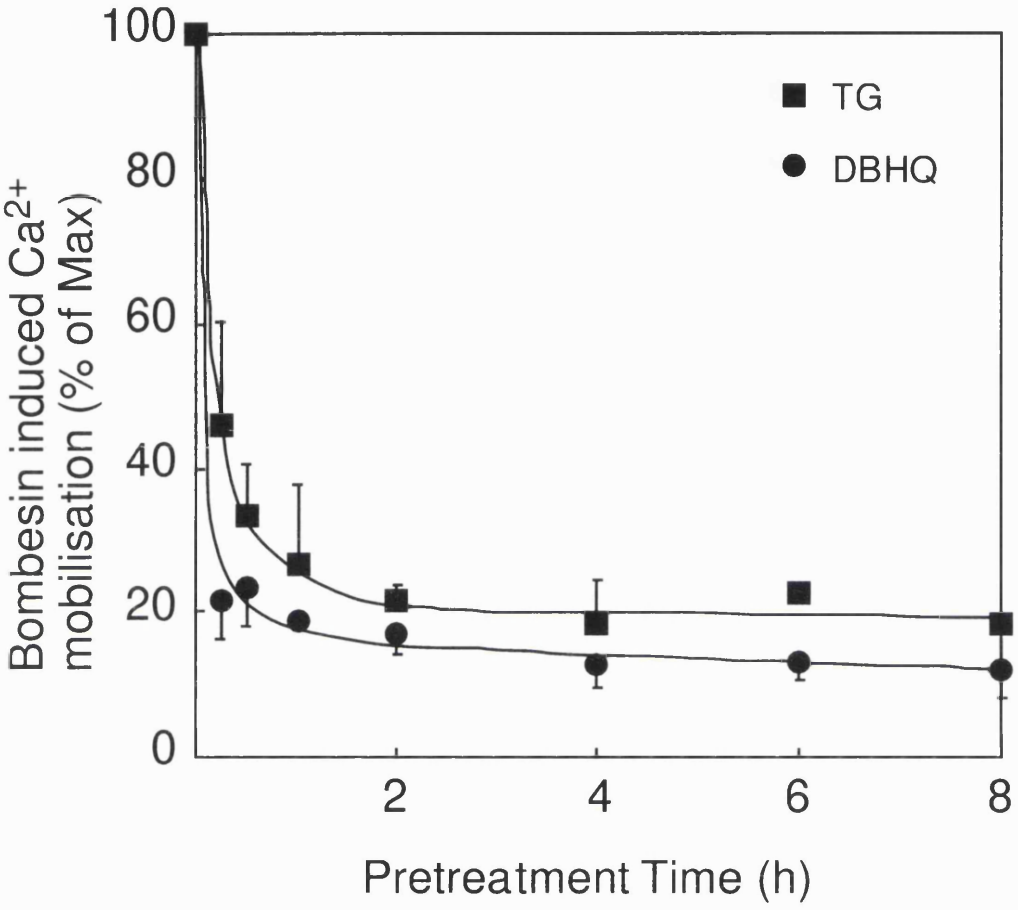

Figure 5.13. Time course of depletion of bombesin induced calcium response by thapsigargin and DBHQ

Confluent, quiescent Swiss 3T3 cells were incubated at 37°C for various times with 0.5 nM thapsigargin (squares) or 7.5 μM DBHQ (circles). The cells were then washed twice with DMEM and loaded with Fura-2/AME, as above, in the presence of 0.5 nM thapsigargin (squares) or 7.5 μM DBHQ (circles). The cells were then washed twice in PBS and scraped into 2 ml electrolyte solution containing 0.5 nM thapsigargin (squares) or 7.5 μM DBHQ (circles). After 1 min 2.5 nM bombesin was added. Values are expressed as a mean percentage of the difference in $[Ca^{2+}]_{cyt}$ induced by 2.5 nM bombesin in untreated cells, with errors as SE from 2 - 3 experiments.

5.4.3. THAPSIGARGIN AND DBHQ DO NOT PREVENT BOMBESIN-INDUCED INOSITOL PHOSPHATE ACCUMULATION

It was verified that treatment with these agents did not inhibit bombesin-induced Ca^{2+} mobilisation by preventing the production of inositol phosphates. This was assessed by collecting inositol phosphates on an anion exchange column from cells that had been labelled overnight with [^{3}H]inositol. Table 5.II shows that pretreatment with thapsigargin or DBHQ did not prevent the production of inositol phosphates, so thapsigargin and DBHQ inhibit Ca^{2+} release by a different mechanism.

Table 5.II. Effect of thapsigargin on total inositol phosphates generated by various mitogens

MITOGEN	- (cpm)	+ TG (cpm)
-	209 ± 21	263 ± 26
20 nM PDB	194 ± 6	199 ± 10
2.5 nM BOM	482 ± 21	560 ± 15
10 nM BOM	822 ± 21	800 ± 48

Confluent, quiescent cultures of Swiss 3T3 cells were equilibrated overnight with 15 μCi/ml *myo*-[2-^{3}H]inositol then pretreated for 8 h without (-) or with 0.5 nM thapsigargin (+ TG). Cells were washed twice, incubated in DMEM/Waymouth's for 10 min, 37°C, with either 20 nM PDB, 2.5 nM or 10 nM bombesin (BOM), in the absence (-) or presence of 0.5 nM thapsigargin (+ TG). Total inositol phosphates were extracted and collected on Dowex AG1-X8 columns, $HCOO^-$ form, and eluted with 1 M NH_4COOH, 0.1 M HCOOH as described in materials and methods. Values are expressed as cpm. The experiment was performed in triplicate, with the error shown as SE.

5.4.4. THAPSIGARGIN AND DBHQ CAUSE PROLONGED DEPLETION OF TOTAL CELLULAR Ca^{2+}

To demonstrate that the decrease in bombesin-induced Ca^{2+} mobilisation was due to depletion of Ca^{2+} stores, cells were loaded with $^{45}Ca^{2+}$ and the total Ca^{2+} remaining in the cells was determined after various treatments. This method measures Ca^{2+} in intracellular stores which is accumulated to millimolar concentrations rather than cytoplasmic Ca^{2+} which is maintained at nanomolar concentrations as described in section 1.3.2.1.

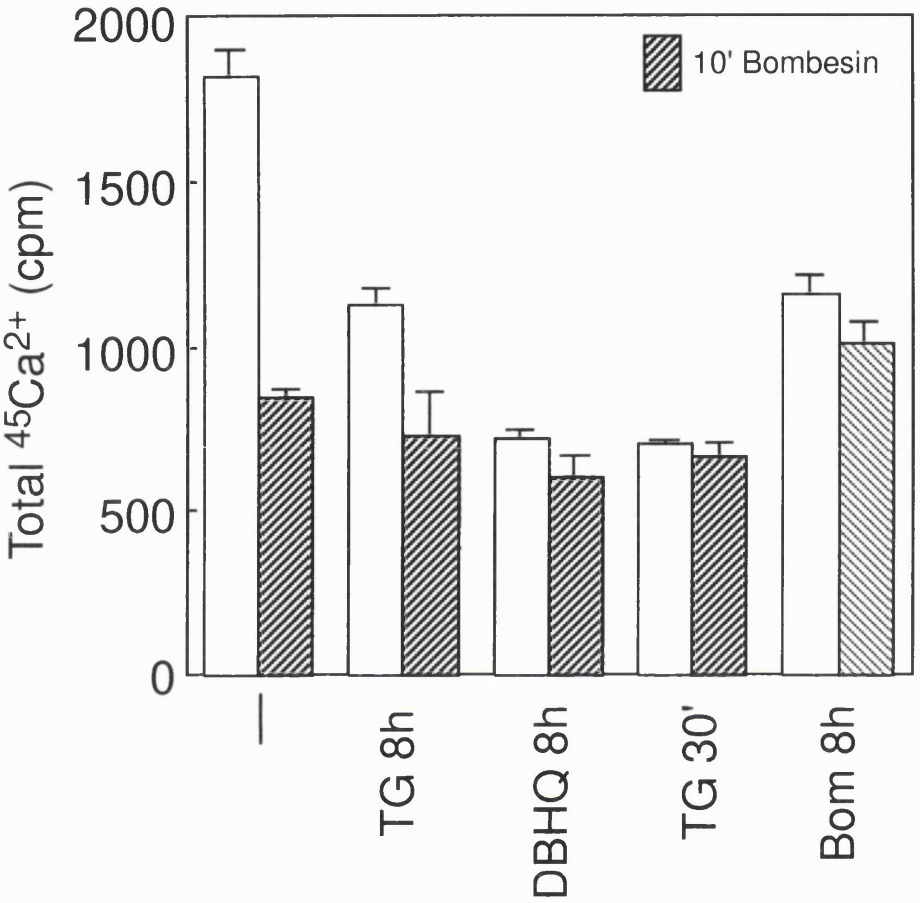

Figure 5.14. Effect of Thapsigargin and DBHQ on total intracellular calcium measured by $^{45}Ca^{2+}$

Confluent, quiescent Swiss 3T3 cells loaded with $^{45}Ca^{2+}$ were then pretreated for 8 h without (-) or with 0.5 nM thapsigargin (TG 8h), 7.5 μM DBHQ (DBHQ 8h) 10 nM bombesin (Bom 8h), or for 30 min with 30 nM thapsigargin (TG 30). Cells were then washed twice with DMEM, the same factors added back as for the pretreatment and then incubated for 10 min at 37°C in the presence (striped bars) or absence (open bars) of 10 nM bombesin. In the case of bombesin pretreatment, due to down regulation of the bombesin response, further stimulation was obtained with 10 min of 20 nM vasopressin (reverse striped bar). Residual $^{45}Ca^{2+}$ in the cells was measured as described in materials and methods. Values are mean of three determinations from one experiment with errors shown as SE.

The results in figure 5.14 demonstrate that 8 h pretreatment by either 0.5 nM thapsigargin or 7.5 μM DBHQ, conditions which are optimum for DNA synthesis, reduced total cellular $^{45}Ca^{2+}$ content to a level comparable to that reached after 10 min exposure to 10 nM bombesin (a maximum concentration). When thapsigargin or DBHQ treated cells were further challenged with 10 nM bombesin for 10 min a small amount of additional $^{45}Ca^{2+}$ was released which is in agreement with the measurements of $[Ca^{2+}]_{cyt}$ shown in figure 5.11. Complete depletion of the $Ins(1,4,5)P_3$ responsive pool was obtained by exposure of cells to 30 nM thapsigargin for 30 min since subsequent exposure to bombesin caused no further reduction in cellular $^{45}Ca^{2+}$. For comparison, an 8 h treatment with 10 nM bombesin induced a depletion in $^{45}Ca^{2+}$ content similar to that achieved by 8 h pretreatment with 0.5 nM thapsigargin. The results in figures 5.11 - 5.14 demonstrate that treatment of Swiss 3T3 cells with thapsigargin or DBHQ, at concentrations that induce synergistic stimulation of DNA synthesis with PDB, cause a partial and persistent depletion in Ca^{2+} from the $Ins(1,4,5)P_3$ responsive Ca^{2+} pool.

5.5. EFFECT OF EXTRACELLULAR CALCIUM ON DNA SYNTHESIS

The Ca^{2+} response to agents that mobilise Ca^{2+} through formation of $Ins(1,4,5)P_3$ is typically biphasic. The first event is a large transient increase in $[Ca^{2+}]_{cyt}$ originating from intracellular stores and the second is a sustained influx of Ca^{2+} through the plasma membrane which causes a prolonged elevation of cytoplasmic Ca^{2+} (Putney and Bird, 1993). Because this sustained Ca^{2+} influx through the plasma membrane is dependent on extracellular Ca^{2+}, the effect of extracellular Ca^{2+} concentration on DNA synthesis induced by a variety of growth promoting factors was examined. The variety of factors will also distinguish between regulatory signals and obligatory events. The results in figure 5.15 show that DBHQ and PDB induced [^{3}H]thymidine incorporation is strikingly sensitive to extracellular Ca^{2+}. The $[Ca^{2+}]_{out}$ that is required to achieve 50% of the maximum response (ED_{50}) induced by PDB and DBHQ was 410 μM. A similar result was obtained for thapsigargin. In sharp contrast, the ED_{50} for PDB and insulin, a combination that induces DNA synthesis but does not stimulate Ca^{2+} mobilisation, was 6 μM. In order to substantiate the involvement of Ca^{2+} influx in the synergistic stimulation of DNA synthesis, the inhibitor econozole was used. This compound inhibits Ca^{2+} influx in several cell types (Alvarez, *et al.*, 1991; Breittmayer, *et al.*, 1993; Randriamampita and Tsien, 1993; Vostal and Fratantoni, 1993) through a mechanism independent of cytochrome *P*-450 inhibition (Vostal and Fratantoni, 1993).

The results in figure 5.16 show that DNA synthesis stimulated by PDB and DBHQ was reduced by 55% in the presence of 1 μM econozole (a concentration which has been shown to inhibit capacitative Ca^{2+} influx, (Alvarez, *et al.*, 1991). In contrast, DNA synthesis induced by PDB and insulin was only reduced by 13%. The results in figures 5.15 and 5.16 demonstrate that the synergistic stimulation of DNA synthesis by DBHQ with PDB requires capacitative Ca^{2+} influx.

Because bombesin is a well known mitogen for Swiss 3T3 cells that mobilises Ca^{2+} (Rozengurt and Sinnett-Smith, 1983; Mendoza, *et al.*, 1986) the contribution of Ca^{2+} influx to bombesin-induced DNA synthesis was examined. This was compared to the combination of bombesin with insulin because insulin elicits intracellular signals other than Ca^{2+}. Figure 5.17 demonstrates that bombesin-induced DNA synthesis is more sensitive to $[Ca^{2+}]_{out}$ (ED_{50} = 20 μM) than in the presence of insulin (ED_{50} = 0.9 μM).

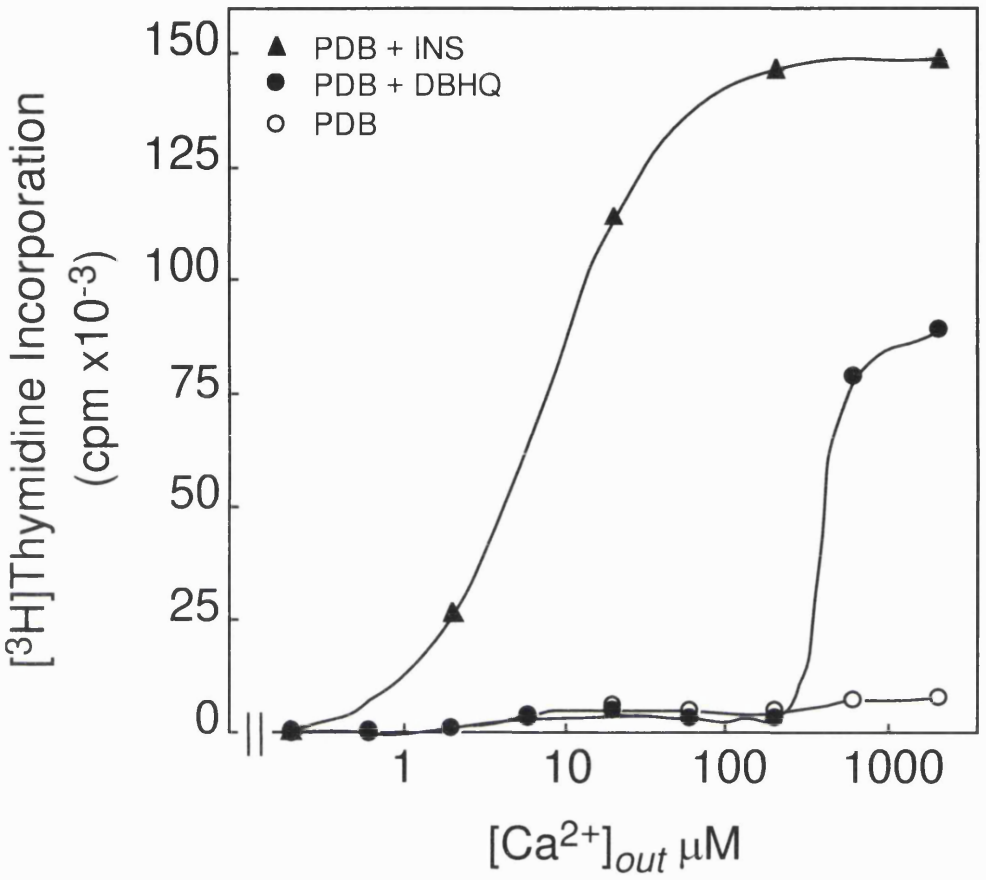

Figure 5.15. Effect of extracellular calcium on [^{3}H]thymidine incorporation induced by different mitogens

Confluent, quiescent cultures of Swiss 3T3 cells were pretreated for 6 - 8 h with 7.5 µM DBHQ (closed circles). Cells were then washed twice with DMEM and incubated in DMEM/Waymouth's medium containing [^{3}H]thymidine with various amounts of Ca^{2+} buffered by 2 mM EGTA in the presence of 80 nM PDB (open circles), 80 nM PDB and 7.5 µM DBHQ (closed circles), or 80 nM PDB and 1 µg/ml insulin (closed triangles). Similar results were obtained in 4 independent experiments.

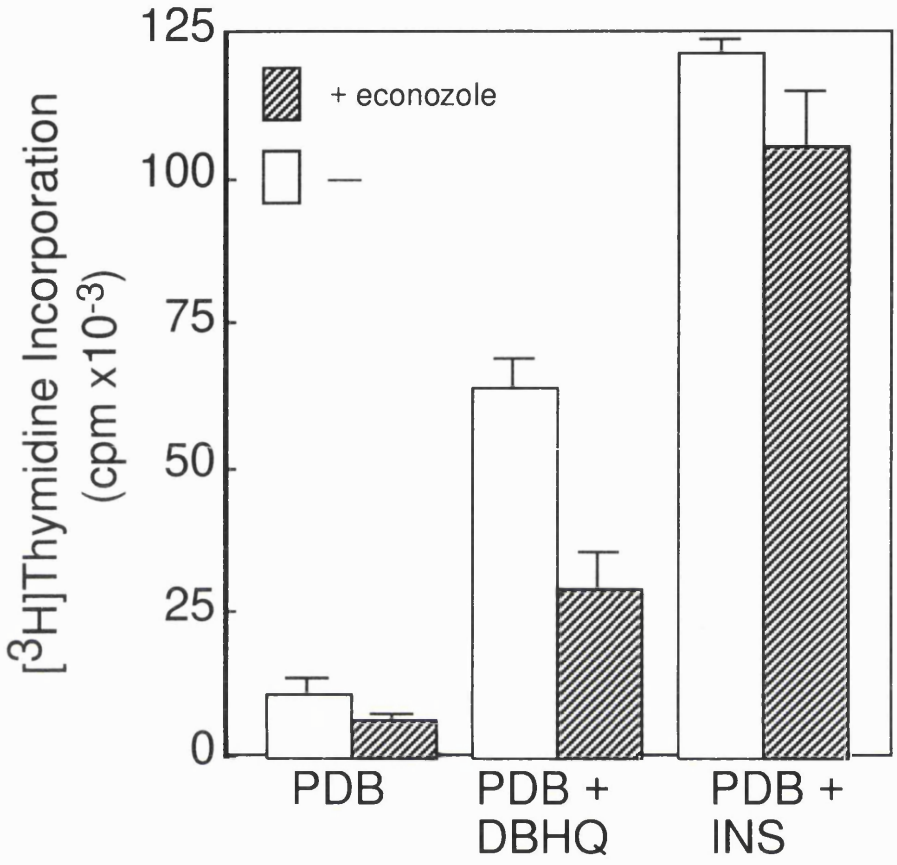

Figure 5.16. Effect of econozole on [^{3}H]thymidine incorporation induced by different mitogens

Confluent, quiescent cultures of Swiss 3T3 cells were pretreated for 6 h with or without 7.5 μM DBHQ. Cells were then washed twice with DMEM and incubated in DMEM/Waymouth's medium containing [^{3}H]thymidine. Cultures were stimulated with either 80 nM PDB (-), 80 nM PDB and 7.5 μM DBHQ (DBHQ), or with 80 nM PDB and 1 μg/ml insulin (INS) in the absence (open bars) or presence (striped bars) of 1 μM econozole. Similar results were obtained in two independent experiments.

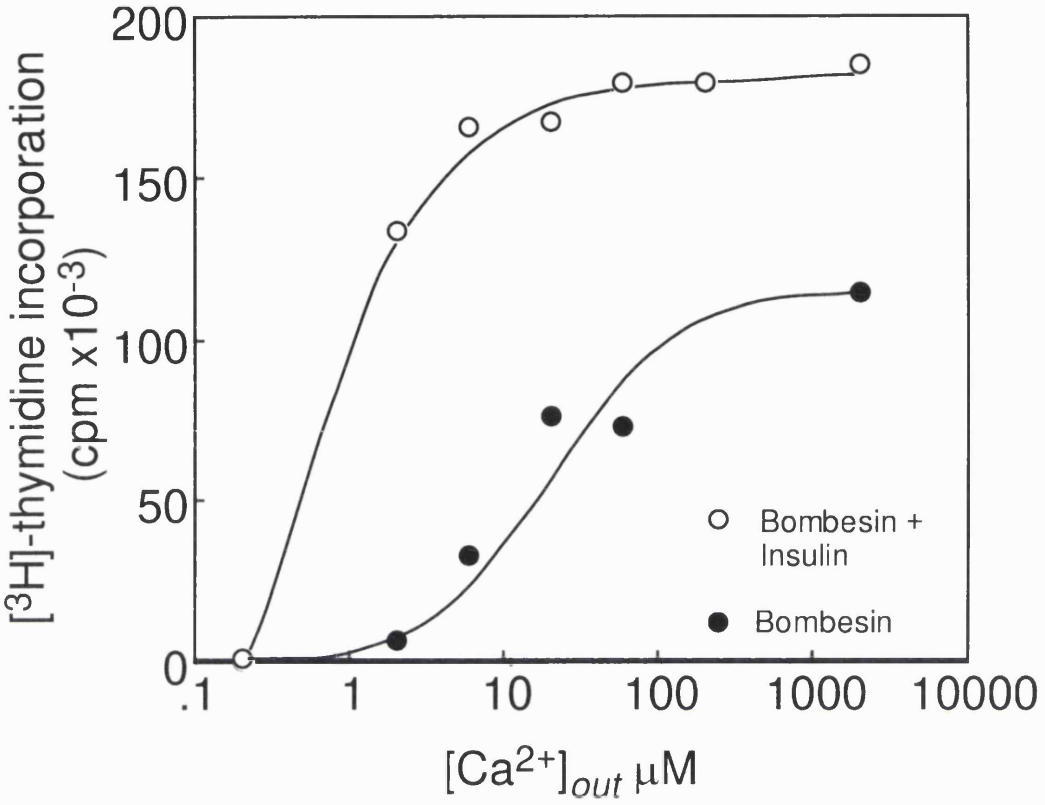

Figure 5.17. Effect of extracellular calcium on [^{3}H]thymidine incorporation induced by bombesin

Confluent, quiescent cultures of Swiss 3T3 cells were washed twice with DMEM and incubated in DMEM/Waymouth's medium containing [^{3}H]thymidine with various amounts of Ca^{2+} buffered by 2 mM EGTA in the presence of 10 nM bombesin and 1 µg/ml insulin (open circles) or 10 nM bombesin (closed circles). Similar results were obtained in 3 independent experiments.

5.6. SUMMARY AND DISCUSSION

The findings presented here demonstrate that thapsigargin and DBHQ synergistically stimulate DNA synthesis with PDB or bombesin in cultures of Swiss 3T3 cells. At the low concentrations that induce DNA synthesis, thapsigargin and DBHQ cause a slow but persistent depletion of the intracellular Ca^{2+} stores The stores remain depleted for at least 8 h and importantly bombesin, a physiological agonist, induces a prolonged depletion for more than 8 h also. This extends earlier measurements which showed that vasopressin caused Ca^{2+} depletion for upto 1 h (Lopez-Rivas and Rozengurt, 1984). This is the first time that either thapsigargin or DBHQ have been shown to stimulate the reinitiation of DNA synthesis in any target cell.

5.6.1. CAPACITATIVE Ca^{2+} ENTRY

It is recognised that depletion of ER Ca^{2+} stores e.g. by thapsigargin, DBHQ or neuropeptides, activates Ca^{2+} influx from the extracellular medium of many cell types (Putney and Bird, 1993). This has been termed capacitative Ca^{2+} entry (Putney, 1986) and is thought to be activated by a diffusible messenger released from the ER when the Ca^{2+} is depleted (Montero, *et al.*, 1992; Parekh, *et al.*, 1993; Randriamampita and Tsien, 1993). How does depletion of intracellular Ca^{2+} stores act in signalling DNA synthesis? Two alternative mechanisms could be envisaged. The diffusible messenger released from the depleted Ca^{2+} stores could, as well as activating Ca^{2+} entry, interact with other signalling components to promote synergistic stimulation of cell cycle entry from G_0. Alternatively, capacitative Ca^{2+} influx may lead to a persistent increase in $[Ca^{2+}]_{cyt}$. An attempt was made to distinguish between these possibilities. If the stimulation of DNA synthesis elicited by thapsigargin and DBHQ depends on Ca^{2+} influx it was reasoned that DNA synthesis should be critically dependent on $[Ca^{2+}]_{out}$. Indeed this was the case for the mitogenic combination of DBHQ and PDB. The ED_{50} of $[Ca^{2+}]_{out}$ required to support DNA synthesis stimulated by DBHQ and PDB was 100-fold higher than that required for DNA synthesis induced by insulin and PDB. The high $[Ca^{2+}]_{out}$ in DBHQ-treated cells is consistent with the apparent K_m (1 mM) of the channel that mediates capacitative Ca^{2+} influx (Mauger, *et al.*, 1984). In support of this, econozole, which inhibits Ca^{2+} influx (Alvarez, *et al.*, 1991; Breittmayer, *et al.*, 1993; Randriamampita and Tsien, 1993; Vostal and Fratantoni, 1993), inhibited DNA synthesis induced by PDB and DBHQ to a much greater extent than DNA synthesis induced by PDB and insulin. Taking these data together it can be concluded that the induction of DNA synthesis by DBHQ and thapsigargin in the presence of PDB is dependent on the activity of the capacitative Ca^{2+} influx pathway.

The results presented in this chapter contrast with the earlier studies on removal of Ca^{2+} from the growth medium (see section 1.3.2.5) which demonstrated that Ca^{2+} was essential, but could not show whether it had a stimulatory role in reinitiating DNA synthesis. The results presented in figures 5.15 - 5.17 clearly demonstrate that $[Ca^{2+}]_{out}$ is differentially required by different combinations of mitogens, defining Ca^{2+} fluxes as a regulatory signal rather than an obligatory event. PDB and either thapsigargin or DBHQ, induce only a subset of potential cellular signalling pathways, i.e. PKC activation and Ca^{2+} mobilisation, and are the most dependent on $[Ca^{2+}]_{out}$. Bombesin, which in addition activates PKC-independent pathways, is less sensitive to $[Ca^{2+}]_{out}$. Insulin induces different signalling pathways such as activation of PI3K (White and Kahn, 1994) and thus insulin together with PDB or bombesin are the least sensitive combinations to $[Ca^{2+}]_{out}$. The inhibition of DNA synthesis seen with all combinations at 0.2 μM $[Ca^{2+}]_{out}$ represents the essential role of Ca^{2+} in cell viability.

Interestingly, signals generated by thapsigargin- or DBHQ-depleted Ca^{2+} stores synergise with submaximal concentrations bombesin even though the bombesin-induced Ca^{2+} spike is attenuated. This would argue against the large transient increase in $[Ca^{2+}]_{cyt}$ being the mitogenic signal and suggest it is the consequence of the depleted stores that stimulates DNA synthesis. It has been shown in T-lymphocytes that it is the sustained influx rather than the intracellular Ca^{2+} spike which is important for IL-2 secretion (Gelfand, *et al.*, 1988). Similarly it is the influx which is necessary for catecholamine release in adrenal chromaffin cells (Cheek, *et al.*, 1993).

5.6.2. SYNERGY WITH PKC

In theory, treatment with thapsigargin or DBHQ leading to persistent depletion of Ca^{2+} stores could enhance the ability of PDB to induce cellular responses through cPKC which contain CalB domains. Several lines of evidence indicate that this is not the case. Specifically, treatment with either thapsigargin or DBHQ did not enhance the ability of PDB to induce a variety of responses such as direct PKC-mediated phosphorylation of 80K/MARCKS, stimulation of the kinase cascade leading to MAP kinase activation and tyrosine phosphorylation of multiple bands identified previously as FAK and paxillin (Sinnett-Smith, *et al.*, 1993; Zachary, *et al.*, 1993). Therefore, it can be concluded that Ca^{2+} and PKC signals act in parallel and converge downstream to induce synergistic stimulation of DNA synthesis.

The effect of $[Ca^{2+}]_{out}$ on bombesin-stimulated signalling has been investigated by Takuwa, *et al.* (1991a). It was shown that S6 kinase activity, MAPK activity, protein phosphorylation and c-*fos* induction were not inhibited by low $[Ca^{2+}]_{out}$. This also indicates that Ca^{2+} influx does not contribute to early events that are activated by PKC.

The only event measured that was shown to be inhibited by low Ca^{2+} was AA release, see below.

5.6.3. CYTOTOXIC EFFECTS

Our results contrast with previous studies that demonstrate that thapsigargin and DBHQ profoundly inhibit cell proliferation or induce cytotoxic effects (Ghosh, *et al.*, 1991; Marks, *et al.*, 1991; Short, *et al.*, 1993). Although different cell types were used, an obvious difference between the present and previous studies is the concentration of thapsigargin employed. In fact, inhibition of DNA synthesis and cytotoxic effects in Swiss 3T3 cells at higher concentrations of thapsigargin and DBHQ were readily obtained. The existence of stimulatory and inhibitory effects could explain the bell-shaped dose-response of these agents on DNA synthesis found in this study. The inhibitory effects are likely to be caused by extensive depletion of Ca^{2+} from the ER which, as well as being a store for hormonally releasable Ca^{2+}, plays an important role in the production of secretory, lysosomal and membrane bound proteins.

Draining of Ca^{2+} from stores by oscillations in $[Ca^{2+}]_{cyt}$ is suggested to induce controlled waves of secretion of proteins from distinct regions of the heterogeneous ER. However, mass perturbation of these stores by excessive amounts of ionophore could lead to indiscriminate vesiculation (Sambrook, 1990).

Sequestered Ca^{2+} rather than $[Ca^{2+}]_{cyt}$ is proposed to control protein synthesis (Brostrom and Brostrom, 1990). It is therefore not suprising that the initial effect of high concentrations of thapsigargin is to inhibit protein synthesis (Price, *et al.*, 1992; Wong, *et al.*, 1993). One of the earliest proteins to be synthesised 2 - 5 h after treatment with thapsigargin or A23187 is Grp78 (Benton, *et al.*, 1989; Price, *et al.*, 1992; Wong, *et al.*, 1993). Grp78 belongs to a family of glucose regulated proteins (Grp) and their induction is stimulated by a variety of stressful conditions such as glucose starvation, blockage of cellular glycosylation, reducing agents, Ca^{2+} ionophores and chelating agents and low extracellular pH (Lee, 1987). Other members of the Grp family are Grp94 (endoplasmin), ERp72 (has sequence identity with PDI) and Grp58 (Lee, 1992). Endoplasmin contains low affinity, high capacity Ca^{2+} binding sites and may bind to malfolded proteins retained in the ER. In the stress response the levels of Grp78 are elevated the most. Grp78 binds to a wide variety of proteins in the ER and assists in an ATP-dependent manner in the translocation, folding and assembly of oligomeric proteins. In normal cells Grp78 is found primarily in an aggregated oligomeric state where it is modified by phosphorylation or ADP-ribosylation. In stress-induced cells where there is an accumulation of non-transportable proteins, Grp78 is predominately monomeric and unmodified. Upon release from associated proteins Grp78 can be reversibly modified (Lee, 1992).

It is believed that reduction of Ca^{2+} in the ER inhibits protein glycosylation and it is the accumulation of underglycosylated proteins that triggers the induction of Grp78 (Lee, 1987). Consistent with this is the demonstration that the glycosylation of α1-antitrypsin was blocked at an early high mannose step when cells were treated with Ca^{2+} ionophore resulting in decreased secretion, whereas the secretion of albumin which is not glycosylated was affected to a lesser extent (Kuznetsov, *et al.*, 1992). The high mannose state was probably due to inhibition of α1,2-mannosidase, which requires Ca^{2+} for activity (Schutzbach and Forsee, 1990).

Control of the filling state of the ER by physiological agonists is carefully balanced but the excessive depletion caused by ionophores or high concentrations of thapsigargin could lead to serious disturbances of protein synthesis, processing and trafficking. This could explain the inhibition of cell proliferation induced by thapsigargin and DBHQ at higher concentrations than those used in the present study. In this respect Ghosh, *et al.* (1991), Marks, *et al.* (1991) and Short, *et al.* (1993) have identified the permissive requirement for Ca^{2+} rather than a regulatory role.

It is hypothesised that thapsigargin and DBHQ, at optimum mitogenic concentrations, reduce the Ca^{2+} content from internal stores in Swiss 3T3 cells to a critical level that leads to entry into DNA synthesis but does not impair essential ER functions.

5.6.4. POSSIBLE PATHWAYS ACTIVATED

Preparation of the ER

The two roles of the ER described above (hormonally-responsive Ca^{2+} store and protein processing organelle) are not mutually exclusive but very much interlinked. It was mentioned above that draining of Ca^{2+} from ER stores by oscillations in $[Ca^{2+}]_{cyt}$ is suggested to induce waves of secretion of proteins (Sambrook, 1990). Consistent with this, decreased Ca^{2+} in the ER results in the dissociation of T cell receptor chain from BiP and subsequent expression on the cell surface (Suzuki, *et al.*, 1991). Similarly Milner, *et al.* (1992) describe that Ca^{2+} regulation of KDEL on the KDEL receptor could regulate retention and secretion of ER luminal proteins.

In Swiss 3T3 cells the mitogens PMT, bombesin and PDGF all reduce the ADP-ribosylation of Grp78 (Staddon, *et al.*, 1992) hence converting it from an inactive to an active conformation. This was attributed to the preparation of the ER for the increase in protein traffic associated with re-entry into the cell-cycle that would require the activity of BiP. This further illustrates the interplay between protein processing in the ER and stimulation by mitogenic agonists that mobilise Ca^{2+}. Interestingly Grp78 is also induced by treatment with insulin (Lee, 1987) and this may also be in preparation for increased protein synthesis, processing and trafficking.

Activation of signal transduction molecules

Ca^{2+} influx is crucial for DNA synthesis induced by thapsigargin or DBHQ with PDB, but the possible pathways activated have not been elucidated. Whatever these pathways are it is evident they cannot initiate DNA synthesis alone but require a synergistic signal from PKC. Clues could come from proteins which bind Ca^{2+}. As already described in section 1.3.2.4, Ca^{2+} is a very localised second messenger, giving rise to Ca^{2+} micro-gradients and possibly nano-gradients around Ca^{2+} channels in membranes. In this respect Petersen, *et al.* (1994) point out that low affinity Ca^{2+} binding proteins such as those with CalB domains including $cPLA_2$, PKCγ and PLCγ, are generally associated with membranes whereas proteins which bind Ca^{2+} with high affinity are in the cytosol. So could treatment with thapsigargin and DBHQ be causing the activation of membrane bound proteins? The results in Chapter 5 showed that thapsigargin and DBHQ did not potentiate PKC-stimulated signals so it is unlikely that species of PLC or PKC contribute to the observed synergy. In contrast there is evidence that suggests the involvement of PLA_2 and AA.

$cPLA_2$ is regulated by Ca^{2+} and MAPK (Clark, *et al.*, 1991; Piomelli, 1993). Also A23187 can induce AA release in Swiss 3T3 cells (Millar and Rozengurt, 1990a). In addition there are many reports that link extracellular Ca^{2+} and/or Ca^{2+} influx to subsequent AA release (Toyoshima, *et al.*, 1982; Balsinde, *et al.*, 1990; Takuwa, *et al.*, 1991a; Takuwa, *et al.*, 1991b; Liu, *et al.*, 1993; Sharma, 1993; Arkinstall, *et al.*, 1994; Tornquist, *et al.*, 1994). Of particular interest is the observation that bombesin-induced AA release requires high $[Ca^{2+}]_{out}$ (Takuwa, *et al.*, 1991b). It is thought that bombesin-induced release of AA in Swiss 3T3 cells results in production of PGE_1 which acts on a G_s-coupled receptor to increase cAMP (Millar and Rozengurt, 1988; Millar and Rozengurt, 1990a). It is of interest to note that cAMP does not increase 80K phosphorylation (Rodriguez-Pena and Rozengurt, 1986), MAPK activation (Withers, *et al.*, 1995) or tyrosine phosphorylation of bands 70,000 - 80,000 and 110,000 - 130,000 (Zachary, *et al.*, 1991a).

An unusual aspect of DNA synthesis induced by thapsigargin and DBHQ is their inability to synergise with insulin. Characterisation of a plethora of growth promoting agents, both physiological and pharmacological, has identified insulin as a factor that is not able to stimulate DNA synthesis by itself but can synergise with almost all other compounds tested including cAMP elevating agents (Rozengurt, 1986). If thapsigargin and DBHQ were acting to increase cAMP levels via AA release and prostaglandin synthesis then it would have been expected that they would synergise with insulin. However, Ca^{2+} influx alone is not enough to fully stimulate PLA_2. Sustained activation of PLA_2 also requires PKC activity because down regulation of PKC by prolonged treatment with PDB abolishes the sustained bombesin-induced PLA_2 activity (Takuwa, *et al.*, 1991a). Hence thapsigargin and DBHQ could not have

synergised with insulin in the absence of PDB. It would then be difficult to show synergism between insulin and PDB in combination with thapsigargin or DBHQ because PDB and insulin is a potent mitogenic combination itself. This illustrates that PKC signals and Ca^{2+} signals could converge on one single enzyme.

CaM and multifunctional CaMK are also potential targets for a Ca^{2+} stimulated signal transduction pathway and it has been demonstrated that bombesin induces CaMK activity (Duan, *et al.*, 1994). CaMK has been proposed to act as frequency decoder for intracellular Ca^{2+} oscillations (Hanson, *et al.*, 1994). Interestingly one of the few studies that has also used 0.5 nM thapsigargin demonstrated that partial inhibition of Ca^{2+}ATPase pumps increased intracellular spiking frequency (Petersen, *et al.*, 1993) suggesting a mechanism whereby treatment with low concentrations of thapsigargin could increase intracellular Ca^{2+} oscillations to a threshold frequency thus activating CaMK.

Chapter 6 - SUMMARY AND PERSPECTIVES

It has been recognised for over a decade that the activation of multiple signal transduction pathways is required for a full mitogenic effect (Rozengurt, 1986). For instance activation of PKC by phorbol ester or the addition of insulin to Swiss 3T3 cells will not stimulate DNA synthesis but phorbol ester together with insulin is a potent mitogenic combination (Dicker and Rozengurt, 1978; Dicker and Rozengurt, 1980). Similarly in Swiss 3T3 cells increasing cAMP does not induce DNA synthesis but elevation of cAMP together with activation of PKC does induce DNA synthesis (Rozengurt, 1986). This identifies the co-operation between two signalling pathways.

This principle can be extended to physiological growth factors. PDGF and bombesin are sole mitogens for Swiss 3T3 cells whereas EGF, vasopressin and bradykinin are not. Thus it was hypothesised that PDGF and bombesin can stimulate multiple, synergistic signalling pathways (Rozengurt, 1986). The mechanisms by which receptor tyrosine kinases can elicit multiple signals involves phosphorylated tyrosine residues which create multiple docking sites for signal transduction molecules. The mechanism by which G protein-coupled receptors can elicit multiple signal transduction pathways is more elusive. Because bombesin is a sole mitogen much attention has been given to elucidating its cellular mechanisms of activation.

Bombesin stimulates multiple signal transduction pathways including PLC-mediated hydrolysis of PIP_2, PKC activation, Ca^{2+} mobilisation, Na^+/H^+ exchange activity and cytoplasmic alkalisation, arachidonic acid release, enhancement of cAMP elevation, MAPK activation and tyrosine phosphorylation of multiple substrates including FAK and paxillin. All of the above events are totally or partially dependent on PKC, this is summarised in table 3.III. However tyrosine phosphorylation was unusual in that it had no PKC or Ca^{2+} dependency. This raised questions concerning the mechanisms by which bombesin could couple to multiple pathways. At least three possible mechanisms can be envisaged: 1) the different pathways could be elicited by distinct receptors; 2) distinct $G\alpha$ subunits could couple to the same receptor; 3) $\beta\gamma$ subunits of heterotrimeric G proteins could elicit distinct pathways from $G\alpha$. It had been suggested that a separate bombesin receptor mediated tyrosine phosphorylation and mitogenesis but no direct evidence was presented (Bold *et. al.*, 1995). Therefore we aimed to characterise signalling pathways elicited by the bombesin/GRP receptor which was transfected into Rat-1 fibroblasts, a cell line that is unresponsive to bombesin.

The bombesin receptor couples to multiple pathways

The results presented in Chapter 3 of this thesis provide compelling evidence that ligand activation of the transfected bombesin/GRP receptor activates tyrosine phosphorylation of FAK, paxillin and other proteins as well as couple to PLC-mediated events and induces cell proliferation. Thus the full signalling and mitogenic potential of bombesin can be mediated by the bombesin/GRP receptor without the need to propose further receptor subtypes. This is the first time that a single subtype of G protein-coupled receptor, defined by transfection, has been shown to couple to PLC-mediated events, tyrosine phosphorylation of FAK and paxillin and stimulate DNA synthesis. This contrasts with the other well known mitogens LPA and thrombin for which receptor identity has not been clearly established. The possible mechanism of coupling to multiple pathways was also discussed.

It had already been shown that tyrosine phosphorylation induced by bombesin and other neuropeptides was unlikely to be downstream of α_q (Sinnett-Smith *et. al.*, 1993) and this is supported by the demonstration that microinjection of activated α_q did not enhance the formation of stress fibres (Buhl, *et al.*, 1995). The possibility of signalling via $\beta\gamma$ subunits is also unlikely because there is very little evidence for the specificity of $\beta\gamma$ combinations on effector activation, see section 1.1.6.4 and microinjection of several $\beta\gamma$ combinations had no effect on stress fibre formation (Buhl, et. al., 1995). The most likely mechanism of bombesin-induced tyrosine phosphorylation of multiple substrates including FAK and paxillin is the coupling of the bombesin/GRP receptor to multiple Gα, potential candidates being α_{12} or α_{13}.

Further experimental work is required to test which Gα the bombesin/GRP receptor couples to. This could be, for example, by using [α-^{32}P]GTP azidoanilide to photolabel the α subunits which are activated. Alternatively specific Gα could be inhibited by microinjection of antibodies against the α subunits and then bombesin-induced stress fibre assembly and tyrosine phosphorylation could be examined by fluorescence microscopy. Antisense vectors against different Gα followed by phosphotyrosine analysis could determine which Gα are necessary for bombesin-induced tyrosine phosphorylation. Also mutational analysis of the bombesin/GRP receptor could identify sites of multiple G protein interaction.

Bombesin stimulates the GTP binding of Ras

It is being increasingly recognised that activation of the MAPK cascade is important on receptor identity and especially on cellular context. This is in contrast to tyrosine phosphorylation which displays similar characteristics when stimulated by a variety of receptors coupled to G_q in various cells. Receptors coupled to G_q generally activate the MAPK pathway in a PKC-dependent fashion. Consistent with this, MAPK activation induced by bombesin in Swiss 3T3 cells is dependent on PKC. However

other reports imply that, like G_i-coupled receptors, G_q-coupled receptors activate MAPK in a Ras dependent fashion. Therefore characteristics of MAPK stimulation were investigated in Rat-1 cells that were transfected by the bombesin/GRP receptor.

Chapter 4 showed that in contrast to earlier studies in Swiss 3T3 cells, bombesin-stimulation of the MAPK cascade was independent of PKC activation in Rat-1 cells. In addition it was shown for the first time that bombesin can stimulate Raf-1 activity and this also occurred in a PKC-independent and pertussis toxin-insensitive fashion. These results led to the examination of Ras activation in Rat-1 cells transfected with the bombesin/GRP receptor. Addition of bombesin to BOR 15 cells caused an increase in the amount of GTP bound to Ras, implicating Ras in the activation of MAPK induced by bombesin in Rat-1 cells. This demonstrates that the variability of bombesin/GRP receptor coupling depends on the cell type that expresses the receptor. The possible upstream pathway leading to Ras activation was discussed. This includes activation of protein tyrosine kinases or lipid kinases and these may have other cellular consequences in addition to the activation of Ras. Similarly Ras has multiple downstream effectors further expanding the repertoire of potential signalling pathways.

Calcium

The first and second parts of the thesis investigated the multiple pathways that bombesin induced and the mechanisms by which bombesin could induce multiple pathways. One of the earliest events induced by bombesin is the mobilisation of Ca^{2+}, and before this work was started it had not been established if Ca^{2+} fluxes initiated another signal transduction pathway that was capable of synergising with the signalling pathways already documented to reinitiate DNA synthesis. Addition of bombesin to Swiss 3T3 cells causes a transient increase in Ca^{2+} followed by a depletion of total cellular Ca^{2+} that lasts at least 1 h. To investigate the effect of Ca^{2+} mobilisation on DNA synthesis we used new tools which were thapsigargin and DBHQ. These agents inhibit the ER Ca^{2+}ATPase causing a depletion of Ca^{2+} from hormonal responsive stores.

Chapter 5 shows that depletion of intracellular Ca^{2+} by low concentrations of thapsigargin or DBHQ, in a manner similar to that induced by bombesin, has profound effects on [^{3}H]thymidine incorporation, shown to be due to reinitiation of DNA synthesis. This contrasted with earlier studies which showed that thapsigargin and DBHQ inhibited cell growth and induced cytotoxic effects and this was attributed to gross perturbation of ER Ca^{2+} stores which are necessary for protein synthesis, processing and trafficking. The presence of functional PKC was crucial but the early events stimulated by PKC were not potentiated after pretreatment with thapsigarin or DBHQ suggesting that PKC signals and Ca^{2+}-induced signals converge further into G_1.

The remarkable sensitivity to $[Ca^{2+}]_{out}$ on DNA synthesis induced by PDB with either thapsigargin or DBHQ suggested that Ca^{2+} influx was crucial. Bombesin was also partially dependent on extracellular Ca^{2+} but in the presence of insulin the requirements for $[Ca^{2+}]_{out}$ were bypassed. Thus Ca^{2+} could be an important signalling pathway participating in bombesin regulated cell proliferation. The observation that different combinations of mitogenic factors are dependent on $[Ca^{2+}]_{out}$ to varying degrees identifies Ca^{2+} influx induced by DBHQ as a regulatory signal rather than an obligatory event. The demonstration that Ca^{2+} can stimulate DNA synthesis is also important for receptor tyrosine kinases which bind to and activate PLCγ.

The pathways activated by thapsigargin and DBHQ are unknown. Identifying the signal transduction pathways would help to further understand the role of Ca^{2+} in initiating DNA synthesis. Starting points could be measurements of CaMK activity or arachidonic acid release. It would also be interesting to monitor $[Ca^{2+}]_{cyt}$ by fluorescence microscopy to see if there is increased Ca^{2+} around the periphery of the cell close to the influx channels or if there is an increase in Ca^{2+} spiking frequency in response to treatment of cells with thapsigargin or DBHQ..

Summary

The results presented in this thesis contribute to a new scheme of signalling pathways stimulated by the bombesin/GRP receptor. This is shown in figure 6.1. Thus bombesin may couple to FAK and paxillin phosphorylation via $G_{12/13}$, couple to $p21^{ras}$ and MAPK activation in Rat-1 cells via βγ subunits and couple to PLCβ-mediated activation of PKC and Ca^{2+} mobilisation the latter of which has been demonstrated to contribute signals to the mitogenic response.

These results further support the concept of multiple synergistic signalling pathways. It has been demonstrated at the molecular level that a single species of receptor can elicit all pathways induced by bombesin, and also at a cellular level that the interaction between PKC and Ca^{2+} is a potent synergistic combination.

Implications

The demonstration that bombesin can couple to multiple pathways that can vary from cell type to cell type, raises important questions to how bombesin signals in *in vivo* rather than in cultured cells. For example, bombesin has long been implicated in lung development and recently it was shown more specifically to induce foetal lung branching (Li, *et al.*, 1994; King, *et al.*, 1995). Bombesin is also implicated in small cell lung cancer (Sethi, *et al.*, 1992). The pathways activated by bombesin in these situations have not been fully characterised, although it has been shown in small cell lung cancer cell lines that bombesin mobilises Ca^{2+} and as demonstrated in Chapter 5, Ca^{2+} fluxes could have an important contribution in reinitiating DNA synthesis.

However, the effect on Ras in these cell lines has not been studied. Thus the work presented in this thesis may be relevant to bombesin/GRP signalling *in vivo*, both in normal and pathological conditions.

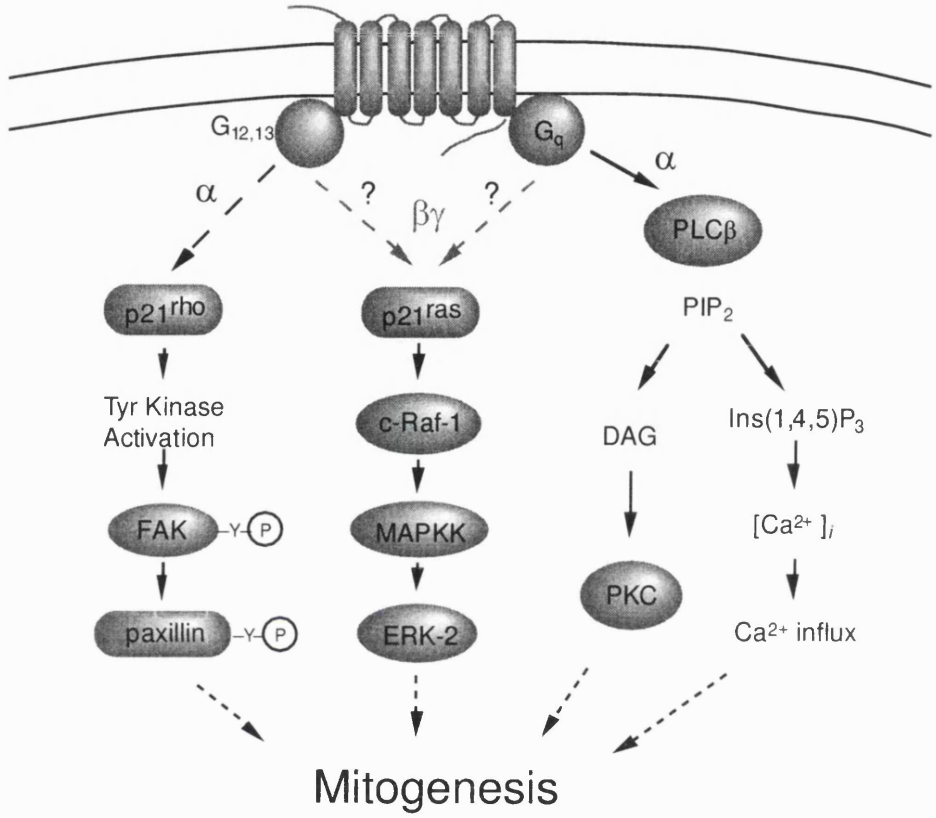

Figure 6.1. The bombesin/GRP receptor couples to multiple signal transduction pathways.

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Thapsigargin and Di-*tert*-butylhydroquinone Induce Synergistic Stimulation of DNA Synthesis with Phorbol Ester and Bombesin in Swiss 3T3 Cells*

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The specific inhibitors of the endoplasmic reticulum Ca^{2+} pump, thapsigargin and 2,5-di-*tert*-butylhydroquinone (DBHQ), stimulated reinitiation of DNA synthesis in synergy with either phorbol 12,13-dibutyrate or bombesin in Swiss 3T3 cells. Maximum stimulation was achieved at 0.5 nM thapsigargin and 7.5 μM DBHQ. Kinetics of [^{3}H]thymidine incorporation were consistent with exit from G_0 and entry into S phase. Autoradiography of labeled nuclei showed that the increase in [^{3}H]thymidine incorporation was due to an increase in the proportion of cells entering into DNA synthesis. Down-regulation or selective inhibition of protein kinase C abolished this synergistic stimulation of DNA synthesis. Thapsigargin and DBHQ did not potentiate protein kinase C-mediated signals such as direct phosphorylation of myristoylated alanine-rich C-kinase substrate, activation of mitogen-activated protein kinase, and tyrosine phosphorylation of bands 110,000–130,000 and 70,000–80,000. Thapsigargin and DBHQ caused a marked reduction in the ability of bombesin to induce a rapid and transient increase in intracellular Ca^{2+} via depletion of total cellular Ca^{2+}, measured by $^{45}Ca^{2+}$ content. The synergistic stimulation of DNA synthesis by DBHQ and phorbol 12,13-dibutyrate was dependent on a high concentration of extracellular Ca^{2+} (ED_{50} = 410 μM) and was preferentially inhibited by the inhibitor of Ca^{2+} influx econozole. This suggests a role for Ca^{2+} entry in growth control. This is the first time that either thapsigargin or DBHQ has been shown to stimulate the reinitiation of DNA synthesis in any target cell.

Fibroblasts reversibly arrested in the G_0 phase of the cell cycle can be stimulated to reinitiate cell proliferation through multiple signal transduction pathways that act in a synergistic and combinatorial fashion (1, 2). The possibility that Ca^{2+} fluxes could play a role in the transduction of mitogenic signals has attracted intense interest. Neuropeptides such as bombesin, vasopressin, endothelin, and bradykinin, which are potent mitogens for Swiss 3T3 cells (1), induce a rapid and transient increase in the cytoplasmic concentration of Ca^{2+} ($[Ca^{2+}]_i$)[1] followed by a persistent depletion of Ca^{2+} from stores in the ER (3–6). This effect is mediated by the second messenger $Ins(1,4,5)P_3$ (7), which is generated in response to receptor-mediated activation of G_q, the GTP-binding protein that couples neuropeptide receptors to phospholipase C β (8, 9). Accordingly, transfection of receptors that are constitutively coupled to G_q (10) or of activated G_q mutants (11) causes transformation of certain cells. Also, microinjection of antibodies raised against G_q blocks the mitogenic activity of bradykinin (12). Furthermore, a bacterial toxin from *Pasteurella multocida*, which causes a striking activation of inositol phosphate production and Ca^{2+} mobilization, also promotes cell proliferation including anchorage-independent growth (13–15). All of these findings suggest, but do not prove, a role for Ca^{2+} fluxes in the mitogenic activation of quiescent fibroblasts because hydrolysis of phosphatidylinositol 4,5-bisphosphate also generates diacylglycerol, which activates PKC, a well known pathway leading to gene expression and cell proliferation (16–19). Furthermore, insulin and epidermal growth factor can stimulate DNA synthesis in Swiss 3T3 cells without inducing Ca^{2+} mobilization (20, 21). Similarly, mutant receptors for platelet-derived growth factor and fibroblast growth factor defective in phospholipase C γ activation have been shown to retain mitogenic activity upon ligand activation (22–25). Thus, the contribution of Ca^{2+} to mitogenic signaling remains unclear.

An approach for assessing the role of Ca^{2+} in the transition from G_0 into DNA synthesis is the use of specific inhibitors of the Ca^{2+} ATPase pump of the ER, which is responsible for the accumulation of Ca^{2+} into these stores. The tumor promoter thapsigargin and the structurally distinct compound DBHQ have been identified as inhibitors of Ca^{2+} accumulation in the ER (26–31). Addition of these compounds to intact cells induces mobilization of Ca^{2+} from internal stores bypassing phospholipase C-mediated formation of $Ins(1,4,5)P_3$. Previous studies have shown that the addition of either thapsigargin or DBHQ, at micromolar concentrations, profoundly inhibited cell proliferation or induced cytotoxic effects (32, 33). These findings are difficult to reconcile with the fact that mitogenic neuropeptides and other growth-promoting factors also cause persistent depletion of Ca^{2+} from the ER.

The experiments presented here were designed to assess the effect of gradual depletion of Ca^{2+} from the ER on the ability of quiescent cells to exit from G_0 and enter DNA synthesis.

EXPERIMENTAL PROCEDURES

Cell Culture—Stock cultures of Swiss 3T3 cells were propagated as previously described (34). For experimental purposes, cells were subcultured in 33- or 100-mm Nunc Petri dishes with DMEM supplemented with 10% FBS and incubated in a humidified atmosphere of 10% CO_2, 90% air at 37 °C until they became confluent and quiescent (6–8 days).

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[1] The abbreviations used are: $[Ca^{2+}]_i$, intracellular calcium concentration; DBHQ, di-(*tert*)-butylhydroquinone; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; $[Ca^{2+}]_{out}$, extracellular calcium concentration; FBS, fetal bovine serum; fura-2/AME, fura 2-tetraacetoxymethyl ester; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; MAP kinase, mitogen-activated protein kinase; 80K/MARCKS, myristoylated alanine-rich C-kinase substrate; PDB, phorbol 12,13-dibutyrate; PBS, phosphate-buffered saline; PKC, protein kinase C; PAGE, polyacrylamide gel electrophoresis; Me_2So, dimethyl sulfoxide; mAb, monoclonal antibodies.

Pretreatments—Pretreatments with thapsigargin and DBHQ were carried out in conditioned medium, which is the medium on 6–8-day-old cells that have attained quiescence and is thus essentially devoid of growth-promoting activity.

[^{3}H]Thymidine Incorporation Assay—Determinations of DNA synthesis were performed as previously described (35). Cultures were washed twice with DMEM and incubated with DMEM/Waymouth's medium (1:1 (v/v)) containing [^{3}H]thymidine (0.25 μCi/ml, 1 μM) and various additions as described in the figure legends. After 40 h, the cultures were washed twice with PBS and incubated in 5% trichloroacetic acid at 4 °C for 30 min to remove acid-soluble radioactivity. Cultures were washed with ethanol, solubilized in 1 ml of 2% Na_2CO_3, 0.1 M NaOH, 1% SDS, and the radioactivity in the acid-insoluble pools was determined by scintillation counting in 6 ml of Ultima Gold (Packard).

Autoradiography of Labeled Nuclei—Labeled nuclei were determined by autoradiography as previously described (35). Cultures were washed twice with DMEM and incubated with DMEM/Waymouth's medium (1:1 (v/v)) containing [^{3}H]thymidine (2.5 μCi/ml) and various additions as described in the figure legends. After 40 h, the medium was removed, and the cultures were fixed in formol saline (1:9 (v/v)). The cultures were washed twice with Tris-saline, pH 7.4, at 4 °C, fixed twice (for 5 and 2 min) with 5% trichloroacetic acid at 4 °C, and washed three times with 70% ethanol. Autoradiographic film was put on the cultures and developed after a 2-week exposure. After counterstaining with Giemsa, the labeled nuclei were counted.

Measurement of Total Inositol Phosphates—Total inositol phosphates were determined as previously described (14). Briefly, cultures of Swiss 3T3 cells labeled for 16–18 h with *myo*-[2-^{3}H]inositol (15 μCi/ml) were washed twice with DMEM/Waymouth's medium (1:1 (v/v)) and incubated in this medium with additions as described in the figure legends. LiCl was added to a final concentration of 20 mM for the last 30 min of the incubation. Inositol phosphates were extracted by replacing the medium with 1 ml of ice-cold 3% $HClO_4$. After 20 min at 4 °C, the extract was neutralized with 0.5 M KOH containing 25 mM HEPES, 5 mM EGTA, and 0.01% phenol red. Precipitated $KClO_4$ was removed by centrifugation. Samples were diluted to 10 ml with water then loaded onto 1 ml of Dowex AG 1-X8 (200–400 mesh, $HCOO^-$ form) in Bio-Rad Econo-columns. Columns were washed with 3 × 10 ml of H_2O and 2 × 10 ml of 60 mM NH_4COOH, 5 mM $Na_2B_4O_7$ and inositol phosphates eluted with 6 ml of 1 M NH_4COOH, 0.1 M HCOOH. An aliquot (1 ml) of eluate was counted in 10 ml of Ultima Gold.

$^{32}P_i$ Labeling of Swiss 3T3 Cells—Quiescent cultures of cells in 33-mm dishes were washed twice with DMEM without phosphate and labeled overnight in this medium with 50 μCi/ml $^{32}P_i$. Cells were treated with various factors as indicated then lysed in 250 μl of ice-cold lysis buffer (10 mM Tris/HCl, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 mM Na_3VO_4, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride, pH 7.6).

Immunoprecipitations—For immunoprecipitation of phosphotyrosyl proteins, quiescent cultures of cells in 100-mm dishes were washed twice with DMEM, treated with factors in 5 ml of DMEM as indicated, and lysed at 4 °C in 1 ml of lysis buffer. Lysates of these cells and those of the $^{32}P_i$-labeled cells were clarified by centrifugation at 15,000 × *g* for 10 min. Tyrosine-phosphorylated proteins were immunoprecipitated for 2 h at 4 °C with agarose-linked anti-Tyr(P) as previously described (36). 80K/MARCKS was immunoprecipitated for 3 h at 4 °C with 80K/MARCKS antiserum (37) and then 1 h with agarose-linked protein A. Immunoprecipitates were washed three times with lysis buffer, extracted in 2 × sample buffer (200 mM Tris/HCl, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol, pH 6.8) and then resolved by one-dimensional SDS-PAGE and analyzed as described in the figure legends.

Western Blotting—After SDS-PAGE, proteins were transferred to Immobilon transfer membranes. Membranes were blocked using 5% non-fat dried milk in PBS, pH 7.2, and incubated for 2 h with either the anti-Tyr(P) mAbs (Py20 and 4G10, 1 μg/ml antibody) or the MAP kinase antiserum, raised against a synthetic peptide to the C-terminal domain (1:1000) in PBS containing 3% non-fat dried milk. Immunoreactive bands were visualized by autoradiography using ^{125}I-labeled sheep anti-mouse IgG (1:1000) for anti-Tyr(P) mAbs and ^{125}I-labeled protein A (1:1000) for the MAP kinase antiserum.

Measurement of Intracellular Calcium—$[Ca^{2+}]_i$ was measured with the fluorescent Ca^{2+} indicator fura-2/AME using procedures previously described (38). Cells on 100-mm dishes were washed twice with DMEM and incubated for 10 min in 5 ml of DMEM containing 1 μM fura-2/AME freshly prepared from a 1 mM stock maintained in Me_2SO. Cells were washed twice with PBS, gently scraped and resuspended in 2 ml of electrolyte solution (150 mM NaCl, 0.5 mM KCl, 0.9 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM glucose, 16 mM HEPES (the same concentration of amino acids as those in DMEM), pH 7.1), then transferred to a 1-cm^2 quartz cuvette, which was kept at 37 °C and stirred continuously throughout the experiment. Fluorescence was monitored in a Perkin-Elmer LS-5 luminescence spectrometer with an excitation wavelength of 336 nm and an emission wavelength of 510 nm. $[Ca^{2+}]_i$ was calculated after sequential addition of Triton X-100 to 0.05% and EGTA to 100 mM to obtain the maximum (F_{max}) and minimum (F_{min}) fluorescence respectively, using the formula $[Ca^{2+}]_i$ in nM = $K(F - F_{min})/(F_{max} - F)$, where F is the fluorescence at the unknown $[Ca^{2+}]_i$, and the value of K is 220 nM for fura-2/AME.

Measurement of Total $^{45}Ca^{2+}$ Content—Confluent quiescent Swiss 3T3 cells were loaded to equilibrium with 4 μCi of $^{45}Ca^{2+}$/ml over 16 h. Cells were then washed twice with DMEM and incubated with factors for 10 min at 37 °C. Cells were rapidly washed with 2 × 5 ml of DMEM with 2 mM EGTA and then 6 × 5 ml of DMEM. Cells were dissolved in 1 ml of 2% Na_2CO_3, 0.1 N NaOH, 1% SDS, and the total amount of $^{45}Ca^{2+}$ in the cells was measured by liquid scintillation counting in 10 ml of Ultima Gold.

Materials—Thapsigargin, GF 109203X, and fura-2/AME were obtained from Calbiochem. DBHQ was obtained from Aldrich. PDB, insulin, bombesin, and econozole were obtained from Sigma. [*methyl*-^{3}H]Thymidine (25 Ci/mmol), *myo*-[2-^{3}H]inositol (16 Ci/mmol), $^{32}P_i$ (10 mCi/ml), ^{125}I-labeled sheep anti-mouse IgG (20 μCi/μg), ^{125}I-labeled protein A (30 mCi/mg), and $^{45}CaCl_2$ (10–40 mCi/mg calcium) were obtained from Amersham International (United Kingdom). MAP kinase antiserum was a generous gift of Dr. Johan Van Lint of The Katholieke Universiteit Leuven. Anti-mouse IgG-agarose-linked anti-Tyr(P) mAb clone Py72 was obtained from the hybridoma development unit, Imperial Cancer Research Fund (London). Py20 anti-Tyr(P) mAb was from ICN (Buckinghamshire, UK). The 4G10 anti-Tyr(P) mAb was from Tissue Culture Supplies (Buckingham, UK). FBS was obtained from Life Technologies, Inc. All other materials were of the purest grade commercially available.

Stock solutions of thapsigargin were made up at 1 mM in Me_2SO and stored at −20 °C. DBHQ was dissolved fresh each time to 100 mM in Me_2SO. Dilutions of compounds in Me_2SO and Me_2SO controls were added to the medium at 1 μl/ml. This concentration of Me_2SO had no measurable effects on our cells.

RESULTS

Effect of Thapsigargin and DBHQ on PDB-stimulated DNA Synthesis—Initial experiments confirmed that thapsigargin (2–10 nM) inhibited DNA synthesis induced by a variety of growth-promoting factors in Swiss 3T3 cells. However, when the dose dependence of this effect was carefully examined, a stimulatory action was discovered at low concentrations of thapsigargin. This prompted a detailed analysis of the effects of Ca^{2+}ATPase pump inhibitors on cellular DNA synthesis.

Quiescent cultures of Swiss 3T3 cells were preincubated with a range of concentrations of DBHQ or thapsigargin for 6 h. The cultures were then exposed to fresh medium containing PDB, the same concentrations of thapsigargin or DBHQ and [^{3}H]thymidine. Cumulative [^{3}H]thymidine incorporation was measured after 40 h of incubation. Fig. 1 shows that both DBHQ and thapsigargin caused a striking stimulation of DNA synthesis in the presence of PDB. The optimum effects were observed at 7.5 μM DBHQ and 0.5 nM thapsigargin where they reached 63 and 62%, respectively, of the maximum response induced by FBS. The stimulation of [^{3}H]thymidine incorporation by these agents was induced within a narrow concentration range. At higher concentrations of DBHQ or thapsigargin, cell toxicity (detachment) was clearly evident after 40 h of incubation. DBHQ and thapsigargin added alone did not induce any [^{3}H]thymidine incorporation in Swiss 3T3 cells (Fig. 1).

To examine the extent to which the pretreatment with 0.5 nM thapsigargin and 7.5 μM DBHQ affected the resulting stimulation of [^{3}H]thymidine incorporation, confluent quiescent cultures of Swiss 3T3 cells were preincubated with these agents for various times. Cultures were then washed, thapsigargin or DBHQ was replaced, and PDB was added. Cumulative [^{3}H]thymidine incorporation was measured after 40 h. The results in

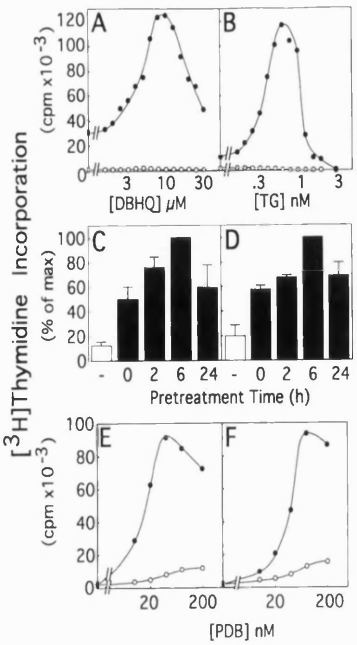

FIG. 1. *A* and *B*, dose-response curve for the stimulation of DNA synthesis by DBHQ (*A*) or thapsigargin (*TG*) (*B*). Confluent quiescent cultures of Swiss 3T3 cells were pretreated with various concentrations of DBHQ (*A*) or thapsigargin (*B*) for 6 h. The cells were washed twice with DMEM and incubated at 37 °C in 2 ml of 1:1 (v/v) DMEM/Waymouth's medium containing 0.25 μCi [^{3}H]thymidine/ml and various concentrations of DBHQ (*A*) or thapsigargin (*B*) in presence (*closed circles*) or absence (*open circles*) of 80 nM PDB. After 40 h, DNA synthesis was assessed by measuring the level of [^{3}H]thymidine incorporated into half of the acid-precipitable material by liquid scintillation counting. Each *point* is the mean of two determinations from one representative experiment. 10% FBS gave an incorporation of 197 × 10^3 cpm (*A*) or 189 × 10^3 cpm (*B*). Similar results were obtained in 17–22 independent experiments. *C* and *D*, effect of the length of preincubation with DBHQ and thapsigargin on the subsequent stimulation of DNA synthesis by these compounds in synergy with PDB. Confluent quiescent cultures of Swiss 3T3 cells were pretreated without (–, *open bars*) or with (*closed bars*) 7.5 μM DBHQ (*C*) or 0.5 nM thapsigargin (*D*) for 0, 2, 6, or 24 h. Then (as above), the cells were washed twice with DMEM and incubated for 40 h in DMEM/Waymouth's medium with [^{3}H]thymidine, 7.5 μM DBHQ (*C*) or 0.5 nM thapsigargin (*D*), and 20 nM (*C*) or 80 nM PDB (*D*). DNA synthesis is expressed as percent of maximum. Each *bar* is the mean of 3–8 determinations. *Error bars* are standard error of the mean (S.E.). *E* and *F*, dose-response curve for the stimulation of DNA synthesis by PDB. Confluent quiescent cultures of Swiss 3T3 cells were pretreated for 6–8 h without (*open circles*) or with (*closed circles*) 7.5 μM DBHQ (*E*) or 0.5 nM thapsigargin (*F*). Then (as above), the cells were washed twice with DMEM and incubated for 40 h in DMEM/Waymouth's medium with [^{3}H]thymidine and various concentrations of PDB in the absence (*open circles*) or presence (*closed circles*) of 7.5 μM DBHQ (*E*) or 0.5 nM thapsigargin (*F*). 10% FBS gave 164 × 10^3 cpm (*E*) and 168 × 10^3 cpm (*F*). Similar results were obtained in three independent experiments.

Fig. 1, *C* and *D*, reveal that DBHQ or thapsigargin synergized with PDB in stimulating DNA synthesis without preincubation, but the effect was enhanced after a pretreatment of 6 h. Similar enhancement of DNA synthesis was seen when the cells were preincubated with thapsigargin or DBHQ for 8 h instead of 6 h (data not shown).

To further substantiate the observed synergy between PDB and DBHQ or thapsigargin, we examined the effect of a range of concentrations of PDB in the presence of a fixed and optimum concentration of either DBHQ (7.5 μM) or thapsigargin (0.5 nM). The results show striking synergistic stimulation of DNA synthesis by PDB in the presence of either DBHQ or thapsigargin (Fig. 1, *E* and *F*, respectively). Maximum effects were produced at a concentration of 80 nM PDB. PDB alone did not induce any significant [^{3}H]thymidine incorporation in Swiss 3T3 cells in agreement with previous results (35, 39).

The synergistic stimulation of DNA synthesis by the combination of PDB with either DBHQ or thapsigargin was also assessed as a function of time (Fig. 2*A*). These compounds stimulated [^{3}H]thymidine incorporation after a lag period of 20–22 h. This is consistent with the duration of the transition from G_0/G_1 to DNA synthesis stimulated by neuropeptide growth factors (*e.g.* bombesin, vasopressin) in Swiss 3T3 cells (34, 35).

To assess whether PDB and thapsigargin or DBHQ synergistically interact in eliciting initiation of DNA synthesis rather than in changing the specific activity of the [^{3}H]thymidine precursor pool, quiescent cultures of Swiss 3T3 cells were treated with PDB and thapsigargin or DBHQ, and incorporation of [^{3}H]thymidine into DNA was quantified by autoradiography of labeled nuclei. As shown in Fig. 2*B*, thapsigargin and DBHQ caused a marked enhancement of the labeling index (from 7% in cultures treated with PDB alone to 44 and 54% in cultures treated also with 0.5 nM thapsigargin and 7.5 μM DBHQ, respectively). Thus, PDB in combination with either thapsigargin or DBHQ synergistically enhances the proportion of cells that enter into DNA synthesis. This was in very good agreement with the cumulative [^{3}H]thymidine incorporation into parallel cultures expressed as a percentage of the response induced by 10% FBS (Fig. 2*C*).

The results shown in Figs. 1 and 2 demonstrate that DBHQ and thapsigargin, at low concentrations, promote synergistic stimulation of DNA synthesis in combination with PDB.

Effect of DBHQ with Other Mitogens—Next, we examined whether DBHQ could act synergistically with signaling pathways other than those mediated by PKC. As shown in Table I, the effect of DBHQ on DNA synthesis in the presence of various growth-promoting factors was determined. The polypeptides, epidermal growth factor and insulin, bind to receptors endowed with intrinsic, ligand-dependent, tyrosine kinase activity that are not linked to phospholipase C γ and hence to PKC activation in Swiss 3T3 cells (20, 21). These polypeptides failed to act synergistically with DBHQ. Similarly, the cAMP-elevating agents forskolin and 3-isobutyl-1-methylxanthine, which are potent mitogens for Swiss 3T3 cultures in the presence of insulin (40), did not increase DNA synthesis in the presence of DBHQ. Interestingly, bradykinin, which causes a rapid but transient activation of PKC (41), also failed to act synergistically with DBHQ.

Bombesin, a potent mitogen for Swiss 3T3 cells, induces persistent stimulation of PKC even at concentrations sub-maximal for inducing DNA synthesis. As shown in Table I and Fig. 3, 2.5 nM bombesin (a concentration that was sub-maximal) acted synergistically with DBHQ in promoting DNA synthesis in Swiss 3T3 cells. This synergistic effect was also demonstrated when DNA synthesis was determined by autoradiography of [^{3}H]thymidine-labeled nuclei (Fig. 3*A*). Bombesin (2.5 nM) was almost as effective as PDB in promoting DNA synthesis in the presence of DBHQ. This synergy was not evident when saturating doses of bombesin (10 nM) were used (data not shown). These results strongly suggest that DBHQ-mediated Ca^{2+} mobilization acts synergistically with agents that induce persistent activation of PKC. Similar results were obtained when thapsigargin was added instead of DBHQ (data not shown).

The preceding results prompted us to verify that the synergistic effects between DBHQ and either PDB or bombesin on stimulation of DNA synthesis are dependent on PKC. It is known that treatment with saturating doses of PDB (800 nM) causes down-regulation of all phorbol ester-sensitive isoforms of PKC present in Swiss 3T3 cells (42), *i.e.* PKCs α, δ, and ε (43). As shown in Fig. 3*C*, down-regulation of phorbol ester-sensitive PKCs strikingly decreased the [^{3}H]thymidine incorporation in-

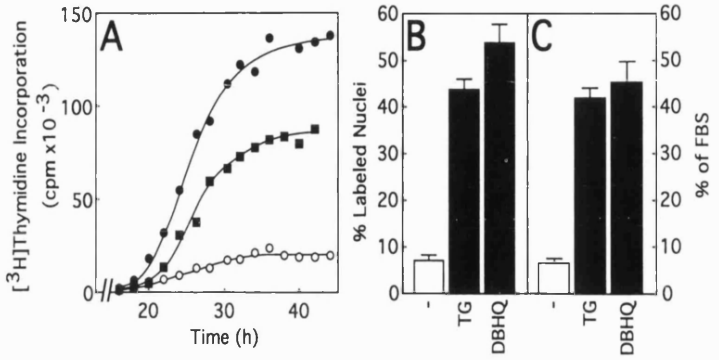

FIG. 2. *A*, time course for incorporation of [^{3}H]thymidine in the presence of thapsigargin and DBHQ. Confluent quiescent cultures of Swiss 3T3 cells were pretreated for 6–8 h without (*open circles*) or with 0.5 nM thapsigargin (*squares*) or 7.5 μM DBHQ (*closed circles*). Then, the cells were washed twice with DMEM and incubated for various times (from 16 to 44 h) in DMEM/Waymouth's medium with [^{3}H]thymidine, 80 nM PDB, and in the absence (*open circles*) or presence of 0.5 nM thapsigargin (*squares*) or 7.5 μM DBHQ (*closed circles*). A similar lag period prior to DNA synthesis was obtained in three independent experiments. *B* and *C*, effect of thapsigargin and DBHQ on DNA synthesis assessed by autoradiography (*B*) and liquid scintillation counting (*C*) of [^{3}H]thymidine incorporation. Confluent quiescent cultures of Swiss 3T3 cells were pretreated for 6–8 h without (*open bars*) or with (*closed bars*) 0.5 nM thapsigargin or 7.5 μM DBHQ. Then, as in Fig. 1, the cells were washed twice with DMEM and incubated for 40 h in DMEM/Waymouth's medium with 2.5 μCi [^{3}H]thymidine/ml (*B*) or 0.25 μCi/ml (*C*) and with 20 nM PDB either in the absence (–, *open bars*) or presence (*closed bars*) of 0.5 nM thapsigargin (*TG*) or 7.5 μM DBHQ. For autoradiography, the experiment was stopped, and cells were fixed by replacing medium with formol saline. Labeled nuclei were visualized by *in situ* autoradiography using Kodak autoradiographic stripping plate AR10. Films were then developed *in situ*. *Values* in *B* are the percent of nuclei that were labeled, counted from eight microscopic fields (total of 65–80 cells per field) per dish. 10% FBS labeled 93% of nuclei. *Values* in *C* are expressed as percentage of the values obtained with 10% FBS and are presented for comparison with the autoradiography results. For both *B* and *C*, *values* are the mean of two dishes with the *error bars* showing the range.

TABLE I

Effect of DBHQ on DNA synthesis induced by various mitogens

Confluent quiescent cultures of Swiss 3T3 cells were pretreated for 8 h without (–) or with (+) 7.5 μM DBHQ. Then, as in Fig. 1, the cells were washed twice with DMEM and incubated for 40 h in DMEM/Waymouth's medium with [^{3}H]thymidine and various mitogens (1 μg/ml insulin (INS), 5 ng/ml EGF, 50 μM forskolin (FOR), 25 μM 3-isobutyl-l-methylxanthine (IBMX), 50 nM bradykinin (BK), 2.5 nM bombesin (BOM), and 20 nM PDB) in the presence (+) or absence (–) of 7.5 μM DBHQ. Values are expressed as percent of [^{3}H]thymidine incorporation obtained with 10% FBS and are the mean, with the error shown as S.E. of 4 independent experiments performed in duplicate or 27 experiments for PDB.

Mitogen(s)	[^{3}H]Thymidine incorporation (of FBS)	
	–	+ DBHQ
	%	
–	1.3 ± 0.9	0.8 ± 0.5
INS	10.4 ± 3.3	17.3 ± 3.8
EGF	6.5 ± 1.3	8.0 ± 2.5
FOR + IBMX	4.2 ± 2.3	4.5 ± 1.0
BK	4.1 ± 1.3	8.8 ± 4.3
BOM	9.1 ± 3.5	36.7 ± 6.5
PDB	7.1 ± 0.7	43.7 ± 3.5

duced by DBHQ and PDB or DBHQ and bombesin.

To substantiate the requirement of PKC activity for synergistic stimulation of DNA synthesis by DBHQ and PDB or bombesin, we used the inhibitor GF 109203X. This staurosporine-related compound has been reported to be a selective inhibitor of PKC in Swiss 3T3 cells (44). Pretreatment of Swiss 3T3 cells with 3.5 μM GF 109203X for 1 h before addition of PDB or bombesin, conditions that have been previously shown to effectively inhibit PKC activity in intact Swiss 3T3 cells (45), also strikingly decreased the [^{3}H]thymidine incorporation induced by DBHQ and PDB or DBHQ and bombesin. The results shown in Fig. 3, *C* and *D*, demonstrate that PKC is necessary for the synergistic stimulation of DNA synthesis induced by DBHQ and either PDB or bombesin.

Thapsigargin and DBHQ Do Not Potentiate PDB-mediated Signal Transduction—Treatment with thapsigargin or DBHQ could enhance PDB-mediated signals or converge with them at a downstream locus further into G_1. To distinguish between these possibilities, we determined whether prior exposure of Swiss 3T3 cells to 0.5 nM thapsigargin or 7.5 μM DBHQ for 6 h had any effect on PDB-mediated events. Fig. 4 (*upper panel*) shows that preincubation with thapsigargin or DBHQ did not affect the ability of PDB to stimulate the phosphorylation of 80K/MARCKS, a prominent and specific substrate of PKC in intact cells (20). Similarly, treatment with thapsigargin or DBHQ did not affect the activation of MAP kinase by exposure to PDB, as judged by a mobility shift assay (Fig. 4, *middle panel*).

Recently, it has been shown that PDB stimulates the tyrosine phosphorylation of multiple substrates including bands of M_r 110,000–130,000 and M_r 70,000–80,000 (45). The novel cytosolic tyrosine kinase p125^{FAK} has been identified as a prominent tyrosine-phosphorylated protein of the M_r 110,000–130,000 band, whereas paxillin corresponds to the M_r 70,000–80,000 heterogenous band (36, 45). As shown in Fig. 4 (*lower panel*), pretreatment with thapsigargin or DBHQ did not change the basal level of tyrosine phosphorylation or affect the PDB-induced increase in tyrosine phosphorylation of the M_r 110,000–130,000 or M_r 70,000–80,000 bands. In accordance with all of these results, treatment of Swiss 3T3 cells with either DBHQ or thapsigargin did not alter the extent to which [^{3}H]PDB bound to intact cells (data not shown). These results indicate that thapsigargin and DBHQ do not directly potentiate PKC-mediated signals.

Effect of Thapsigargin and DBHQ on Intracellular Calcium Stores—Thapsigargin inhibits the activity of the ER Ca^{2+}ATPase, leading to a depletion of the intracellular Ca^{2+} stores (26, 27, 29–31). However, most previous studies on the effects of thapsigargin on cell proliferation and Ca^{2+} sequestering in the ER have used high concentrations of this compound (0.1–10 μM). Here, we found synergistic stimulation of DNA synthesis at sub-nanomolar concentrations. Consequently, it was important to examine the effects of low concentrations of thapsigargin and DBHQ on intracellular Ca^{2+}. Specifically, if treatment with mitogenic concentrations of thapsigargin or DBHQ inhibits the accumulation of Ca^{2+} into the Ins(1,4,5)P_3-sensitive pool, it should prevent the increase in $[Ca^{2+}]_i$ induced by a subsequent addition of Ca^{2+}-mobilizing agents.

Fig. 5*A* shows that treatment with 0.5 nM thapsigargin or 7.5 μM DBHQ markedly reduced the ability of 2.5 nM bombesin to induce a rapid and transient increase in the $[Ca^{2+}]_i$ from Swiss

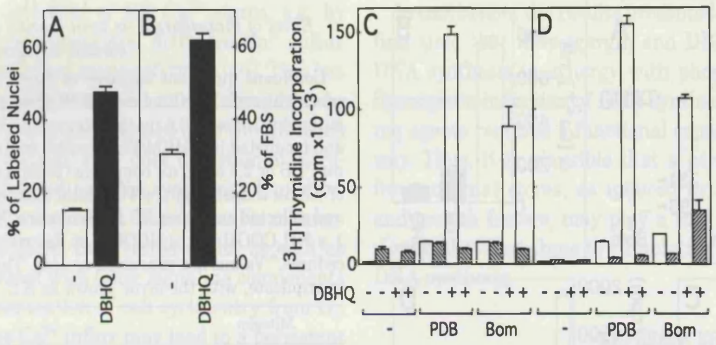

FIG. 3. *A* and *B*, effect of DBHQ on DNA synthesis induced by bombesin assessed both by autoradiography (*A*) and liquid scintillation counting (*B*) of [^{3}H]thymidine incorporation as comparison. Confluent quiescent cultures of Swiss 3T3 cells were pretreated for 6–8 h without (*open bars*) or with 7.5 μM DBHQ (*closed bars*). Cells were then washed twice with DMEM and incubated for 40 h in DMEM/Waymouth's medium with 2.5 μCi [^{3}H]thymidine/ml (*A*) or 0.25 μCi/ml (*B*) and with 2.5 nM bombesin in the absence (*open bars*) or presence of 7.5 μM DBHQ (*closed bars*). *Values* in *A* are the percent of nuclei that were labeled, counted from eight microscopic fields (total of 65–80 cells per field) per dish. 10% FBS labeled 93% of nuclei. *Values* in *B* are expressed as mean percentage of the values obtained with 10% FBS with error as S.E. Effect of down-regulating (*C*) or inhibiting (*D*) PKC on [^{3}H]thymidine incorporation induced by DBHQ and either PDB or bombesin. *C*, confluent quiescent cultures of Swiss 3T3 cells were treated in the absence (*open bars*) or presence (*striped bars*) of 800 nM PDB for 48 h to down-regulate PKC. Then, the cells were pretreated without (–) or with 7.5 μM DBHQ (+). After 6 h, the cells were washed twice with DMEM and incubated in DMEM/Waymouth's medium with [^{3}H]thymidine in the absence (–) or presence of 80 nM PDB or 1 nM bombesin (*Bom*) and without (–) or with (+) 7.5 μM DBHQ. *D*, confluent quiescent cultures of Swiss 3T3 cells were pretreated without (–) or with (+) 7.5 μM DBHQ. After 6 h, the cells were washed twice with DMEM, incubated in DMEM/Waymouth's medium with [^{3}H]thymidine, and DBHQ was replaced. Then, cells were treated without (*open bars*) or with 3.5 μM GF 109203X (*striped bars*) for 1 h before the addition of nothing (–), 80 nM PDB, or 1 nM bombesin.

3T3 cells. Preincubation with 0.5 nM thapsigargin or 7.5 μM DBHQ for 6–8 h inhibited bombesin-induced increase in $[Ca^{2+}]_i$ by 79 ± 2% (n = 7) and 90 ± 1% (n = 9), respectively (Fig. 5*B*).

Next, we examined the reduction in bombesin-induced Ca^{2+} mobilization by thapsigargin or DBHQ as a function of length of pretreatment time. Fig. 5*C* demonstrates that maximum inhibition of the increase in $[Ca^{2+}]_i$ was reached within 1 h of exposure to thapsigargin or DBHQ. We verified that treatment with these agents did not prevent the production of inositol phosphates in response to bombesin (Table II).

To further demonstrate that the decrease in bombesin-induced Ca^{2+} mobilization was due to depletion of Ca^{2+} stores, cells were loaded with $^{45}Ca^{2+}$, and the total Ca^{2+} remaining in the cells was determined after various treatments. This method measures Ca^{2+} in intracellular stores, which is accumulated to millimolar concentrations rather than cytoplasmic Ca^{2+}, which is maintained at nanomolar concentrations. The results in Fig. 5*D* demonstrate that 8 h of pretreatment by either 0.5 nM thapsigargin or 7.5 μM DBHQ, conditions that are optimum for DNA synthesis, reduced total cellular $^{45}Ca^{2+}$ content to a level comparable with that reached after 10 min of exposure to 10 nM bombesin, a maximum concentration. When thapsigargin- or DBHQ-treated cells were further challenged with 10 nM bombesin for 10 min, a small amount of additional $^{45}Ca^{2+}$ was released, which is in agreement with the measurements of $[Ca^{2+}]_i$ shown in Fig. 5*B*. Complete depletion of the Ins(1,4,5)P_3-responsive pool was obtained by exposure of cells to 30 nM thapsigargin for 30 min since subsequent exposure to bombesin caused no further reduction in cellular $^{45}Ca^{2+}$. For comparison, an 8-h treatment with 10 nM bombesin induced a depletion in $^{45}Ca^{2+}$ content similar to that achieved by an 8-h pretreatment with 0.5 nM thapsigargin. The results in Fig. 5 demonstrate that treatment of Swiss 3T3 cells with thapsigargin or DBHQ, at concentrations that induce synergistic stimulation of DNA synthesis with PDB, causes a partial and persistent depletion in Ca^{2+} from the Ins(1,4,5)P_3-responsive Ca^{2+} pool.

Effect of Extracellular Calcium on DNA Synthesis—The Ca^{2+} response to agents that mobilize Ca^{2+} through formation of Ins(1,4,5)P_3 is typically biphasic. The first event is a large transient increase in $[Ca^{2+}]_i$ originating from intracellular stores, and the second is a sustained influx of Ca^{2+} through the plasma membrane, which causes a prolonged elevation of cytoplasmic Ca^{2+} (46). Because this sustained Ca^{2+} influx through the

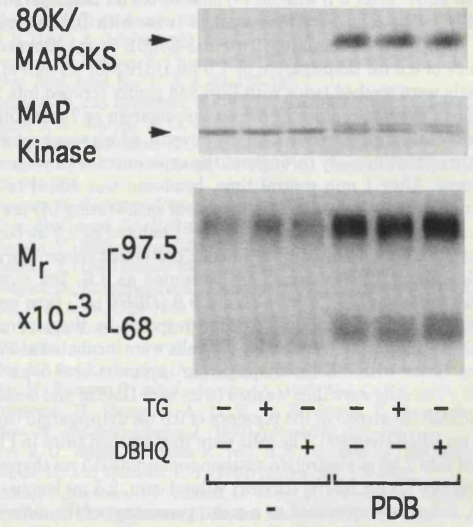

FIG. 4. **Effect of thapsigargin and DBHQ on PDB-mediated signal transduction.** *Upper panel*, 80K/MARCKS phosphorylation. Confluent quiescent cultures of Swiss 3T3 cells were loaded with $^{32}P_i$ overnight and then pretreated for 6 h without or with 0.5 nM thapsigargin or 7.5 μM DBHQ as indicated. Cells were incubated 10 min at 37 °C with or without 80 nM PDB as indicated. Cells were lysed and immunoprecipitated with 80K/MARCKS antiserum and resolved by SDS-PAGE. Bands were visualized by autoradiography. *Middle panel*, MAP kinase activation. Confluent quiescent cultures of Swiss 3T3 cells were pretreated for 6 h with or without 0.5 nM thapsigargin or 7.5 μM DBHQ as indicated. Cells were then washed twice with DMEM and incubated in DMEM for 10 min at 37 °C without (–) or with 0.5 nM thapsigargin (*TG*) or 7.5 μM DBHQ in the presence or absence of 80 nM PDB as indicated for 5, 20, or 60 min. Cells were lysed with 2 × sample buffer, and proteins were separated by SDS-PAGE. Proteins were transferred to Immobilon membranes and Western blotted with MAP kinase antiserum. Bands were visualized by autoradiography. Results shown are after 20 min of stimulation. Similar results were obtained when PDB was added for 5 or 60 min instead of 20 min (results not shown). *Lower panel*, tyrosine phosphorylation of proteins M_r 110,000–130,000 and M_r 70,000–80,000. Confluent quiescent cultures of Swiss 3T3 cells were pretreated for 6 h with or without 0.5 nM thapsigargin or 7.5 μM DBHQ as indicated. Then, cells were washed twice with DMEM and incubated in DMEM for 10 min at 37 °C with or without (–) 0.5 nM thapsigargin or 7.5 μM DBHQ and in the presence or absence of 80 nM PDB as indicated. Cells were lysed and immunoprecipitated with agarose-linked anti-Tyr(P) mAb and resolved by SDS-PAGE. Phosphotyrosyl proteins were transferred to Immobilon membranes and Western blotted with 4G10 and Py20 anti-Tyr(P) mAbs. Bands were visualized by autoradiography.

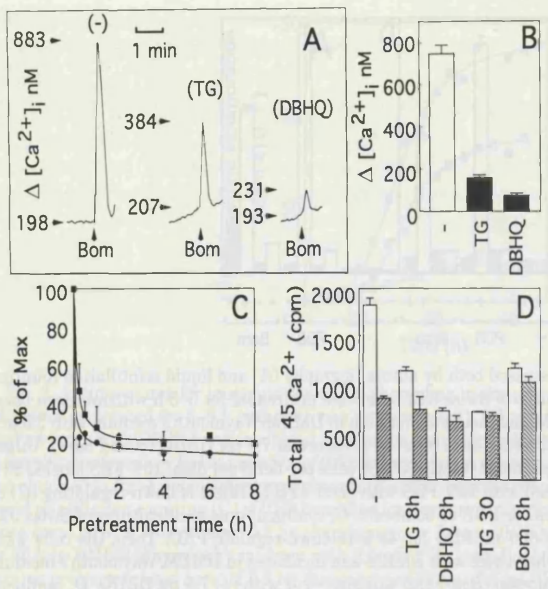

FIG. 5. **Effect of Thapsigargin and DBHQ on hormonal responsive calcium pool in Swiss 3T3 cells.** *A* and *B*, reduction of bombesin (*Bom*)-induced Ca^{2+} mobilization by thapsigargin (*TG*) and DBHQ. Confluent, quiescent Swiss 3T3 cells in 100-mm Nunc dishes were incubated at 37 °C for 8 h without (–) or with 0.5 nM thapsigargin or 7.5 μM DBHQ. The cells were then washed twice with DMEM and incubated with DMEM containing 1 μM fura-2/AME in the absence (–) or presence of 0.5 nM thapsigargin or 7.5 μM DBHQ for 10 min at 37 °C. The cells were washed twice with PBS and gently scraped into 2 ml of electrolyte solution containing 0.5 nM thapsigargin or 7.5 μM DBHQ as necessary and transferred to a quartz cuvette, which was kept at 37 °C and stirred continuously throughout the experiment. Fluorescence was monitored. After 1 min control time, bombesin was added to 2.5 nM (shown by *arrow*). *Values* on the *left* side of each tracing (*A*) are $[Ca^{2+}]_i$ in nM. *B* shows the mean increase in $[Ca^{2+}]_i$ induced by 2.5 nM bombesin in control cells (–, *open bars*) or in cells pretreated (*closed bars*) with thapsigargin or DBHQ. *Errors* are presented as S.E. The number of samples are $n = 17$ (–), $n = 7$ (*TG*), $n = 9$ (*DBHQ*). *C*, time course of depletion of bombesin-induced calcium response by thapsigargin and DBHQ. Confluent quiescent Swiss 3T3 cells were incubated at 37 °C for various times with 0.5 nM thapsigargin (*squares*) or 7.5 μM DBHQ (*circles*). The cells were then washed twice with DMEM and loaded with fura-2/AME (as above) in the presence of 0.5 nM thapsigargin (*squares*) or 7.5 μM DBHQ (*circles*). The cells were then washed twice in PBS and scraped into 2 ml of electrolyte solution containing 0.5 nM thapsigargin (*squares*) or 7.5 μM DBHQ (*circles*). After 1 min, 2.5 nM bombesin was added. *Values* are expressed as a mean percentage of the difference in $[Ca^{2+}]_i$ induced by 2.5 nM bombesin in untreated cells, with errors as S.E. from 2–3 experiments. *D*, effect of thapsigargin and DBHQ on total intracellular calcium measured by $^{45}Ca^{2+}$. Confluent quiescent Swiss 3T3 cells loaded with $^{45}Ca^{2+}$ were then pretreated for 8 h without (–) or with 0.5 nM thapsigargin (*TG 8h*), 7.5 μM DBHQ (*DBHQ 8h*), 10 nM bombesin (*Bom 8h*), or for 30 min with 30 nM thapsigargin (*TG 30*). Cells were then washed twice with DMEM, the same factors added back as for the pretreatment, and then incubated for 10 min at 37 °C in the presence (*striped bars*) or absence (*open bars*) of 10 nM bombesin. In the case of bombesin pretreatment, due to down-regulation of the bombesin response, further stimulation was by 10 min of 20 nM vasopressin (*reverse striped bar*). Residual $^{45}Ca^{2+}$ in the cells was measured as described under "Experimental Procedures." *Values* are mean of three determinations with errors shown as S.E.

plasma membrane is dependent on extracellular Ca^{2+}, we examined the effect of extracellular Ca^{2+} concentration on DNA synthesis induced by a variety of growth-promoting factors. The results in Fig. 6*A* show that DBHQ and PDB-induced [^{3}H]thymidine incorporation is strikingly sensitive to extracellular Ca^{2+}. The concentration of Ca^{2+} in the medium ($[Ca^{2+}]_{out}$) that is required to achieve 50% of the maximum response (ED_{50}) induced by PDB and DBHQ was 410 μM. A similar result was obtained for thapsigargin (data not shown.) In sharp contrast, the ED_{50} for PDB and insulin, a combination that induces DNA synthesis but does not stimulate Ca^{2+} mobilization, was

TABLE II

Effect of thapsigargin on total inositol phosphates generated by various mitogens

Confluent quiescent cultures of Swiss 3T3 cells were equilibrated overnight with 15 μCi/ml *myo*-[2-^{3}H]inositol and then pretreated for 8 h without (–) or with 0.5 nM thapsigargin (+ TG). Cells were washed twice and incubated in DMEM/Waymouth's for 10 min at 37 °C with either 20 nM PDB or 2.5 or 10 nM bombesin (BOM) in the absence (–) or presence of 0.5 nM thapsigargin (+ TG). Total inositol phosphates were extracted and collected on Dowex AG 1-X8 columns, $HCOO^-$ form, and eluted with 1 M NH_4COOH, 0.1 M HCOOH as described under "Experimental Procedures." Values are expressed as cpm. The experiment was performed in triplicate, with the error shown as S.E.

Mitogen	–	+ TG
	cpm	*cpm*
–	209 ± 21	263 ± 26
20 nM PDB	194 ± 6	199 ± 10
2.5 nM BOM	482 ± 21	560 ± 15
10 nM BOM	822 ± 21	800 ± 48

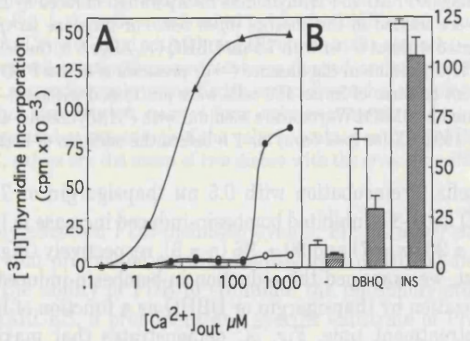

FIG. 6. *A*, effect of extracellular calcium on [^{3}H]thymidine incorporation. Confluent quiescent cultures of Swiss 3T3 cells were pretreated for 6–8 h with 7.5 μM DBHQ (*closed circles*). Cells were then washed twice with DMEM and incubated in DMEM/Waymouth's medium containing [^{3}H]thymidine with various amounts of Ca^{2+} buffered by 2 mM EGTA in the presence of 80 nM PDB (*open circles*), 80 nM PDB, and 7.5 μM DBHQ (*closed circles*), or 80 nM PDB and 1 μg/ml insulin (*closed triangles*). Similar results were obtained in four independent experiments. *B*, effect of econozole on [^{3}H]thymidine incorporation induced by different mitogens. Confluent quiescent cultures of Swiss 3T3 cells were pretreated for 6 h with or without 7.5 μM DBHQ. Cells were then washed twice with DMEM and incubated in DMEM/Waymouth's medium containing [^{3}H]thymidine. Cultures were stimulated with either 80 nM PDB (–), 80 nM PDB, and 7.5 μM DBHQ or with 80 nM PDB and 1 μg/ml insulin (*INS*) in the absence (*open bars*) or presence (*striped bars*) of 1 μM econozole.

6 μM. To substantiate the involvement of Ca^{2+} influx in the synergistic stimulation of DNA synthesis, we used the inhibitor econozole. This compound inhibits Ca^{2+} influx in several cell types (47–50) through a mechanism independent of cytochrome P-450 inhibition (49). The results in Fig. 6*B* show that DNA synthesis stimulated by PDB and DBHQ was reduced by 55% in the presence of 1 μM econozole (a concentration that has been shown to inhibit capacitative Ca^{2+} influx (47)). In contrast, DNA synthesis induced by PDB and insulin was only reduced by 13%. The results in Fig. 6 demonstrate that the synergistic stimulation of DNA synthesis by DBHQ with PDB requires capacitative Ca^{2+} influx.

DISCUSSION

The findings presented here demonstrate that thapsigargin and DBHQ synergistically stimulate DNA synthesis with PDB or bombesin in cultures of Swiss 3T3 cells. At the low concentrations that induce DNA synthesis, thapsigargin and DBHQ cause a slow but persistent depletion of the intracellular Ca^{2+} stores. To our knowledge, this is the first time that either thapsigargin or DBHQ has been shown to stimulate the reinitiation of DNA synthesis in any target cell.

It is recognized that depletion of ER Ca^{2+} stores, *e.g.* by thapsigargin, DBHQ, or neuropeptides, activates Ca^{2+} influx from the extracellular medium of many cell types (46). This has been termed capacitative Ca^{2+} entry (51) and is thought to be activated by a diffusable messenger released from the ER when the Ca^{2+} is depleted (48, 52, 53). How does depletion of intracellular Ca^{2+} stores act in signaling DNA synthesis? Two alternative mechanisms could be envisaged. The diffusable messenger released from the depleted Ca^{2+} stores could, as well as activating Ca^{2+} entry, interact with other signaling components to promote synergistic stimulation of cell cycle entry from G_0. Alternatively, capacitative Ca^{2+} influx may lead to a persistent increase in $[Ca^{2+}]_i$. We attempted to distinguish between these possibilities. If the stimulation of DNA synthesis elicited by thapsigargin and DBHQ depends on Ca^{2+} influx, we reasoned that DNA synthesis should be critically dependent on $[Ca^{2+}]_{out}$. Indeed, this was the case for the mitogenic combination of DBHQ and PDB. The ED_{50} of $[Ca^{2+}]_{out}$ required to support DNA synthesis stimulated by DBHQ and PDB was 100-fold higher than that required for DNA synthesis induced by insulin and PDB. The high $[Ca^{2+}]_{out}$ in DBHQ-treated cells is consistent with the apparent K_m (in the millimolar range) of the channel that mediates capacitative Ca^{2+} influx (54). In support of this, econozole, which inhibits Ca^{2+} influx (47–50), inhibited DNA synthesis induced by PDB and DBHQ to a much greater extent than DNA synthesis induced by PDB and insulin. Taking these data together, we conclude that the induction of DNA synthesis by DBHQ and thapsigargin in the presence of PDB is dependent on the activity of the capacitative Ca^{2+} influx pathway.

In theory, treatment with thapsigargin or DBHQ leading to persistent depletion of Ca^{2+} stores could enhance the ability of PDB to induce cellular responses through PKC. Several lines of evidence indicate that this is not the case. Specifically, treatment with either thapsigargin or DBHQ did not enhance the ability of PDB to induce a variety of responses such as direct PKC-mediated phosphorylation of 80K/MARCKS, stimulation of the kinase cascade leading to MAP kinase activation and tyrosine phosphorylation of multiple bands previously identified as $p125^{FAK}$ and paxillin (36, 45). We conclude that Ca^{2+} and PKC signals act in parallel and converge downstream to induce synergistic stimulation of DNA synthesis.

Our results contrast with previous studies demonstrating that thapsigargin and DBHQ profoundly inhibit cell proliferation or induce cytotoxic effects (32, 33). Although different cell types were used, an obvious difference between the present and previous studies is the concentration of thapsigargin employed. In fact, we can readily obtain inhibition of DNA synthesis and cytotoxic effects in Swiss 3T3 cells at higher concentrations of thapsigargin and DBHQ. The existence of stimulatory and inhibitory effects could explain the bell-shaped dose response of these agents on DNA synthesis found in this study. The inhibitory effects are likely to be caused by extensive depletion of Ca^{2+} from the ER, thereby interfering with its function. The high Ca^{2+} content in the lumen of the ER is thought to play a critical role in the post-translational processing and trafficking of newly synthesized secretory, lysosomal, and membrane proteins (55–57). Furthermore, Ca^{2+} sequestered in the ER appears to be required for maintaining a normal rate of protein synthesis (58, 59). The disruption of these important processes by extensive depletion of ER Ca^{2+} could explain the inhibition of cell proliferation induced by thapsigargin and DBHQ at higher concentrations than those used in the present study. We hypothesize that thapsigargin and DBHQ, at optimum mitogenic concentrations, reduce the Ca^{2+} content from internal stores in Swiss 3T3 cells to a critical level that leads to entry into DNA synthesis but does not impair essential ER functions.

In conclusion, the results presented here demonstrate for the first time that thapsigargin and DBHQ cause stimulation of DNA synthesis in synergy with phorbol ester and bombesin. Synergistic induction of DNA synthesis by these Ca^{2+}-mobilizing agents requires a functional capacitative Ca^{2+} influx pathway. Thus, it is plausible that a persistent depletion of Ca^{2+} from internal stores, as induced by mitogenic neuropeptides and growth factors, may play a role as one of the synergistic signals that contribute to stimulating the transition from G_0 to DNA synthesis.

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The bombesin/GRP receptor transfected into Rat-1 fibroblasts couples to phospholipase C activation, tyrosine phosphorylation of p125^{FAK} and paxillin and cell proliferation

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Bombesin elicits multiple signalling pathways in various cell types. It is not clear, however, whether these responses are mediated by a single receptor subtype or by different subtypes that couple preferentially to specific pathways. To resolve this we transfected the mouse bombesin/GRP receptor into Rat-1 fibroblasts and investigated the pathways activated by bombesin. Expression of the transfected receptors was verified by binding of [^{125}I]GRP and two clones were selected, BOR5 and BOR15. Bombesin stimulation of BOR5 and BOR15 cells caused intracellular Ca^{2+} mobilisation and increased the phosphorylation of 80K/MARCKS, a prominent protein kinase C substrate. The transfected receptor conferred a proliferative response to bombesin demonstrated by incorporation of [^{3}H]thymidine after 18 h and an increase in total cell numbers after 1–2 days. In BOR5 and BOR15 cells, bombesin rapidly stimulated the tyrosine phosphorylation of multiple proteins M_r 110 000–130 000 and 70 000–80 000 including p125^{FAK} and paxillin, at low concentrations (half maximum 0.3 nM). The specific bombesin/GRP receptor antagonist, D-F$_5$-Phe^6, D-Ala^{11}-Bombesin(6–13)OMe, inhibited all the above responses. These results show that phospholipase C activation, cell growth and tyrosine phosphorylation emanate from a single class of bombesin receptor.

Keywords: neuropeptides; growth factors; Ca^{2+} mobilisation; protein kinase C

Introduction

Neuropeptides stimulate DNA synthesis and cell proliferation in cultured cells and are implicated as cellular growth factors in a variety of fundamental biological processes including development, tissue regeneration, inflammation and tumourigenesis (Rozengurt, 1986; Zachary *et al.*, 1987; Rozengurt, 1995a). In particular, the peptides of the bombesin family including Gastrin Releasing Peptide (GRP) are potent mitogens for Swiss 3T3 cells (Rozengurt and Sinnett-Smith, 1983; Zachary and Rozengurt, 1985) and various tumour cell types including small cell lung cancer (Cuttitta *et al.*, 1985; Carney *et al.*, 1987; Sethi *et al.*, 1991). Bombesin binds to specific high affinity receptors on the cell surface (Zachary and Rozengurt, 1985, 1987a; Battey *et al.*, 1991) and elicits a variety of early biochemical responses including rapid phospholipase C (PLC) mediated hydrolysis of polyphosphoinositides leading to mobilisation of intracellular calcium and activation of protein kinase C (PKC) (Zachary *et al.*, 1986; Nanberg and Rozengurt, 1988; Rozengurt, 1992).

Tyrosine phosphorylation has been implicated in the intracellular signalling of bombesin and other neuropeptides (Rozengurt, 1995b). Bombesin stimulates tyrosine phosphorylation of multiple proteins in Swiss 3T3 cells, including broad bands migrating with the apparent molecular masses of 110 000–130 000 and 70 000–80 000 (Zachary *et al.*, 1991a,b). More recently the focal adhesion associated proteins p125 focal adhesion kinase (p125^{FAK}) and paxillin have been identified as prominent tyrosine phosphorylated proteins in Swiss 3T3 cells after stimulation by bombesin (Zachary *et al.*, 1992, 1993; Sinnett-Smith *et al.*, 1993). p125^{FAK} is a novel cytosolic tyrosine kinase that lacks Src Homology 2 (SH2) and SH3 domains (Hanks *et al.*, 1992; Schaller *et al.*, 1992) but associates with signalling proteins including pp60^{src} (Cobb *et al.*, 1994) and paxillin (Hildebrand *et al.*, 1995). Recent molecular cloning of paxillin revealed a multidomain protein that may function as an adaptor capable of associating with p125^{FAK}, Crk, Src and vinculin (Turner and Miller, 1994; Salgia *et al.*, 1995). Bombesin induces tyrosine phosphorylation of p125^{FAK} and paxillin via a PKC and Ca^{2+}-independent pathway that requires the integrity of the actin cytoskeleton (Sinnett-Smith *et al.*, 1993; Zachary *et al.*, 1993) and functional p21^{rho} (Rankin *et al.*, 1994; Seckl *et al.*, 1995). Tyrphostin, an inhibitor of tyrosine kinases, markedly reduces the level of p125^{FAK} tyrosine phosphorylation and DNA synthesis induced by bombesin in Swiss 3T3 cells suggesting that the tyrosine kinase pathway plays a role in signalling the mitogenic response elicited by bombesin in these cells (Seckl and Rozengurt, 1993).

At present three mammalian members of the bombesin-like receptor family have been cloned and shown to belong to the G protein-linked receptor superfamily. They are the bombesin/GRP-preferring (Spindel *et al.*, 1990; Battey *et al.*, 1991), the neuromedin B (NmB)-preferring (Corjay *et al.*, 1991; Wada *et al.*, 1991) and BRS-3 (Gorbulev *et al.*, 1992; Fathi *et al.*, 1993), a bombesin-like receptor whose physiological ligand is not yet known. It has been generally assumed that all the early signals and the subsequent mitogenic response elicited by bombesin are mediated by occupancy of the bombesin/GRP receptor. This assumption has recently been questioned. Bold *et al.* (1994) reported that antagonists that inhibit bombesin-induced Ca^{2+} mobilisation do not prevent

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bombesin-induced cell growth in human gastric adenocarcinoma cells. However tyrphostin blocked bombesin-induced proliferation of cells. Bold *et al.* (1994). suggested that a bombesin receptor subtype couples to Ca^{2+} mobilisation and a different bombesin receptor subtype, not yet identified, couples to the protein tyrosine phosphorylation pathway and cell growth. However, no direct evidence supporting this hypothesis has been provided.

The experiments presented here were designed to elucidate whether the bombesin/GRP-preferring receptor could couple to both PLC activation as well as to $p125^{FAK}$ and paxillin tyrosine phosphorylation. Initially, we show that expression of the bombesin/GRP preferring receptor in Rat-1 cells, a cell line that can be reversibly arrested in the G_0/G_1 phase of the cell cycle, confers a proliferative response to bombesin in the receptor transfected cells. Using this model system, we found that agonist activation of the bombesin/GRP receptor elicits both PLC activation as well as tyrosine phosphorylation of multiple proteins including $p125^{FAK}$ and paxillin. Our results demonstrate, for the first time, that the bombesin/GRP receptor couples to both signalling pathways and to cell proliferation.

Results

Expression of the bombesin/GRP receptor in Rat-1 cells

The mouse bombesin/GRP-preferring receptor cDNA cloned into the mammalian expression vector pCD2, and under the transcriptional control of the SV40 early region promoter, was used for transfection experiments. Rat-1 fibroblasts were chosen as the recipient cell line because these cells can be reversibly arrested in the G_0/G_1 phase of the cell cycle (Higgins *et al.*, 1992) and do not express endogenous functional bombesin/GRP receptors (see below). After transfection, 21 G418 resistant colonies were isolated and propagated; six of these cell lines expressed the bombesin/GRP-preferring receptor compared with the control, untransfected Rat-1 cells.

The functional expression of bombesin/GRP receptors in the transfected Rat-1 cells was verified using ^{125}I-labelled GRP. Figure 1A shows that the transfectants have specific binding sites for [^{125}I]GRP. Parental Rat-1 cells did not specifically bind [^{125}I]GRP. The apparent affinity of [^{125}I]GRP for the transfected receptor in BOR 5 and BOR 15 cells (K_d = 1–2 nM) was similar to the endogenous receptor expressed in Swiss 3T3 cells (K_d = 0.8 nM). The number of receptors on the transfected cells was 30–60% lower than that of Swiss 3T3 cells, about 10^5 per cell (Zachary and Rozengurt, 1985), under identical experimental conditions (data not shown). When BOR 5 cells were incubated with [^{125}I]GRP, cell associated radioactivity increased rapidly reaching a maximum value after 30 min and decreasing afterwards (Figure 1B). This is consistent with internalisation and degradation of the peptide as reported previously in Swiss 3T3 cells (Zachary and Rozengurt, 1987b) and in transformed rat kidney cells (Grady *et al.*, 1995).

Addition of unlabelled GRP, bombesin, litorin, the structurally related neuromedin B (NmB), or the bombesin C-terminal fragment (amino acids 8–14) inhibited specific [^{125}I]GRP binding to BOR 5 cells in a concentration dependent manner (Figure 1C). The IC_{50} values for bombesin, GRP and litorin were approximately 1–3 nM. In contrast NmB inhibited binding with much lower affinity (IC_{50} = 500 nM). Figure 1 shows that BOR 5 has specific binding sites with high affinity for bombesin/GRP and low affinity for NmB demonstrating that the transfected receptor in Rat-1 cells showed the characteristic ligand recognition of the bombesin/GRP-preferring receptor.

The transfected bombesin/GRP receptor functionally couples to PLC-activated signalling pathways

In order to verify that the transfected bombesin/GRP receptor expressed in Rat-1 cells could couple to PLC, we measured the effect of bombesin on the intracellular calcium concentration ($[Ca^{2+}]_i$) and on the phosphor-

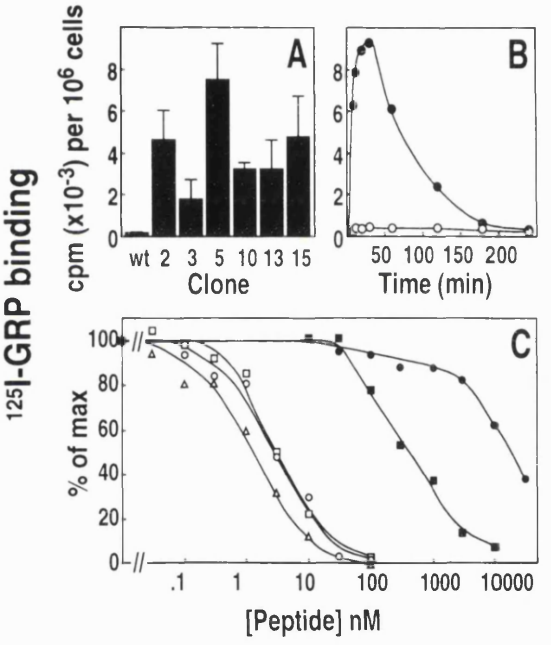

Figure 1 (**A**) Specific [^{125}I]GRP binding to different clones. Confluent, quiescent cultures of transfected and parental (WT) Rat-1 cells were washed twice with DMEM and incubated for 30 min at 37°C in DMEM containing [^{125}I]GRP (0.05 nM, 0.01 μCi and 0.95 nM cold GRP) and 1 mg/ml BSA. Cultures were then placed on ice and rapidly washed 4 × with PBS containing 1 mg/ml BSA. Cultures were dissolved in 0.1 N NaOH/2% $NaCO_3$/1% SDS and counted in a gamma counter. Results are expressed as specific binding per 10^6 cells. Non-specific binding was determined in the presence of 1 μM bombesin. Specific binding was 60–80% of the total binding. (**B**) Time-course of [^{125}I]GRP binding to BOR 5 cells. Confluent, quiescent cultures of BOR 5 cells were washed twice with DMEM and incubated at 37°C in DMEM with [^{125}I]GRP (as above) in the presence of 1 mg/ml BSA (closed circles). Non-specific binding was determined by adding 1 μM bombesin (open circles). The binding of [^{125}I]GRP to BOR 5 cells was terminated after various times as indicated. (**C**) Competition binding of [^{125}I]GRP to BOR 5 cells. Confluent, quiescent cultures of BOR 5 cells were washed twice with DMEM and incubated for 30 min at 37°C in DMEM with [^{125}I]GRP (as above) and various concentrations of competing peptides in the presence of 1 mg/ml BSA. Peptides were bombesin (open squares), GRP (open circles), litorin (open triangles), NmB (closed squares) and bombesin fragment 8–14 (closed circles). Cultures were then placed on ice and washed and counted as above. Results are expressed as a percentage of the total specific binding of [^{125}I]GRP

ylation of the prominent PKC substrate 80K/MARCKS (Myristylated Alanine Rich C Kinase Substrate) (Rodriguez-Pena and Rozengurt, 1986; Brooks *et al.*, 1991).

Bombesin increased $[Ca^{2+}]_i$ in a concentration dependent manner in BOR 5 cells (Figure 2A). The maximum increase, elicited by 100 nM bombesin, was 4.0-fold over the basal level (186 nM ± 5.5, $n = 62$). In contrast bombesin caused no increase in $[Ca^{2+}]_i$ in parental Rat-1 cells even up to 1 μM (data not shown). Addition of the specific bombesin/GRP receptor antagonist, D-F_5-Phe^6, D-Ala^{11}-Bombesin(6-13)OMe (Coy *et al.*, 1992), inhibited bombesin-induced increases in $[Ca^{2+}]_i$ (Figure 2B) but had no effect on the increase in $[Ca^{2+}]_i$ induced by endothelin which binds to receptors endogenously expressed in Rat-1 cells. Studies of Ca^{2+} mobilisation with cultures of BOR 15 cells produced exactly the same results (data not shown).

Addition of bombesin to cultures of BOR 5 cells prelabelled with $^{32}P_i$ markedly increased the phosphorylation of the prominent PKC substrate, 80K/MARCKS, in a concentration dependent manner (Figure 2C). The level of phosphorylation was comparable to that induced by 200 nM PDB, a direct activator of PKC. In contrast 80K/MARCKS was not phosphorylated in response to bombesin (up to 100 nM) in parental Rat-1 cells. The specific PKC inhibitor GF 109203X (Toullec *et al.*, 1991) prevented bombesin stimulation of 80K/MARCKS phosphorylation substantiating the role of PKC in the action of bombesin, (data not shown). The results in Figure 2 therefore show that the transfected bombesin/GRP receptor is functionally coupled to intracellular signalling pathways initiated by PLC activation.

Expression of the bombesin/GRP receptor confers a growth response to bombesin in Rat-1 cells

Next, we examined whether agonist activation of the transfected bombesin/GRP receptor leads to reinitiation of DNA synthesis in Rat-1 cells. Quiescent cultures of BOR 5 cells were incubated with bombesin and at various times after stimulation the cells were labelled with [^{3}H]thymidine for 3 h. The rate of [^{3}H]thymidine incorporation reached a maximum between 18–24 h after the addition of bombesin (Figure 3A). This is consistent with previous results

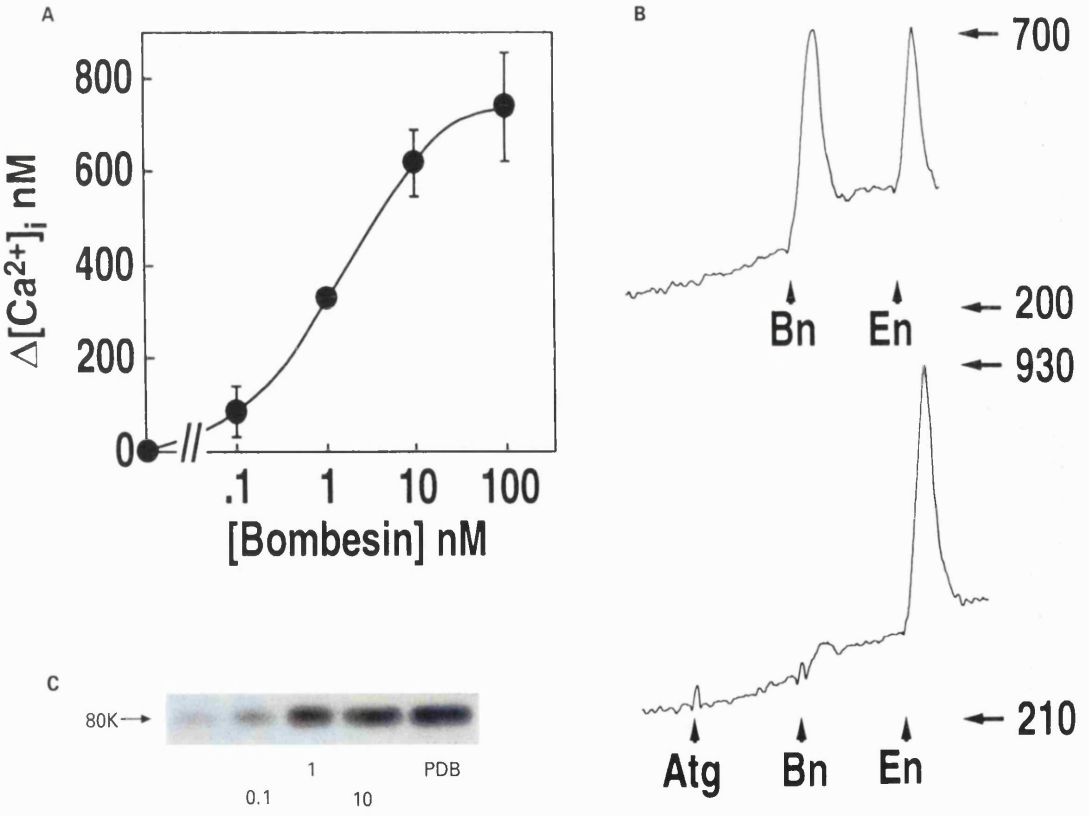

Figure 2 (**A** and **B**) Bombesin-induced increase in $[Ca^{2+}]_i$ in BOR 5 cells. Confluent, quiescent cultures of BOR 5 cells were washed twice in DMEM and detached from the dishes by a 10 min incubation in DMEM with 2.5 μg/ml dispase. Cells were loaded with 1 μM fura-2/AME while in suspension. Loaded cells were pelleted and resuspended in 2 ml electrolyte solution and transferred to a quartz cuvette. Cells were kept at 37°C and stirred continuously while fluorescence was monitored. After a 1 min control period different concentrations of bombesin were added. (**A**). Results are expressed as the difference in $[Ca^{2+}]_i$ between unstimulated and stimulated levels. (**B**). After 3 min of monitoring fluorescence bombesin was added to 1 nM (Bn) and at 5 min endothelin was added to 50 nM (En). At 1 min the bombesin antagonist D-F_5-Phe^6, D-Ala^{11}-Bombesin(6–13)OMe was added to 10 nM as indicated (Atg). Shown are the traces from one representative experiment with $[Ca^{2+}]_i$ in nM on right. (**C**). Phosphorylation of 80K/MARCKS. Confluent, quiescent cultures of BOR 5 cells were washed twice in DMEM without phosphate and incubated in this medium with 5 μCi carrier-free $^{32}P_i$ at 37°C for 18 h. Various concentrations of bombesin (0.1 nM, 1 nM, 10 nM) or 200 nM PDB were added for 10 min. Cultures were put on ice and rapidly washed with cold DMEM without phosphate and lysed with 250 μl lysis buffer. 80K/MARCKS was immunoprecipitated and resolved on a 8% polyacrylamide gel. ^{32}P-labelled 80K was visualised by autoradiography

of bombesin-induced DNA synthesis in Swiss 3T3 cells (Rozengurt and Sinnett-Smith, 1983).

DNA synthesis induced by bombesin in cultures of BOR 5 cells was inhibited by the specific bombesin/GRP receptor antagonist, D-F_5-Phe^6, D-Ala^{11}-Bombesin(6–13)OMe, (Figure 3B) with half maximum and maximum inhibition at 1 nM and 30 nM respectively. When BOR 5 cultures were incubated with various concentrations of bombesin, maximum [^{3}H]thymidine incorporation was elicited at 1 nM (data not shown). These results substantiate that bombesin-stimulated reinitation of DNA synthesis in BOR 5 cells was elicited through a bombesin/GRP preferring receptor.

The mitogenic activity of bombesin in cultures of BOR 5 cells could also be demonstrated by monitoring cell number (rather than [^{3}H]thymidine incorporation into acid precipitable material) over a period of several days (Figure 3C). The addition of bombesin to BOR 5 in serum-free medium increased cell number compared with control cultures as early as day 1 of incubation and reached a maximum after 3 days of bombesin addition. In other experiments we also verified that bombesin induced DNA synthesis and cell proliferation in cultures of BOR 15 cells. Thus, the transfected bombesin/GRP receptor is coupled to signal tranduction pathways which stimulate DNA synthesis and subsequently cell proliferation.

Bombesin induces tyrosine phosphorylation of multiple proteins in BOR 5 and BOR 15 cells

In view of the preceding results we examined whether bombesin can stimulate tyrosine phosphorylation in Rat-1 cells transfected with the bombesin/GRP receptor. The experiments were performed with cultures of both BOR 5 and BOR 15 cells. Lysates of quiescent cultures of BOR 5 and BOR 15 cells treated with bombesin were immunoprecipitated with the anti-phosphotyrosine (anti-Tyr(P)) monoclonal antibody (mAb) Py72 and the immunoprecipitates were analysed by Western blotting with anti-Tyr(P) mAb 4G10. As shown in Figure 4, bombesin induced a dose-dependent increase in the phosphotyrosine content of multiple components including bands migrating with an apparent M_r of 110 000–130 000 and 70 000–80 000. Scanning densitometry of these bands revealed that half maximum and maximum tyrosine phosphorylation was achieved at bombesin concentrations of 0.1–0.3 nM and 1–3 nM, respectively. In contrast, parental Rat-1 cells did not show any increase in tyrosine phosphorylation in response to bombesin (100 nM) in these and the following experiments (data not shown).

The time course of tyrosine phosphorylation induced by bombesin in Rat-1 cells transfected with the bombesin/GRP receptor is shown in Figure 5. The increase in tyrosine phosphorylation of the M_r 110 000–130 000 and the M_r 70 000–80 000 bands was detectable within seconds and reached a maximum after 1 min of incubation with 10 nM bombesin in cultures of both BOR 5 and BOR 15 cells. Increased protein tyrosine phosphorylation persisted for at least 3 h.

To further substantiate the involvement of the bombesin/GRP receptor in tyrosine phosphorylation we used the bombesin/GRP receptor-specific antagonist D-F_5-Phe^6, D-Ala^{11}-Bombesin(6–13)OMe. We have already shown that this antagonist inhibits bombesin-induced Ca^{2+} mobilisation, 80K/MARCKS phosphorylation (Figure 2B) and DNA synthesis (Figure 3B). We therefore examined whether it can also inhibit bombesin-induced tyrosine phosphoryla-

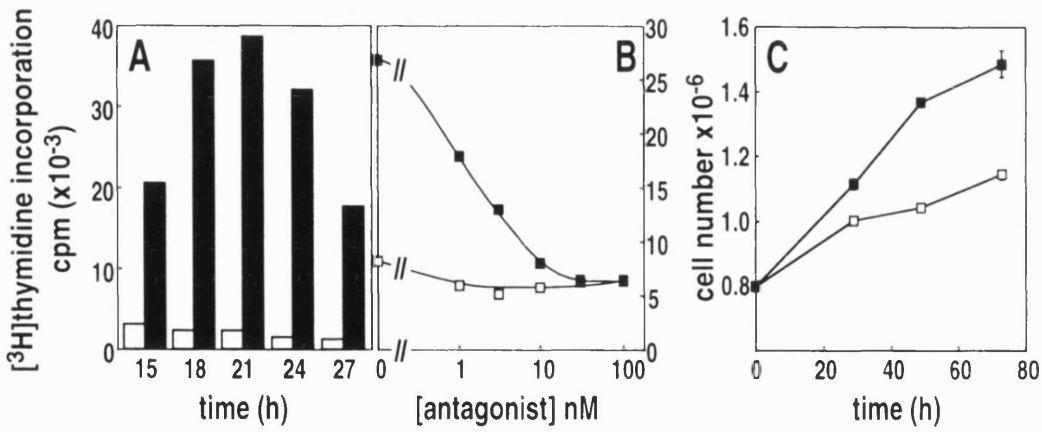

Figure 3 (**A**) Kinetics of [^{3}H]thymidine incorporation induced by bombesin. Confluent, quiescent cultures of BOR 5 cells were washed twice in DMEM and incubated at 37°C in DMEM/Waymouth's [1:1] with (solid bars) or without (open bars) 2 nM bombesin. After the times indicated below each bar [^{3}H]thymidine was added for 3 h and the incorporation was stopped as described in Materials and methods. Results are expressed as the mean c.p.m. from two dishes from a representative experiment. (**B**) Effect of antagonist on bombesin-induced [^{3}H]thymidine incorporation. Confluent, quiescent cultures of BOR 5 cells were washed twice in DMEM and incubated at 37°C in DMEM/Waymouth's [1:1] with various concentrations of antagonist D-F_5-Phe^6, D-Ala^{11}-Bombesin(6–13)OMe in the presence (closed squares) or absence (open squares) of 1 nM bombesin. [^{3}H]thymidine was added after 18 h and its incorporation was stopped after 6 h of additional incubation as described in Materials and methods. Results are expressed as the mean c.p.m from two dishes from a representative experiment. (**C**). Cell proliferation induced by bombesin. Confluent, quiescent cultures of BOR 5 cells were washed twice in DMEM and incubated at 37°C DMEM/Waymouth's [1:1] with (closed squares) or without (open squares) 1 nM bombesin. Cells were trypsinised off the dishes and counted with a Coulter counter at 0, 24, 48 and 72 h after stimulation. Each value is the mean ± SEM of five cultures. Where error bar is not shown, it is smaller than the symbol

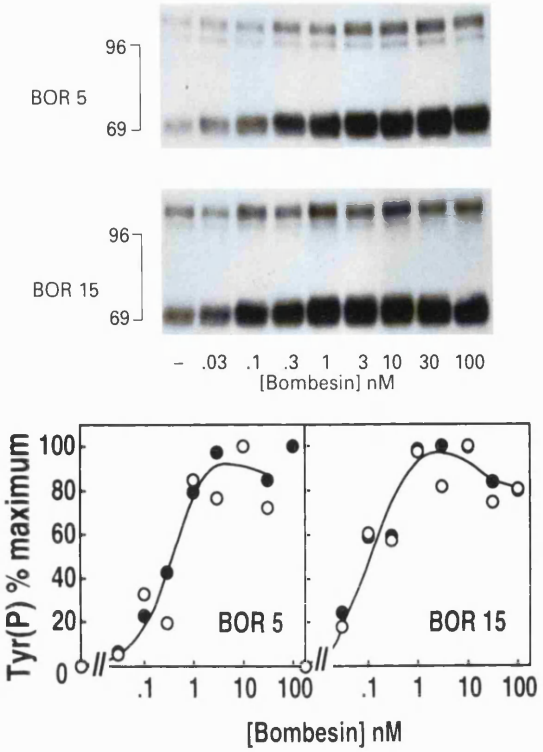

Figure 4 Bombesin-induced tyrosine phosphorylation of multiple proteins. Confluent, quiescent cultures of BOR 5 and BOR 15 cells were washed twice in DMEM and incubated at 37°C in DMEM with various concentrations of bombesin for 10 min. Cells were lysed and tyrosine phosphorylated proteins immunoprecipitated with anti Tyr(P) mAb Py72. Immunoprecipitates were resolved by SDS–PAGE and Western blotted with 4G10 anti-Tyr(P) mAb. Bands were visualised by autoradiography. Autoradiograms were scanned with an LKB Ultrascan XL densitometer to quantify tyrosine phosphorylation in terms of peak area. The 70 000–80 000 band is depicted by closed circles and the 110 000–130 000 band by open circles. Results shown are the average of three independent experiments. Values are expressed as a percentage of the maximum tyrosine phosphorylation after subtraction of the control

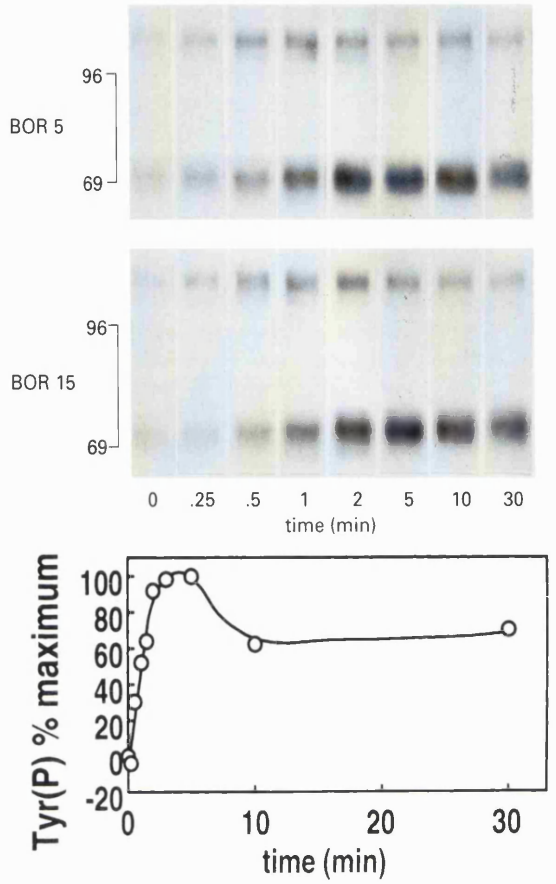

Figure 5 Kinetics of bombesin induction of tyrosine phosphorylation of multiple proteins. Upper Section. Confluent, quiescent cultures of BOR 5 and BOR 15 cells were washed twice in DMEM and incubated at 37°C in DMEM with 10 nM bombesin for various times. Cells were lysed and tyrosine phosphorylated proteins immunoprecipitated with Py72 mAb. Immunoprecipitates were resolved by SDS–PAGE and Western blotted with 4G10 anti-Tyr(P) mAb. Lower section. Autoradiograms were scanned with an LKB Ultrascan XL densitometer to quantify tyrosine phosphorylation in terms of peak area. Results shown are of the 70 000–80 000 band from a representative experiment of BOR 15 which was repeated at least three times with similar results. Values expressed as a percentage of the maximal tyrosine phosphorylation after subtraction of the control

tion. Cultures of BOR 5 and BOR 15 cells were pretreated with the bombesin/GRP receptor-specific antagonist for 10 min then stimulated by bombesin or endothelin and cell lysates were analysed for tyrosine phosphorylated proteins. Figure 6A shows that the antagonist completely blocked bombesin-induced tyrosine phosphorylation of the M_r 110 000–130 000 and the M_r 70 000–80 000 bands. In contrast, the antagonist did not interfere with the stimulation of tyrosine phosphorylation of similar bands induced by endothelin.

It has been shown that bombesin induces tyrosine phosphorylation in Swiss 3T3 cells through a PKC-independent pathway that requires the integrity of the actin cytoskeleton (Sinnett-Smith *et al.*, 1993). We examined whether the stimulation of tyrosine phosphorylation by agonist activation of the transfected bombesin/GRP receptor exhibit similar signalling characteristics. We verified that protein tyrosine phosphorylation was not downstream of PKC. Although the selective PKC inhibitor bisindolylmaleimide (GF 109203X) (Toullec *et al.*, 1991) did prevent PDB-induced tyrosine phosphorylation of M_r 110 000–130 000 and 70 000–80 000 bands, it did not prevent bombesin-induced tyrosine phosphorylation in the transfected Rat-1 cells (data not shown). We also examined the effect of cytochalasin D, which selectively disrupts the network of actin filaments, on bombesin-induced tyrosine phosphorylation. Figure 6B shows that pretreating BOR 5 and BOR 15 cultures with cytochalasin D for 2 h, dramatically inhibited the tyrosine phosphorylation of all bands stimulated by bombesin. In contrast, cytochalasin D did not inhibit EGF-induced tyrosine phosphorylation of its receptor (data not shown) indicating that this drug specifically inhibits the tyrosine phosphorylation of bands M_r 110 000–130 000 and 70 000–80 000. Thus, bombesin-induced tyrosine phosphorylation in BOR 5 and BOR 15 cells is dependent on the integrity of the actin cytoskeleton.

Bombesin stimulates tyrosine phosphorylation of p125^{FAK} and paxillin in BOR 5 and BOR 15 cells

The cytosolic tyrosine kinase p125^{FAK} and the adaptor protein paxillin which localise to focal adhesion plaques have been identified as prominent tyrosine phosphorylated proteins in bombesin-stimulated Swiss 3T3 cells. It was important to therefore assess whether agonist activation of the transfected bombesin/GRP receptor stimulates tyrosine phosphorylation of p125^{FAK} and paxillin in Rat-1 cells. Lysates of bombesin-treated cultures of BOR 5 and BOR 15 were immunoprecipitated with anti-Tyr(P) mAb and the immunoprecipitates were analysed by Western blotting with anti-p125^{FAK} mAb. Figure 7 shows that bombesin induced tyrosine phosphorylation of p125^{FAK} in a concentration dependent manner; half maximum and maximum effects were achieved at 0.3 nM and 3 nM respectively.

Parallel cultures of BOR 5 and BOR15 cells treated with various concentrations of bombesin were lysed and the lysates were immunoprecipitated with anti-paxillin mAb. The immunoprecipitates were analysed by Western blotting with anti-Tyr(P) mAb. Figure 7 shows that bombesin caused tyrosine phosphorylation of paxillin in a concentration dependent manner. The concentrations of bombesin that induced half-maximum and maximum stimulation were identical to those required to induce tyrosine phosphorylation of p125^{FAK} (i.e. 0.3 and 3 nM respectively).

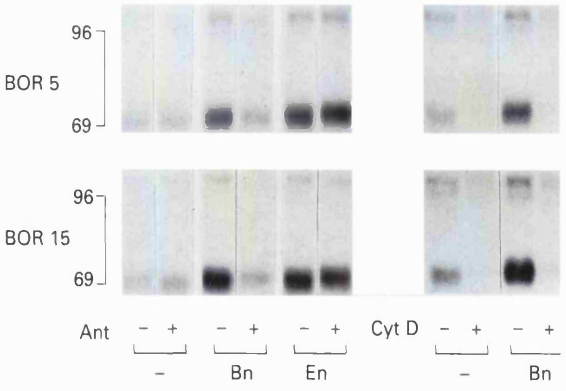

Figure 6 Left side. Effect of antagonist on bombesin-induced tyrosine phosphorylation of multiple proteins. Confluent, quiescent cultures of BOR 5 and BOR 15 cells were washed twice in DMEM and incubated at 37°C in DMEM with (+) or without (−) 100 nM D-F$_5$-Phe^6, D-Ala^{11}-Bombesin(6–13)OMe (Ant) for 10 min. Then 10 nM bombesin (Bn) or 50 nM endothelin (En) was added as indicated for 10 min. Cells were lysed and tyrosine phosphorylated proteins immunoprecipitated with Py72 mAb and Western blotted with 4G10 anti-Tyr(P) mAb. Bands were visualised by autoradiography. Right side. Effect of Cytochalasin D on bombesin-induced tyrosine phosphorylation of multiple proteins. Confluent, quiescent cultures of BOR 5 and BOR 15 cells were washed twice in DMEM and incubated at 37°C in DMEM with (+) or without (−) 2 μM cytochalasin D (Cyt D) for 2 h. Then 10 nM bombesin (Bn) was added as indicated for 10 min. Cells were lysed and tyrosine phosphorylated proteins immunoprecipitated with Py72 mAb and Western blotted with 4G10 anti-Tyr(P) mAb. Bands were visualised by autoradiography

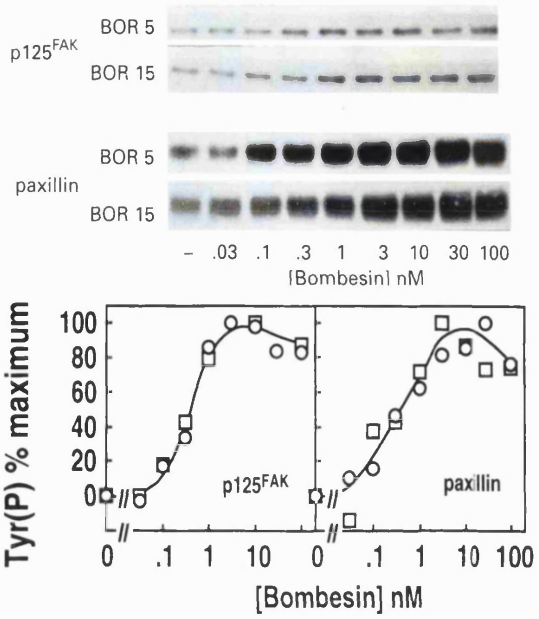

Figure 7 Bombesin-induced tyrosine phosphorylation of p125^{FAK} and paxillin. Upper section, p125^{FAK}. Confluent, quiescent cultures of BOR 5 and BOR 15 cells were washed twice in DMEM and incubated at 37°C in DMEM with various concentrations of bombesin for 10 min. Cells were lysed and tyrosine phosphorylated proteins immunoprecipitated with Py72 mAb. Immunoprecipitates were resolved by SDS-PAGE and Western blotted with anti-p125^{FAK} mAb. Paxillin. Confluent, quiescent cultures of BOR 5 and BOR 15 cells were washed twice in DMEM and incubated at 37°C in DMEM with various concentrations of bombesin for 10 min. Cells were lysed and paxillin immunoprecipitated with anti-paxillin mAb 165 and Western blotted with 4G10 anti-Tyr(P) mAb. Lower section. Autoradiograms were scanned with an LKB Ultrascan XL densitometer to quantify tyrosine phosphorylation in terms of peak area. BOR 5 is represented by squares and BOR 15 by circles. Results shown are from a representative experiment which was repeated at least three times with similar results. Values expressed as a percentage of the maximum tyrosine phosphorylation after subtraction of the control

Discussion

The experiments presented here were designed to determine whether the bombesin/GRP preferring receptor couples to both PLC activation and tyrosine kinase pathway. To examine this possibility, the cDNA encoding the bombesin/GRP receptor has been expressed in Rat-1 fibroblasts, a cell line that can be reversibly arrested in the G_0/G_1 phase of the cell cycle. The relative abilities of bombesin related peptides to displace ^{125}I-GRP in these transfected Rat-1 cells are consistent with the binding properties of endogenous bombesin/GRP receptors found in Swiss 3T3 fibroblasts and other cell types. Furthermore the apparent affinity of GRP for the transfected receptor is similar to that for the endogenous receptor. It should be pointed out that the Rat-1 cell lines transfected with the bombesin/GRP receptor used in this study (BOR 5 and BOR 15) expressed fewer receptors per cell than Swiss 3T3 cells. This is of importance in our analysis of cellular responses because overexpression of G protein linked receptors could overcome the specificity of coupling to effector systems (Ashkenazi *et al*., 1987).

Thus, the clones selected in this study, BOR 5 and BOR 15, provide a useful system to examine the signalling pathways activated by the bombesin/GRP receptor.

Our results demonstrate that agonist binding to the transfected bombesin/GRP receptor elicits multiple responses in Rat-1 cells: (1) mobilisation of Ca^{2+} from internal stores leading to a rapid increase in $[Ca^{2+}]_i$ and increased phosphorylation of the 80K/MARCKS protein mediated by PKC, (2) reinitiation of DNA synthesis and cell proliferation in serum free medium and (3) tyrosine phosphorylation of multiple proteins including broad bands of M_r 110 000–130 000 and 70 000–90 000. We identified two major substrates in the transfected cells as $p125^{FAK}$ and paxillin. None of these responses were elicited by bombesin in the parental Rat-1 cells.

The characteristics of protein tyrosine phosphorylation induced by agonist binding to the bombesin/GRP receptor transfected into Rat-1 cells were examined in detail in two clones, BOR 5 and BOR 15. An increase in the tyrosine phosphorylation of multiple proteins including $p125^{FAK}$ and paxillin occurred at low concentrations of bombesin, the half maximum being 0.3 nM. This is similar to that observed in Swiss 3T3 cells (half maximum 0.08–0.3 nM) (Zachary *et al.*, 1992; Sinnett-Smith *et al.*, 1993). Both Swiss 3T3 cells (Zachary *et al.*, 1991a) and transfected Rat-1 cells show similar time dependence of tyrosine phosphorylation, increases are detected within seconds of bombesin addition, reach a maximum at 1 min and remain elevated for hours. Bombesin stimulation of tyrosine phosphorylation in BOR 5 and BOR 15 cells, as in Swiss 3T3 cells, is not prevented by the specific PKC inhibitor GF 109203X showing that this pathway is not downstream of PKC. $p125^{FAK}$ and paxillin are localised in the focal adhesions which form at the termini of actin stress fibres and disruption of the actin cytoskeleton by cytochalasin D prevents tyrosine phosphorylation of these proteins in Swiss 3T3 cells (Sinnett-Smith *et al.*, 1993; Zachary *et al.*, 1993). In BOR 5 and BOR 15 cells bombesin-induced tyrosine phosphorylation of multiple proteins was also prevented by cytochalasin D. Therefore the protein tyrosine phosphorylation pathway activated by the transfected bombesin/GPR receptor in Rat-1 cells shows the same characteristics as that induced by bombesin stimulation of Swiss 3T3 cells.

In the transfected Rat-1 cells the increases in Ca^{2+} mobilisation, protein tyrosine phosphorylation and DNA synthesis were inhibited by the specific bombesin/GRP receptor antagonist, D-F_5-Phe^6, D-Ala^{11}-Bombesin(6–13)OMe (Coy *et al.*, 1992), further substantiating that all these responses emanate from the same receptor. This is the first report demonstrating that ligand activation of a transfected bombesin/GRP receptor elicits multiple signalling pathways and leads to cell proliferation.

The results presented here raise important questions regarding the mechanism(s) by which a single seven transmembrane receptor subtype can couple to multiple signalling pathways. While the mechanism by which bombesin/GRP receptor and other seven-transmembrane domain receptors couple to PLC through heterotrimeric G proteins of the G_q subfamily is increasingly understood (Lee and Rhee, 1995), little is known about the pathways coupling the bombesin/GRP receptor to tyrosine phosphorylation of $p125^{FAK}$ and paxillin. Recent work has implicated activation of the monomeric G protein of the $p21^{rho}$ subfamily as one step in the signalling pathway leading to tyrosine phosphorylation of these proteins (Rankin *et al.*, 1994; Seckl *et al.*, 1995). Thus, our results suggest that the bombesin/GRP receptor couples to both heterotrimeric (G_q) and monomeric ($p21^{rho}$) G proteins.

The demonstration that certain seven transmembrane domain receptors couple to $p21^{ras}$ via $\beta\gamma$ subunits of the heterotrimeric G protein of the G_i subfamily (Koch *et al.*, 1994) suggests a possible coupling mechanism between the bombesin/GRP receptor and $p21^{rho}$. By analogy, the $\beta\gamma$ subunits generated by activation of G_q/G_{11} could be preferentially coupled to activation of $p21^{rho}$ and thereby to the tyrosine phosphorylation pathway whereas the α subunit of G_q stimulates PLC (Lee and Rhee, 1995). Alternatively, one domain of the bombesin/GRP receptor may couple to G_q and thereby to PLC whereas a separate domain could lead to activation of $p21^{rho}$ and to the tyrosine phosphorylation of $p125^{FAK}$ and paxillin through interaction with a different heterotrimeric G-protein. In this context the recent demonstration that $G\alpha_{12}$ and $G\alpha_{13}$, but not $G\alpha_q$, regulate Rho-dependent actin polymerisation is highly relevant (Buhl *et al.*, 1995). This suggests that the bombesin/GRP receptor could couple to Rho and tyrosine phosphorylation of $p125^{FAK}$ and paxillin via different heterotrimeric G-proteins than those which couple it to PLC. Further experimental work will be required to distinguish between these models of signal transduction.

In conclusion, our results provide compelling evidence that ligand activation of the transfected bombesin/GRP receptor activates tyrosine phosphorylation of $p125^{FAK}$, paxillin and other proteins as well as couple to PLC-mediated events and induce cell proliferation.

Materials and methods

Transfection of Rat-1 cells

A 1.4 kb insert containing the complete cDNA coding region of the murine bombesin/GRP preferring receptor cloned into the mammalian expression vector pCD2-neo (BNR-pCD2-neo) and under the transcriptional control of the SV40 early region promoter, was kindly provided by Dr Jim Battey (Laboratory of Biological Chemistry, National Cancer Institute, Bethesda, Maryland, USA). For transfection, Rat-1 cells were plated at a density of 1 × 10^6 cells/100 mm dish. The following day, cultures were washed twice in Dulbecco's Modified Eagle's Medium (DMEM) and 20 μg DNA from BNR-pCD2-neo expression vector, mixed with 40 μg lipofectin reagent, was added to the cultures in 5 ml DMEM at 37°C. After 14 h incubation, 5 ml of 20% foetal bovine serum (FBS) in DMEM were added and incubation extended for a further day. Then, cultures were trypsinised, divided into four and selected in DMEM supplemented with 1 mg/ml neomycin derivate G418. The medium was changed every 3–4 days. After 2–3 weeks, resistant colonies were cloned by ring isolation and propagated in DMEM supplemented with 5% FBS and 0.5 mg/ml G418.

Cell culture

Stock cultures of Rat-1 cells were maintained as described previously (Higgins *et al.*, 1992) except that transfected cell lines were propagated in the presence of 0.5 mg/ml G418. For experimental purposes, 5×10^4 or 3×10^5 cells were subcultured in 33 or 100 mm Nunc Petri dishes respectively with DMEM supplemented with 5% FBS and 0.5 mg/ml G418 as necessary and incubated in a humidified atmosphere of 10% CO_2, 90% air at 37°C until they became 50–70% confluent (2–3 days), then switched down to 0.5% FBS for 3–4 days until they were quiescent.

[^{125}I]GRP binding assay

Confluent, quiescent, cultures of Rat-1 cells in 33 mm dishes were washed twice with DMEM and incubated for the indicated times at 37°C in DMEM with [^{125}I]GRP (0.05 nM, 0.01 μCi per ml and 0.95 nM cold GRP) in the presence of 1 mg/ml bovine serum albumin (BSA). For the competition studies various concentrations of competing peptides (bombesin, GRP, litorin, NmB and bombesin fragment 8–14) were also added to the binding medium. Non-specific binding was determined by adding 1 μM bombesin. Cultures were then placed on ice and rapidly washed four times with ice cold phosphate buffered saline (PBS) containing 1 mg/ml BSA. Cultures were dissolved in 0.1 N NaOH/2% $NaCO_3$/1% SDS and counted in a gamma counter. Results are expressed as specific binding per 10^6 cells. Specific binding in the transfected cells was 60–80% of the total binding. No statistically significant binding was oberved in untransfected cells in this assay which is able to detect as little as 200 receptors per cell.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured with the fluorescent Ca^{2+} indicator fura-2/acetoxymethylester (fura-2/AME). Confluent, quiescent, cultures of transfected Rat-1 cells in 100 mm dishes were washed twice with DMEM and incubated for 10 min at 37°C in 2.5 mg/ml Dispase/DMEM to digest the intercellular matrix. Cells ($4-5 \times 10^6$) in single cell suspension were then incubated at 37°C, 10 min, in 5 ml of DMEM containing 1 μM fura-2/AME freshly prepared from a 1 mM stock maintained in dimethylsulphoxide. Cells were collected by centrifugation and resuspended in 2 ml electrolyte solution (150 mM NaCl, 0.5 mM KCl, 0.9 mM $MgCl_2$, 1.8 mM $CaCl_2$, 25 mM glucose, 16 mM HEPES, the same amino acid composition as DMEM, pH 7.1) and transferred to a 1 cm^2 quartz cuvette which was maintained at 37°C and stirred continuously throughout the experiment. Fluorescence was monitored in a Perkin–Elmer LS-5 luminescence spectrometer with an excitation wavelength of 336 nm and emission wavelength of 510 nm. Intracellular calcium concentration was calculated by sequential addition of Triton X-100 to 0.05% and EGTA to 100 mM to obtain the maximum (F_{max}) and minimum (F_{min}) fluorescence respectively, using the formula $[Ca^{2+}]_i$ in nM = $K(F - F_{min})/(F_{max} - F)$, where F is the fluorescence at the unknown $[Ca^{2+}]_i$ and the value of K is 220 nM for fura2/AME.

$^{32}P_i$ labelling of Swiss 3T3 cells

Confluent, quiescent, cultures of cells in 33 mm dishes were washed twice with DMEM without P_i and labelled overnight in this medium with 50 μCi/ml $^{32}P_i$. Cells were treated with various factors as indicated then lysed in 250 μl of ice cold lysis buffer (10 mM Tris/HCl, 5 mM EDTA, 50 mM NaCl, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na_3VO_4, 1% Triton X-100 and 1 mM phenylmethylsulphonyl fluoride, pH 7.6).

Immunoprecipitations

For immunoprecipitation of phosphotyrosyl proteins and paxillin, confluent, quiescent, cultures of Rat-1 cells in 33 mm dishes (2.5×10^6 cells total) were washed twice with DMEM, treated with factors in 5 ml of DMEM as indicated and lysed at 4°C in 1 ml lysis buffer. Lysates of these cells and those of the $^{32}P_i$-labelled cells were clarified by centrifugation at 15 000 *g* for 10 min. For immunoprecipitation of tyrosine phosphorylated proteins or paxillin, clarified lysates were incubated with anti-Tyr(P) mAb Py72 for 4 h at 4°C or with anti-paxillin mAb 165 for 2 h at 4°C as indicated. These immune complexes were precipitated with anti-mouse IgG (whole molecule)–agarose for 1 h at 4°C. For immunoprecipitation of 80K/MARCKS clarified lysates were incubated for 3 h at 4°C with 80K/MARCKS antiserum (Brooks *et al.*, 1991) then 1 h with Protein A – agarose. Immunoprecipitates were washed three times with lysis buffer, extracted in 2 × sample buffer (200 mM Tris-HCl, 6% SDS, 2 mM EDTA, 4% 2-mercaptoethanol, 10% glycerol, pH 6.8) and then resolved by one-dimensional SDS-polyacrylamide gel electrophoresis (SDS–PAGE) and analysed as described in the figure legends.

[^{3}H]thymidine incorporation assay

Confluent, quiescent, cultures of Rat-1 cells in 33 mm dishes were washed twice with DMEM and incubated at 37°C with DMEM/Waymouth's medium [1:1(vol/vol)] and various additions as described in the figure legends. After times specified [^{3}H]thymidine (0.25 μCi/ml, 1 μM) was added for 3 h. Cultures were then washed twice with PBS and incubated in 5% trichloroacetic acid at 4°C for 30 min to remove acid-soluble radioactivity. Cultures were washed with ethanol, solubilised in 1 ml of 2% Na_2CO_3, 0.1 M NaOH, 1% SDS and the radioactivity in the acid-insoluble pools determined by scintillation counting in 6 ml Ultima Gold (Packard).

Measurement of cell proliferation

Confluent, quiescent, cultures of Rat-1 cells in 33 mm dishes were washed twice with DMEM and incubated at 37°C in DMEM/Waymouth's with additions as specified in the figure legends. After various times cell number was determined by removing the cells from the dish with a trypsin solution (0.05% trypsin in Ca^{2+} and Mg^{2+} free PBS with EDTA) and counting a portion of the resulting cell suspension in a Coulter counter.

Western blotting

After SDS–PAGE, proteins were transferred to Immobilon transfer membranes. Membranes were blocked using 5% non-fat dried milk in PBS, pH 7.2, and incubated for 2 h with anti-Tyr(P) mAb (4G10 1 μg/ml) or anti-p125^{FAK} mAb (1:1000) as indicated in PBS containing 3% non-fat dried milk. Immunoreactive bands were visualised by autoradiography using ^{125}I-labelled sheep anti-mouse IgG (1:1000).

Materials

FBS was obtained from Gibco BRL, Life Technologies, GRP, BSA, litorin, NmB, bombesin fragment amino acids 8–14, endothelin, insulin, bombesin, anti-mouse IgG (whole molecule)–agarose and Protein A–agarose were obtained from Sigma. Dispase was obtained from Boehringer Mannheim. Fura-2/AME was obtained from Calbiochem. The bombesin/GRP receptor-specific antagonist D-F_5-Phe^6, D-Ala^{11}-Bombesin(6–13)OMe was a kind gift of Dr David H Coy of Peptide Research Laboratories, Tulane University Medical Center, New Orleans, Louisi-

ana, USA. [*methyl*-^{3}H]thymidine (25 Ci/mmol), $^{32}P_i$ (10 mCi/ml), ^{125}I-labelled sheep anti-mouse IgG (20 μCi/μg) and (3-[^{125}I]iodotyrosyl^{15}) Gastrin releasing peptide (2000 Ci/mmole) were obtained from Amersham International, UK. Anti-Tyr(P) mAb clone Py72 was obtained from the hybridoma development unit, Imperial Cancer Research Fund, London, United Kingdom. The 4G10 anti-Tyr(P) mAb was from Tissue Culture Supplies, Buckingham, UK. Anti FAK mAb was obtained from AFFINITI Research Products Ltd, Nottingham, UK. Anti-paxillin mAb 165 was from ICN, High Wycombe, UK. All other materials were of the purest grade commercially available.

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