The Role of GTP-binding Proteins in Exocytosis

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Abstract

Streptolysin-O permeabilised rat mast cells release their granule contents by exocytosis in response to micromolar levels of Ca^{2+} and GTP-γ-S, the dual-effectors. Provision of ATP shifts the sensitivity of secretion to these dual-effectors to lower concentrations. ATP can also maintain the responsiveness of permeabilised cells to the dual effectors, restore responsiveness in permeabilised cells and delay the onset of exocytosis.

Other non-adenine nucleoside triphosphates can also increase the sensitivity to the dual-effectors. This is an indirect effect, involving the phosphorylation of endogenous ADP to generate ATP, a reaction catalysed by nucleoside diphosphate kinase.

When rat mast cells are permeabilised in iso-osmotic solutions composed of zwitterions, secretion occurs in response to either Ca^{2+} or GTP-γ-S when ATP is also provided. The response to GTP-γ-S does not require ATP (although its presence does enhance the response) or Ca^{2+}, but is dependent on Mg^{2+}. The response to Ca^{2+} is totally dependent on the presence of ATP or other nucleoside triphosphates. It can be inhibited by GDP, and it would appear ATP is being used as the substrate for generation of GTP from endogenous GDP. This being the case, secretion appears to be dependent on provision of guanine nucleotide and, by implication, a GTP-binding protein. The presence of Ca^{2+} is not necessary, although when present it modulates the response to guanine nucleotides.

Studying the kinetics of secretion reveals that Ca^{2+} and GTP-γ-S stimulated secretion is modulated by Mg^{2+}. This modulation bears similarity to the effects of Mg^{2+} on the G_s regulated adenylyl cyclase system. The results are interpreted in terms of the interaction of Mg^{2+} with GTP-binding proteins.

Using in-situ oxidisation and reduction of radioactive guanine nucleotides it is possible to label GTP-binding proteins inside Streptolysin-O permeabilised cells. I have labelled GTP-binding proteins in rat hepatocytes, and am able to detect changes in labelling of G_s in response to stimulation with glucagon. It is hoped that this method will allow identification of GTP-binding proteins involved in exocytosis in rat mast cells.

Abbreviations used in this thesis

2`-deoxyGDP	2`-Deoxyguanosine diphosphate
AA	Arachidonic acid
ADP	Adenosine 5' diphosphate
$AMGC_{16}$	Stearyl methyl glycerol
AppNHp	Adenosine 5'-(ßγ-imido)triphosphate
ATP-γ-S	Adenosine 5'-*O*-3-(thiotriphospate)
ATP	Adenosine 5' triphospate
BSA	Bovine serum albumin
cAMP	cyclic Adenosine 5' monophosphate
CDP	Cytosine 5' diphosphate
CTP	Cytosine 5' triphosphate
DAG	Diacyl glycerol
DFP	Diisopropyl fluorophosphate
DMSO	Dimethyl sulfoxide
EGTA	Ethylene glycol-O,O'-bis(2-aminoethyl)-N',N,N',N'-tetraacetic acid
GABA	γ-aminobutyric acid
GAP	GTPase activating protein
GDP-ß-S	Guanosine 5'-*O*-3-(thiodiphosphate)
GDP	Guanosine 5' diphosphate
$GppCH_2P$	Guanosine 5'-(α,ß-methylene)triphosphate
GppNHp	Guanosine 5'-(ßγ-imido)triphosphate
GTP-γ-S	Guanosine 5'-*O*-3-(thiotriphosphate)
GTP	Guanosine 5' triphospate
HEDTA	N-(2-hydroxyethyl)ethylenediamine-N,N',N'-triacetic acid
hexosaminidase	N-acetyl-ß-D-glucosaminidase
hnRNP	heterogeneous nuclear ribonucleoprotein particle
HVD	High voltage discharge
IgE	Immunoglobulin E
IP_3	myo-Inositol 1,4,5 trisphosphate
ITP	Inosine 5' triphospate
mRNA	messenger Ribonucleic acid
NADPH	ß-Nicotinamide adenine dinucleotide phosphate (reduced form)
pCa	$-Log_{10}[Ca^{2+}]$
PIPES	piperazine-N,N'-bis(2-ethane-sulfonic acid)
PKC	Protein kinase C
PLA_2	Phospholipase A_2

PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
SL-O	Streptolysin O
UDP	Uridine 5' diphosphate
UTP	Uridine 5' triphosphate
XTP	Xanthosine 5' triphospate

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Chapter 1

Introduction

1.1 In the beginning...

1.1.1 Ca^{2+} and other signals for secretion

Viewed historically, nearly all experience of stimulus secretion coupling points to the involvement of Ca^{2+} as a prime regulator [Locke, 1914; Mann et al., 1939; Harvey & MacIntosh, 1940]. A rise in intracellular Ca^{2+}, due to its entrance through membrane channels or release from intracellular stores, precedes the onset of exocytosis in the majority of secretory cells. Moreover, artificial elevation of cytosol Ca^{2+} (eg by the use of ionophores [Foreman et al., 1973; Selinger et al., 1974; Prince et al., 1973; Gomperts, 1984; Cockcroft & Gomperts, 1988]) can induce exocytosis from appropriate cells. However, in spite of the overwhelming evidence for Ca^{2+} in secretion, there is remarkably little information about what it is that Ca^{2+} does or what its cytosolic targets might be [Almers, 1990].

Neither is it clear whether an alteration of cytosol Ca^{2+} is the only, or even the most important signal transduction system that might be set in train by the activation of receptors. It is important to be aware of other possible signal transduction systems. These are often less apparent, either because it is not possible to measure or control their concentration (eg cyclicAMP), or because they involve no change in the concentration of a messenger molecule, but instead undergo a change in affinity for a substance with a fixed concentration (eg GTP-binding proteins). Despite the prominence of Ca^{2+} in the control of regulated exocytosis, there are several reasons to believe that other signal transduction systems do have a role to play.

This is clearly evident in RBL-2H3 cells (a mast cell-line), in which Ca^{2+} ionophore (A23187) does not properly mimic the effect of IgE stimulation (the physiological stimulus to secretion) [Beaven et al., 1984; Cunha-Melo et al., 1989]. Even when ionophore is supplemented with the phorbol ester PMA (an activator of Protein kinase C, PKC), the response is only marginally enhanced. If the PKC pathway is inhibited by staurosporine to the point that ionophore and PMA no longer stimulate secretion, the cross-linking of IgE receptors is still capable of triggering secretion. In this case stimulation of the PLC pathway, leading to hydrolysis of phosphatidylinositol bisphosphate, with the release of IP_3 (raising intracellular Ca^{2+}) and diacylglycerol (activating PKC), is not enough. The IgE receptor must be linked to some other messenger system.

1.1.1.1 Metabolic inhibition

Another reason for believing that signal transduction systems other than Ca^{2+} are important in many cell types are the effects of metabolic inhibition. This results not only in the inability of receptor directed agonists to stimulate secretion, but also renders the cells unresponsive to Ca^{2+} ionophores [Fewtrell et al., 1981; Foreman et al., 1973; Johansen, 1987]. The effects of metabolic inhibition are generally ascribed to depletion of the cellular ATP concentration, the primary effect of such treatment, and consequent loss of phosphorylation state. An important secondary effect is that the level of GTP will be similarly depleted, with the consequence that all processes regulated by GTP-binding proteins will become uncoupled from their upstream regulators.

1.1.1.2 Metabolic depletion of GTP

This idea can be tested by treating cells with mycophenolic acid or ribavirin, compounds which deplete cellular GTP levels whilst maintaining those of ATP. These are inhibitors of inosine monophosphate dehydrogenase (which catalyses the conversion of inosine to hypoxanthine), and hence of the purine nucleoside salvage pathway [Yamada et al., 1988]. These compounds inhibit the activation of adenylyl cyclase in intact cells [Johnson & Mukku, 1979]. Intact mast cells or RBL-2H3 cells (mast cell-line) treated with these compounds fail to respond not only to IgE-directed ligands [Marquardt et al., 1987; Wilson et al., 1989; Mulkins et al., 1992], but also to Ca^{2+} ionophores [Wilson et

al., 1988], indicating the need for GTP at a stage after that at which Ca^{2+} is required. Here at least there is indirect evidence of regulation of secretion by a GTP-binding protein, a signal transduction system that operates in respect to its ligand by a change in affinity, not concentration. The concentration of free GTP in cells remains more or less constant, and activation requires a change in the behaviour of the GTP-binding protein.

1.1.1.3 GTP-binding proteins as signal transducers

There are many other lines of evidence indicating an involvement of GTP-binding proteins in both constitutive and regulated secretion. Before reviewing this evidence I shall summarise the current state of our knowledge about GTP-binding proteins. I hope that this will help the reader to form a judgement on how good this evidence is, and where the gaps in our knowledge of the involvement of GTP-binding proteins in the control of secretion lie. To be able to make such an assessment it is important to be familiar with the hallmarks of regulation by GTP-binding proteins. Equally important is to be able to distinguish between the involvement of different classes of GTP-binding protein. To this end I have also noted specific differences in behaviour that are known to exist between the two major families of GTP-binding proteins. I have chosen to refer to these proteins as GTP-binding proteins for the reason that although the term GTPase does indeed "evoke the crucial biochemical activity of these proteins" [Bourne et al., 1990] it soon becomes somewhat confusing when discussing the hydrolysis of GTP.

1.2 Hallmarks of GTP-binding proteins

The proteins that bind GTP comprise a superfamily which selectively bind guanine nucleotides and have the intrinsic ability to hydrolyse bound GTP. Two major subsets of the GTP-binding protein superfamily are pertinent to this discussion. These are the monomeric GTP-binding proteins (20-30 KDa for p21ras and $p21^{Arf}$ like proteins and 42-53 KDa for transcription factors) and the trimeric GTP-binding proteins (composed of 40-50 KDa α-subunit, 36 KDa ß-subunit and 8-10 KDa γ-subunit). There are other proteins that selectively bind GTP, such as tubulin, guanylyl cyclases and GTP utilising kinases, but these are beyond the scope of this discussion.

All GTP-binding proteins, by definition, selectively bind guanine nucleotides and can exist in two interconvertible states, which are the basis of their ability to act as molecular switches.
1) GTP bound, generally considered to be the active species.
2) GDP bound, the inactive form.

A third state also exists when no nucleotide is bound, but this occurs only transiently in cells when GDP is exchanged for GTP.

The activity of the GTP-binding protein is therefore determined by the ratio of GTP-bound ("on") to GDP-bound protein ("off"), and at least three potential mechanisms exist to control this ratio in cells.

1) Accessory proteins which stimulate the binding of GTP in exchange for GDP, termed Guanine Nucleotide Release Proteins (GnRP's).

2) The intrinsic ability of GTP-binding proteins to hydrolyse GTP to GDP, thus inactivating themselves. Accessory proteins, particularly for the monomeric GTP-binding proteins, exist to stimulate this activity.

1 and 2 above constitute the classic GTPase cycle. Unidirectionality is ensured by the hydrolysis of the bound GTP, but there now appear to be certain cases (see 3 below) where this is not irreversible as once thought.

3) Direct phosphorylation of bound GDP to GTP by nucleoside diphosphate kinase.
This direct reversal of the GTPase cycle has so far only been shown to occur in the GTP-binding proteins ARF [Randazzo et al., 1991] and possibly also G_o and G_s [Kikkawa et al., 1990; Kikkawa et al., 1991], and it is not yet known if it is a general activation pathway for other GTP-binding proteins. This being the case I shall limit my discussion of GTP-binding protein activation to nucleotide exchange and GTPase activity.

Given the mechanisms above there are clearly two possibilities that will lead to an increase in the proportion of protein in the GTP bound state, ie activate the GTP-binding protein switch. The first is that a GnRP could stimulate exchange of GTP for GDP. Secondly, the intrinsic GTPase activity could be inhibited, either directly or by preventing stimulation by accessory proteins.

1.3 Guanine Nucleotide Binding and Exchange

1.3.1 Exchange factors for αßγ heterotrimeric GTP-binding proteins

The evidence available at present suggests that both classes of GTP-binding proteins utilise the GnRP activation mechanism. In the case of trimeric GTP-binding proteins, the GnRP is the cell surface receptor, so exchange only occurs in cells when the receptor is stimulated. Due to the wide availability of cloned proteins (and their sequences), much is known about the interaction of the trimeric GTP-binding proteins with their receptors. The receptor will only interact with the GTP-binding protein when all three subunits are united, and this interaction probably causes the release of the α-subunit from the ßγ complex when nucleotide exchange has taken place. The activated receptor markedly increases the rate of dissociation of GDP from the GTP-binding protein and it also increases the rate (eight fold) and extent of GTP binding to the α-subunit of the GTP-binding protein [Asano et al., 1984; Asano & Ross, 1984]. The limiting step in the activation of the GTP-binding protein by its receptor is not GDP release, but a subsequent step [Brandt & Ross, 1986].

1.3.1.1 Mg^{2+} and guanine nucleotide binding on trimeric GTP-binding proteins

Mg^{2+} is an important co-factor in the binding of guanine nucleotides to, and the subsequent activation of α-subunits [Northup et al., 1982; Higashijima et al., 1987c; Codina et al., 1984; Sternweis et al., 1981; Hanski et al., 1981; Higashijima et al., 1987d; Codina et al., 1984]. For stimulation of G_s by GTP-γ-S in the absence of an activated receptor, millimolar levels of Mg^{2+} (50-100mM Mg^{2+} for maximal activation) are required to stimulate binding. The receptor acts to reduce the requirement for Mg^{2+} by several thousand fold [Iyengar & Birnbaumer, 1982]. In the absence of Mg^{2+}, the binding of GTP-γ-S to α-subunits becomes reversible and it rapidly dissociates [Higashijima et al., 1987d].

1.3.2 Exchange factors for small monomeric GTP-binding proteins

Monomeric GTP-binding proteins such as p21ras and those which control protein synthesis are also linked to specific exchange proteins. For the ribosomal EF-Tu, exchange is stimulated by EF-Ts [Kaziro, 1978] in cells

actively synthesizing protein. Several proteins that can catalyse nucleotide exchange on low molecular weight monomeric GTP-binding proteins have been identified [West et al., 1990; Wolfman & Macara, 1990; Huang et al., 1990; Downward et al., 1990b; Isomura et al., 1990; Hiraoka et al., 1992] and also a cytoplasmic protein that can inhibit GDP dissociation from $p21^{rho}$ and $p21^{rac}$ [Kuroda et al., 1989; Hiraoka et al., 1992; Fukumoto et al., 1990]. This suggests that upstream control of monomeric GTP-binding proteins may involve several different sorts of exchange protein. In streptolysin-O (a bacterial toxin which can permeabilise cells) permeabilised T-cells, nucleotide exchange on $p21^{ras}$ appears to be constitutively activated, with a half life of 1 minute [Downward et al., 1990a]. Whether all $p21^{ras}$-like monomeric GTP-binding proteins possess constitutively active exchange factors is not clear, and our understanding of the control of these GnRP's is still far from adequate [Downward, 1990; Hall, 1990; Downward et al., 1992].

As activation of $p21^{ras}$ in mammalian cells is associated with entry into the cell cycle [Mulcahy et al., 1985] (and in some systems differentiation [Bar-Sagi & Feramisco, 1985]), one might imagine that exchange on $p21^{ras}$ would be associated with growth factor receptor stimulation, as is the case for the related yeast RAS genes. Certainly, stimulation of cells by a wide range of growth factors leads to activation of $p21^{ras}$ [Satoh et al., 1990a; Satoh et al., 1991; Gibbs et al., 1990; Burgering et al., 1991; Satoh et al., 1990b] (as measured by an increase in the percentage of protein isolated in the GTP-bound form). Although the link between these receptors and $p21^{ras}$ activation is not yet clear, all the receptors share tyrosine kinase activity. This fits nicely with the finding that GAP, the GTPase Activating Protein for p21ras [Trahey & McCormick, 1987] (and proteins associated with it) are phosphorylated on tyrosine residues [Molloy et al., 1989; Ellis et al., 1990; Kaplan et al., 1990] and GAP can be found associated with both the PDGF receptor [Kaplan et al., 1990; Moran et al., 1991] and insulin receptor [Brott et al., 1991]. Although stimulation of these receptors appears to lead to $p21^{ras}$ activation, the functional effect of the tyrosine phosphorylations and association with the receptor remains to be clarified. There is evidence that the mechanism of activation in T-cells is inhibition of GAP activity by a PKC pathway rather than guanine nucleotide exchange [Downward et al., 1990a]. Since $p21^{ras}$ can still be activated in T-cells (and other cell types) even when PKC is

inhibited this does not appear to be the only or a universal mechanism [Downward et al., 1992] (see section below on GTPase activity).

1.3.2.1 Mg^{2+} and guanine nucleotide binding on monomeric GTP-binding proteins

Mg^{2+} is again important in the binding of guanine nucleotides to monomeric GTP-binding proteins. In the absence of Mg^{2+} the affinity of isolated $p21^{ras}$ [Hall & Self, 1986] for GTP (relative to GDP) is markedly enhanced due to a large increase in the off-rate of GDP [Feuerstein et al., 1987]. This mechanism appears to be applicable to most [Shoji et al., 1989; Kuroda et al., 1989; Wagner et al., 1987; Burstein & Macara, 1992; Frech et al., 1990], though not all [Kabcenell et al., 1990] monomeric GTP-binding proteins.

1.3.3 Identifying GTP-binding protein regulated processes

Despite the uncertainty of how monomeric GTP-binding proteins arrive at a GTP bound state, since all GTP-binding proteins appear to be active in the GTP bound form, it is clear that the hallmark of the involvement of a GTP-binding protein in a control pathway is the stimulation of a process by, or the necessity for, GTP. GDP (or its non-metabolisable analogues) may also inhibit the process. The structure of the receptors which catalyse exchange on the trimeric GTP-binding proteins are well defined and as far as we know identification of a receptor with such a structure also points to the involvement of a trimeric GTP-binding protein. The ability of pertussis toxin to disrupt the interaction of certain trimeric GTP-binding proteins with their receptors [Katada & Ui, 1982] and of diphtheria [Pappenheimer & Gill, 1973] and Botulinum C toxin [Sekine et al., 1989] to interfere with the effector interactions of certain monomeric GTP-binding proteins also allows identification of processes controlled by GTP-binding proteins. It is possible that when more GnRP's for monomeric GTP-binding proteins have been identified, these too will be found to have common structural motifs, enabling identification of monomeric GTP-binding protein regulated pathways from upstream proteins.

1.3.4 Differences between trimeric and monomeric GTP-binding protein guanine nucleotide binding

There are two criteria that may allow differentiation between monomeric and trimeric GTP-binding proteins on the basis of guanine nucleotide binding and activation. Firstly the contrasting effects of Mg^{2+} on the binding of guanine nucleotides to monomeric and trimeric GTP-binding proteins. For trimeric GTP-binding proteins, exclusion of Mg^{2+} permits the dissociation of GTP-γ-S [Higashijima et al., 1987d] and only when Mg^{2+} is provided does activation becomes rapid and irreversible. High concentrations of Mg^{2+} (1-100 mM, depending on the α-subunit tested) can actually promote activation of G_s [Brandt & Ross, 1985], G_i [Higashijima et al., 1987d] and G_o [Katada et al., 1986] by promoting GDP and ßγ dissociation. In contrast to this, the absence of Mg^{2+} actually increases the affinity of many monomeric GTP-binding proteins for GTP (relative to GDP) [Shoji et al., 1989; Kuroda et al., 1989; Wagner et al., 1987; Burstein & Macara, 1992; Frech et al., 1990] due to a marked increase in the off-rate of GDP [Feuerstein et al., 1987]. Thus the absence of Mg^{2+} promotes activation of monomeric GTP-binding proteins, apparently by the same mechanism whereby exchange factors activate $p21^{ras}$. Also worth noting is the observation that for those monomeric GTP-binding proteins in which it has been measured, GTP binds with higher affinity than its non-hydrolysable analogues ($p21^{ras}$: [Feuerstein et al., 1989] and $p21^{RalA}$: [Frech et al., 1990]. For trimeric GTP-binding proteins, GTP-γ-S binds with the highest affinity.

Second is the ability of F^- (almost certainly complexed with Al^{3+} as AlF_4^-) to stimulate GDP-bound trimeric GTP-binding proteins [Sternweis & Gilman, 1982], probably by mimicking the terminal phosphate of GTP [Bigay et al., 1985]. Fluoride is not able to stimulate monomeric GTP-binding proteins in the same way [Kahn, 1991].

1.4 GTP Hydrolysis

1.4.0.1 Trimeric GTP-binding proteins

For the trimeric GTP-binding proteins the GTPase undoubtedly provides the off switch, and the intrinsic rate of GTP hydrolysis is high [Bourne et al., 1990]. For some trimeric GTP-binding proteins (transducin (G_t) [Arshavsky

& Bownds, 1992], G_o [Berstein et al., 1992]), there also appears to be an effector stimulated increase in GTPase activity. The intrinsic GTPase activity appears to act as a time-switch dictating the length of time that the α subunit remains active, interaction with the effector decreasing this active time even further for some α-subunits. Inhibition of the GTPase by cholera toxin [Cassel & Selinger, 1977], Mg^{2+} deprivation [Brandt & Ross, 1986] or use of non-hydrolysable GTP analogues renders the GTP-binding protein constitutively active. There is no condition other than the presence of GTP (ie the substrate) on the protein that has to be met for the GTPase to be active, although interaction with the effector increases the rate of GTP hydrolysis even further for some α-subunits.

1.4.1 Conditional activation of GTPase activity

1.4.1.1 Example of elongation factor

In contrast with the trimeric GTP-binding proteins, the GTPase mechanism of ribosomal EFTu is strictly conditional on the correct assembly of ribosomal subunits or matching of RNA codons [Kaziro, 1978]. The basal GTPase activity of EFTu is almost nonexistent, and only if the association of the two components is correct does GTP hydrolysis occur. Hydrolysis allows the amino acid linked to the incoming tRNA to be joined to the growing polypeptide chain, and the EFTu to disassociate [Tanaka et al., 1977]. The GTP-binding protein controls the fidelity of the reaction (by the so called kinetic proof reading mechanism), and inhibition of the GTPase activity prevents the EFTu dissociating and inhibits further protein synthesis.

1.4.1.2 Gaps, gaps and gaps

The control of GTP hydrolysis in p21ras like monomeric GTP-binding proteins is not yet entirely clear. The intrinsic rates of GTP hydrolysis on these proteins are very slow [Hall, 1990; Downward, 1990] (though there appear to be some exceptions: Rac1 [Menard et al., 1992]). There are proteins which act to stimulate the intrinsic GTPase activity, called GTPase Activating Proteins (GAP's) [Trahey & McCormick, 1987] and distinct classes of GAP proteins for each family of monomeric GTP-binding proteins [Garrett et al., 1989; Hall, 1992a]. The important question is whether these proteins are also the downstream effectors (thus, making

the GTPase conditional on the correct downstream association, like the EFTu family) or purely negative regulators (stimulating the GTPase to limit the activation of a separate target by the GTPase, like the trimeric family).

1.4.1.3 GAP: negative regulator and/or downstream effector?

There is good evidence that GAP does function as a negative regulator. In T-cells, inhibition of GAP (by a PKC dependent mechanism) causes activation [Downward et al., 1990a] and in $p21^{ras}$ transformed fibroblasts, transfection of GAP causes the cells to revert to their normal phenotype [Zhang et al., 1990]. However the weight of evidence now points to it also fulfilling the role of a downstream effector [Hall, 1992a]. For some time this argument rested on three pieces of evidence: 1) The fact that oncogenic mutants of $p21^{ras}$ still associated with GAP (despite its failure to stimulate GTPase activity), 2) that GAP interacted with the mutationally mapped effector domain of $p21^{ras}$ and 3) that regions inessential for transformation by $p21^{ras}$ were also inessential for interaction with GAP [Adari et al., 1988; Cales et al., 1988]. The case has been considerably strengthened by recent experiments. Firstly the finding that $p21^{ras}$ and GAP together can act to inhibit atrial K^+ channels [Yatani et al., 1990]. This suggested that a complex of $p21^{ras}$ and GAP is the effector, and further that $p21^{ras}$ might induce a conformational change in GAP allowing it to interact with target proteins [Martin et al., 1992].

Two possible target proteins for GAP have been cloned, p62 and p190. These two proteins are associated with GAP and growth factor receptors and are also tyrosine phosphorylated [Settleman et al., 1992; Wong et al., 1992]. p62 bears sequence homology to the hnRNP's (heterogeneous nuclear Ribonucleoprotein Particles) involved in mRNA splicing and it also binds nucleic acid. This points to both $p21^{ras}$ and tyrosine phosphorylation as regulators of post-transcriptional processing [Wong et al., 1992]. p190 is even more revealing in its sequence homologies. It contains motifs for a GTP-binding protein, rho-GAP activity and most importantly, a transcriptional repressor. If this protein can be shown to regulate gene transcription it will be the much longed for link between the plasma membrane and nucleus. Better still, it would provide a link between tyrosine kinase growth factor receptors, $p21^{ras}$ and mRNA production. All this evidence firmly suggests that GAP is the effector of $p21^{ras}$, and therefore that GTP hydrolysis on $p21^{ras}$ is conditional. If this is the case,

then the activity of p21^{ras} could be mechanistically similar to the way elongation factors operate, ensuring the correct assembly of a macromolecular signalling assembly in response to growth factor stimulation. A similar idea has been proposed for the action of p21 ^{rac} in the activation of the neutrophil NADPH oxidase [Abo et al., 1991], the role of p21^{rho} in actin assembly [Hall, 1992b] and the role of arf in coat assembly in transport vesicles [Serafini et al., 1991].

Despite this apparent clarification of the mystery of p21 ^{ras}/GAP interaction, there is another layer of complexity. More proteins have now been cloned that possess GAP activity, and several of these are implicated as the site of causative genetic lesions in malignancy. This again emphasises the importance of GTP hydrolysis on p21^{ras} in the regulation of cell growth and differentiation. The first of these was neurofibromin (the product of the NF1 gene), which contains a domain with rasGAP activity [Ballester et al., 1990; Martin et al., 1990; Xu et al., 1992]. Since neurofibromin levels in cells from type 1 neurofibromatosis patients are reduced (or absent) [Li et al., 1992; Basu et al., 1992; DeClue et al., 1992], NF1 is implicated as a tumour suppressor gene, acting to maintain p21^{ras} in the GDP bound state. Despite normal levels of rasGAP in these cells, there is an abnormally high proportion of p21^{ras} in the GTP-bound form. This suggests NF1 is a negative regulator of p21^{ras}, maintaining p21^{ras} inactive and preventing it from stimulating rasGAP, the putative effector. Since neurofibromin is such a large multi domain protein, this rather simplistic explanation is unlikely to be the whole story.

The existence of multiple GAPs for monomeric GTP-binding proteins is not restricted to p21^{ras}. There is also a growing family of rhoGAP related proteins. These include p190 (already mentioned above), p85, the regulatory subunit of PI kinase [Otsu et al., 1991], the break point cluster region gene (which is disrupted in Philadelphia chromosome translocations causing various leukaemias) [Diekmann et al., 1991] and the protein n-chimerin [Diekmann et al., 1991] (localised in the brain in areas involved in complex learning behaviour). The elucidation of the interplay between these various GAP's is the next step in further understanding the control of p21^{ras}-like proteins.

1.4.2 Conditional GTPase activation by the arf proteins

Other ras-like GTP-binding proteins, such as the arf family involved in vesicle trafficking, seem to have strictly conditional GTPase activity [Bourne, 1988; Rothman & Orci, 1992]. This has been shown by the fact that GTP-γ-S freezes endosomal fusion [Mayorga et al., 1989], secretory vesicle formation [Tooze et al., 1990], vesicle trafficking from the endoplasmic reticulum to the Golgi [Beckers & Balch, 1989] and between Golgi stacks [Melançon et al., 1987]. In the Golgi this leads to an accumulation of coated vesicles and would appear to be indicative of a conditional GTPase activity which requires hydrolysis of the bound GTP to release coat proteins before the vesicles can fuse to their target membranes.

1.4.3 Differences between GTP hydrolysis on trimeric and monomeric GTP-binding proteins

The conditionality of GTP hydrolysis is therefore one possible means of distinguishing between the involvement of monomeric and trimeric GTP-binding proteins. The sensitivity of cellular processes to inhibition of GTP hydrolysis should allow differentiation between regulation by monomeric (conditional GTPase) and trimeric (unconditional GTPase) GTP-binding proteins. Inhibition of GTP hydrolysis can be achieved in a number of ways, such as removal of Mg^{2+}, the use of toxins (eg cholera toxin for trimeric proteins) or the use of non-hydrolysable analogues of GTP (eg GTP-γ-S). Unconditional GTPase activity will be stimulated by such treatment and conditional GTPase activity frozen by it. Clearly in the case of the $p21^{ras}$ proteins this distinction does not prove that useful, as inhibition of the GTPase leads to persistent stimulation of cell division.

Having covered both the shared and unique characteristics of monomeric and trimeric GTP-binding proteins, I shall now review the evidence for the role of GTP in constitutive and regulated secretion with a view to nominating the class identities of the GTP-binding proteins involved.

1.5 GTP-binding proteins in vesicle trafficking

GTP-binding proteins are certainly involved in constitutive secretion although the exact role they play in the regulation of this process is still

under debate [Rothman & Orci, 1992; Bourne, 1988]. Until recently only GTP-binding proteins of the monomeric family were implicated in the control of vesicle trafficking. Now it appears that the ordered delivery of vesicles between intracellular compartments requires both monomeric and trimeric GTP-binding proteins [Burgoyne, 1992].

1.5.0.1 Rab n'Arf

At least two sub-families (rab and arf) of monomeric GTP-binding proteins appear to regulate steps in the constitutive secretory pathway. The involvement of these GTP-binding proteins was uncovered by genetic studies of secretory mutants in yeast [Novick et al., 1981] and biochemical work in cell free reconstitution systems from animal cells [Fries & Rothman, 1980]. The arf family functions in a way similar to that of EFTu, and appears to regulate the assembly of coats on transport vesicles [Serafini et al., 1991]. These coats form as vesicles bud from the donor compartment, and the vesicles must shed their coats before they can fuse with the acceptor compartment. GTP-γ-S or AlF_4^-, which cause persistent activation of GTP-binding proteins, prevent uncoating and in this way freeze transport [Melançon et al., 1987; Orci et al., 1989]. Presumably GTP-bound arf drives the coat assembly at the donor membrane, and some GAP type protein stimulates GTP hydrolysis to allow uncoating at the acceptor membrane [Rothman & Orci, 1992]. In yeast, the Sar1p protein appears to fulfil a similar function [D'Enfert et al., 1991].

The other family of GTP-binding proteins involved in vesicle trafficking are the members of the rab family. These were first discovered by genetic experiments in yeast [Salminen & Novick, 1987], and then sequencing of the mutated genes in these non-secreting yeast mutants revealed homology to ras type GTP-binding proteins. There are a growing number of members of the rab family which are localised to distinct membrane compartments [Tooze et al., 1991; Chavrier et al., 1991]. They appear to function in the docking and fusion of vesicles with the acceptor membrane. One possibility is that they drive assembly of the vesicle docking or fusion apparatus in a manner similar to that of arf in coat assembly [Segev, 1991; Rexach & Schekman, 1991]. In particular the yeast SEC4 protein appears to require the ability to hydrolyse GTP in order to function correctly. Mutations of the gene possessing no GTPase activity cause secretion to stop, and vesicles to accumulate inside the yeast

[Walworth et al., 1989; Walworth et al., 1992], suggesting a cyclical mode of action where fusion requires GTP hydrolysis. The multitude of rab proteins and their precise individual locations suggests that these GTP-binding proteins may serve as specific targeting signals, only allowing vesicles to dock with their correct acceptor membranes [Rothman & Orci, 1992; Bourne, 1988]. Accurate association then initiates GTP hydrolysis on rab (which can then dissociate and be recycled) and the fusion apparatus to be activated. There is also evidence for a role for rab1b in vesicle budding as well as docking [Plutner et al., 1991], perhaps reflecting the need to 'tag' vesicles as they leave their donor compartments, or a wider role of rab function than just targeting.

1.5.0.2 Trimeric GTP-binding proteins in the constitutive pathway of secretion

A role for trimeric GTP-binding proteins in the overall regulation of vesicle transport in the constitutive pathway has recently been proposed [Barr et al., 1991; Stow et al., 1991; Donaldson et al., 1991]. These trimeric GTP-binding proteins appear to be able to stimulate and inhibit the bulk flow through the constitutive pathway, which possibly explains how cells can shut down secretion when mitosis occurs [Hesketh et al., 1984].

From this involvement of both monomeric and trimeric GTP-binding proteins in a single process it would seem that the trimeric GTP-binding proteins regulate the overall working of the system, transducing information from (unknown) intracellular signals about the growth status of the cell, whereas the monomeric proteins generally fulfil the role of ensuring accurate assembly of macromolecular complexes. There may be similar roles for monomeric GTP-binding proteins in retrograde transport from the Golgi to the endoplasmic reticulum [Tan et al., 1992], nuclear envelope assembly [Boman et al., 1992a] (possibly arf [Boman et al., 1992b]) and protein translocation across the ER [Rapiejko & Gilmore, 1992].

1.6 GTP-binding proteins in Regulated Secretion

Just as the role of GTP-binding proteins in constitutive secretion has become clear through the use of cell free reconstitution systems, the role of GTP-binding proteins in regulated secretion has been uncovered by the

use of permeabilised cell systems. To be able to identify GTP-binding proteins involved in regulated secretion it is necessary to gain access to the interior of the cell. Simply stimulating cells with cell surface receptors will not reveal the presence (or absence) of late acting GTP-binding proteins controlling secretion. By creating lesions in the plasma membrane it is possible to test whether the presence of GTP (or its non-hydrolysable analogues) either modulates or is essential for exocytosis to occur, which is the primary test for the involvement of GTP-binding proteins. There are a large number of systems in which this appears to be the case, and these are reviewed below. However, despite the considerable amount of data on the effects of guanine nucleotides on secretion, the identity of those GTP-binding proteins involved in regulated secretion remains obscure.

1.6.0.1 Gaining access to the cytosol

Access to the cytosol can be achieved in a number of ways. Lesions can be created in the plasma membrane with bacterial toxins (eg streptolysin-O, staphylococcal α-toxin), detergents (eg digitonin, saponin) and High Voltage Discharge (HVD) or by use of the patch pipette in whole cell mode. These processes create lesions of differing size (leading to differential loss of cytosol components), differing duration (some are irreversible, others are time dependent or controllable) and perturb the plasma membrane to varying extents. These differences often lead to conflicting or divergent results between preparations, and have been a source of much argument as to which method of permeabilisation is "the best". The answer is that each preparation has its own merits and drawbacks. Much can be learned about the control of secretion by comparing results obtained by the use of different methods of permeabilisation. The patch pipette method is worthy of special note since its excellent time resolution and the ability to make precise electrical measurements has allowed the dissection of the kinetics of secretion to the level of single granule fusions.

1.6.0.2 Requirement for ATP

The majority of permeabilised cell systems contain ATP (generally at mM concentrations) in their intracellular buffers (platelets [Knight et al., 1984], pancreatic acini [Kitagawa et al., 1990], chromaffin cells [Baker & Knight, 1978], parathyroid [Oetting et al., 1986], insulinoma [Vallar et al., 1987]

and others reviewed elsewhere [Gomperts, 1990a; Gomperts, 1990b]). ATP is generally included in the buffer as the substrate for a stimulus-induced phosphorylation event comprising a step in the pathway or to allow the maintenance of a basal phosphorylation state. The actual dependence of secretion on the presence of ATP has not been tested in many of these systems. A detailed study has been carried out in bovine Adrenal Chromaffin cells, and it has been shown that contrary to earlier findings [Baker & Knight, 1978], ATP is not required for exocytosis stimulated immediately after permeabilisation [Holz et al., 1989]. The role of ATP appears to be maintenance of responsiveness for longer periods of time, which has also been shown in rat mast cells [Howell et al., 1989; Lillie et al., 1991].

Another possible role for ATP, which has not generally been considered, is that it can also provide a substrate for nucleoside diphosphate kinase in the maintenance of GTP. Thus the provision of ATP to permeabilised cells in secretion assays could potentially mask the involvement of a GTP-binding protein.

1.6.0.3 GTP-binding protein activation of phospholipases

When the involvement of a GTP-binding protein is implicated it is important to establish that the guanine nucleotide is required at a late stage in the mechanism, since trimeric GTP-binding proteins (G_q, G_z and others) can also couple surface receptors to phospholipid metabolism which could likewise be a determinant of secretion. Two phospholipases in particular (PLC and PLA_2) have been implicated in the regulation of secretion and are thought to be controlled by plasma membrane GTP-binding proteins. PLC is controlled by G_q (and possibly G_z) and its activation results in hydrolysis of phosphatidylinositol bisphosphate to inositol 1,4,5 triphosphate (IP_3) and diacylglycerol (DAG). IP_3 mediates intracellular Ca^{2+} release and DAG, with Ca^{2+}, activates Protein kinase C (PKC). PLA_2 is thought to be controlled by an as yet unidentified GTP-binding protein. Its activation leads mainly to hydrolysis of phosphatidyl choline releasing arachidonic acid (AA) which is further metabolised by the lipoxygenase and cycloxygenase pathways to prostaglandins, leukotrienes and thromboxanes. One further complication is the ability of AA to substitute for DAG in the activation of PKC-γ [McPhail et al., 1984], thus activation of PLA_2 may also activate PKC.

1.6.0.4 GTP-binding proteins and the cytoskeleton

It is now becoming apparent that as well as tubulin, other GTP-binding proteins (particularly $p21^{rho}$ [Ridley & Hall, 1992] and $p21^{rac}$ [Ridley et al., 1992]) [Paterson et al., 1990; Hall, 1992b; Bengtsson et al., 1990; Abo et al., 1991] and GTP utilising microtubule motors [Obar et al., 1991; Shpetner & Vallee, 1992] are essential regulators and components of the cytoskeleton. Whilst detailed discussion of these proteins is beyond the scope of this introduction, it is worth noting that the cytoskeleton can affect regulated exocytosis in many cells [Cheek & Burgoyne, 1991], and application of guanine nucleotides could potentially affect the cytoskeleton [Hall, 1992b]. Further clarification of this possible interaction will require detailed studies in secretory cells.

1.6.1 Neurosecretory Systems

1.6.1.1 Neurotransmitter Release

In classical neurosecretion there is no evidence for the involvement of a GTP-binding protein in the control of fusion. The time course of such secretion is far too rapid (less than a millisecond) to involve such a lengthy (several 100 milliseconds) mechanism. Monomeric GTP-binding proteins (Rab3a) have been shown to be present on synaptic vesicle membranes [Fischer von Mollard et al., 1990], and to dissociate during secretion [Fischer von Mollard et al., 1991]. Since such GTP-binding proteins are also implicated in the biogenesis of secretory vesicles [Tooze et al., 1990], it is possible that these are vestiges of the vesicles' production. In this system Ca^{2+} appears to be the sole regulator of the process of exocytosis, though the identity of its intracellular target remains obscure. However, one does not have to move very far from nerves to find a secretory cell that is at least modulated by guanine nucleotides.

1.6.1.2 Adrenal Chromaffin Cells

The adrenal medulla is composed primarily of secretory cells derived from the neural crest, the chromaffin cells. These cells secrete catecholamines (plus ATP, bioactive peptides and other proteins) in response to a rise in intracellular Ca^{2+}, which is provided by stimulation of nicotinic Acetyl Choline receptors leading to the opening of voltage gated Ca^{2+} channels.

In permeabilised cells guanine nucleotides can both modulate Ca^{2+} dependent secretion and stimulate secretion in a Ca^{2+} independent manner. The results obtained from such experiments appear to depend both on whether the cells are fresh or cultured (chromaffin cells change their properties when cultured, and often give results conflicting with those found in freshly isolated cells [Burgoyne, 1991]) and on the method of permeabilisation used (possibly reflecting the differential loss of cytosolic proteins [Burgoyne, 1991]). Experiments using HVD-permeabilised bovine chromaffin cells indicated a negative influence of both GppNHp and GTP-γ-S [Knight & Baker, 1985] on Ca^{2+} induced exocytosis, when the guanine nucleotides were preincubated with the permeabilised cells. Under the same conditions, Ca^{2+} stimulated secretion from chicken chromaffin cells was enhanced by both GppNHp and GTP-γ-S [Knight & Baker, 1985], thus it is not clear what role guanine nucleotides may play in electropermeabilised chromaffin cells.

When digitonin permeabilised cultured bovine cells are used, GppNHp [Bittner et al., 1986] or GTP, XTP, ITP and GppNHp [Morgan & Burgoyne, 1990a]) can elicit rather low levels (3-5%) of Ca^{2+} independent secretion (5mM EGTA, ~pCa9). All these nucleotides are potential activating ligands for GTP-binding proteins [Bilezikian & Aurbach, 1974], but despite being a potent activator of GTP-binding proteins, GTP-γ-S has only a very small effect on the system. Interestingly, when the same preparation is pre-incubated with GTP-γ-S and then stimulated with Ca^{2+}, secretion is inhibited [Holz et al., 1989], similar to the result obtained in electropermeabilised cells (see above). It may well be that the pre-incubation of the permeabilised cells causes the discrepancy in the effects of guanine nucleotides, possibly due to protein leakage.

The site of action of the proposed GTP-binding protein is certainly not PLA_2, since the rank order of nucleotides stimulating Ca^{2+} independent exocytosis (for maximal stimulation, XTP > ITP > GppNHp > GTP-γ-S) is quite distinct from the order for activation of PLA_2 [Morgan & Burgoyne, 1990a]. A role for the activation of PLC by guanine nucleotides (due to activation of G_p) has not been entirely ruled out, as the probable concentration of Ca^{2+} in the "Ca^{2+} free" conditions was about pCa8, possibly not sufficiently low to ensure full suppression of PLC activity. One of the main effects of PLC activation would be stimulation of PKC, one action of which is thought to be the enhancement of sensitivity for Ca^{2+}

[Knight & Baker, 1983]. However, phorbol esters alone are unable to elicit secretion, and the effect of the guanine nucleotides is therefore unlikely to involve activation of protein kinase C. Since exocytosis can proceed without discernible release of arachidonate in permeabilised chromaffin cells [Morgan & Burgoyne, 1990b; Morgan & Burgoyne, 1990a], activation of the GTP-binding protein controlling PLA_2 is also ruled out, even though activation of PLA_2 can modulate secretion [Frye & Holz, 1984].

This evidence appears to support a role for a late acting GTP-binding protein in an alternative exocytotic pathway in the chromaffin cell. A further indication of GTP-binding protein involvement in the late stages of the pathway is offered by the finding that pretreatment with pertussis toxin enhances the effective affinity for Ca^{2+} for exocytosis from digitonin permeabilised chromaffin cells [Ohara-Imaizumi et al., 1990; Sontag et al., 1991]. This effect of pertussis toxin may be via G_o, as anti-G_o antibodies mimic the effect of pertussis toxin [Ohara-Imaizumi et al., 1992]. The granule membrane of the chromaffin cell has been shown to contain GTP-binding proteins of both monomeric (*rab3A*) and trimeric (pertussis toxin substrates) families [Darchen et al., 1990; Toutant et al., 1987]. Whether these are vestiges of the formation of the granule or are actively involved in the control of secretion remains to be seen.

1.6.2 Pituitary

1.6.2.1 Melanotrophs

Secretion of α-MSH from the melanotrophs of the rat pituitary gland requires the presence of extracellular Ca^{2+}. These cells are electrically excitable and depolarisation leads to the entry of Ca^{2+}, inducing exocytosis as demonstrated by simultaneous fura-2 and capacitance measurements with the patch pipette [Thomas et al., 1990]. Stimulation of intact cells with ß-adrenergic agonists enhances Ca^{2+} induced secretion by elevating cyclicAMP, presumably by acting through G_s [Tsuruta et al., 1982]. In HVD permeabilised cells stimulated to secrete by raising cytosolic Ca^{2+}, cyclic AMP has no effect unless GTP (or one of its analogues) is also provided [Yamamoto et al., 1987]. Neomycin, an inhibitor of inositide-specific phospholipase C, does not inhibit the GTP dependent stimulation and Mg^{2+} is also a requirement for the potentiation, but not for the exocytotic reaction. These three findings may indicate a

role for a GTP-binding protein in the late stages of exocytosis for the reasons that GTP is required downstream of cAMP, activation of G_p (and PLC) do not appear to be involved and the requirement for Mg^{2+} is characteristic of guanine nucleotide binding by trimeric GTP-binding proteins.

1.6.2.2 Lactotrophs

When Ca^{2+} is intracellularly applied from a patch pipette it stimulates exocytosis (as measured by an increase in membrane capacitance of 20-50%) from bovine anterior pituitary lactotrophs [Mason et al., 1988; Zorec et al., 1988]. If GTP-γ-S and Ca^{2+} (1 μM) are both provided in the pipette, the amplitude of the exocytotic response is decreased but the rate is increased [Sikdar et al., 1989]. A plausible interpretation of these results is that GTP-γ-S stimulates a GTP-binding protein involved in the translocation of granules to the plasma membrane or assembly of the fusion apparatus, but because it cannot be hydrolysed it cannot be recycled for further rounds of exocytosis, thus decreasing the amplitude of the response.

1.6.2.3 Gonadotrophs

α-toxin permeabilised pituitary cells can be stimulated to secrete lutenizing hormone (LH) by elevating Ca^{2+}, and at resting levels of Ca^{2+} (pCa7), by introduction of cyclicAMP, GTP-γ-S or GppNHp. Since cAMP and guanine nucleotides have the same effect, it is likely that this is mediated by G_s and the generation of cyclicAMP, not a late acting GTP-binding protein. However, treatment of the permeabilised cells with GTP-γ-S before stimulation gives a very different response. In this case subsequent secretion stimulated by any one of Ca^{2+}, cAMP, PMA or PMA and cAMP together is inhibited [van der Merwe et al., 1991; Davidson et al., 1991]. GppNHp is without effect, but the action of GTP-γ-S is similar to that observed in the cell-free membrane traffic systems. It is possible that there is a late acting monomeric GTP-binding protein which must cycle between GTP and GDP-bound states to ensure continuous delivery of vesicles to the plasma membrane, or alternatively, that there is a late acting inhibitory GTP-binding protein.

1.6.3 Endocrine Secretory Systems

1.6.3.1 Parathyroid

Secretion of PTH from the parathyroid is unusual in that it occurs in response to a decrease in extracellular Ca^{2+} concentration [Sherwood et al., 1966; Brown et al., 1987; Brown, 1991]. Hormone secretion occurs as extracellular Ca^{2+} is reduced to less than 1.25mM [Fitzpatrick et al., 1986a], with a corresponding decline in the level of intracellular Ca^{2+} [Muff & Fischer, 1986]. However, it is unlikely that the secretory response is elicited simply as a consequence of reducing intracellular Ca^{2+} concentration since dopamine, noradrenaline and Li^{+} are all capable of inducing secretion, and do so without any alteration in the concentration of cytosol Ca^{2+} [Nemeth et al., 1986].

There is evidence for the involvement of GTP-binding proteins at both early and late stages in the control of secretion in these cells. In parathyroid cells treated with pertussis toxin, exocytosis becomes insensitive to the inhibitory effects of high extracellular Ca^{2+} and appears to proceed uncontrolled. The application of Ca^{2+} ionophore inhibits release regardless of whether cells have been treated with the toxin or not [Fitzpatrick et al., 1986a], so it is possible that the pertussis substrate is linked to some form of extracellular Ca^{2+} sensitive receptor, possibly associated with production of inositol phosphates [Brown et al., 1987]. The finding that PT treatment also renders the system insensitive to the inhibitory effects of Ca^{2+} channel agonists [Fitzpatrick et al., 1986b] could be interpreted as evidence that the regulatory GTP-binding protein might interact with a Ca^{2+} channel.

Evidence for a late acting GTP-binding protein has been found in HVD permeabilised cells, where GppNHp (a non-metabolisable analogues of GTP) is capable of inducing exocytosis at pCa9, ie in a Ca^{2+} independent manner [Oetting et al., 1986]. Surprisingly, it was also found that exocytosis from permeabilised cells can be stimulated by elevation of cytosol Ca^{2+} (from pCa7 to pCa5), although the extent of Ca^{2+} induced secretion is much less than that induced by guanine nucleotides [Oetting et al., 1987]. When loaded with GppNHp (10^{-5}M), exocytosis remains maximal as the level of Ca^{2+} is raised up to 200nM. Above this concentration of Ca^{2+} it declines steeply, approaching a baseline response

at pCa6 [Oetting et al., 1986], mimicking the normal response of intact cells.

1.6.3.2 Renal juxtaglomerular cells

Secretion of renin from juxta-glomerular cells is also inhibited by high extracellular Ca^{2+} [Fray et al., 1987; Park et al., 1986]. In HVD permeabilised renal juxta-glomerular cells there is evidence for a late acting GTP-binding protein since both Ca^{2+} and a non-hydrolysable guanine nucleotide are required for secretion of renin [Newton & Knight, 1991]. If Ca^{2+} is raised above pCa6 in the permeabilised cells secretion is inhibited, the same result as is obtained in intact cells when extracellular Ca^{2+} is raised.

1.6.3.3 Insulin Secreting cells

In insulin secreting cells there is evidence for GTP-binding proteins both promoting and inhibiting exocytosis. In permeabilised islet cells [Wollheim et al., 1987] and insulinoma RINm5F and HIT-T15 cells [Vallar et al., 1987; Ullrich et al., 1990], secretion can be induced either by Ca^{2+}, or by non-hydrolysable analogues of GTP at low Ca^{2+} concentrations. In RIN5mF cells, GTP-γ-S remains effective as Ca^{2+} is suppressed to pCa9, conditions under which the activation of G_p-linked PLC and release of IP_3 are unlikely to occur. This therefore supports a role for a separate late acting GTP-binding protein. Stimulation of α_2-adrenergic or somatostatin receptors can inhibit Ca^{2+} induced exocytosis in permeabilised RINm5F cells [Ullrich & Wollheim, 1988]. This inhibition requires the presence of GTP, and is prevented by pre-treatment with pertussis toxin. Although these receptors remain able to inhibit adenylyl cyclase (presumably via G_i) in permeabilised pancreatic islet cells [Jones et al., 1987] and RINm5F cells [Ullrich & Wollheim, 1988] there is no correlation between the level of cyclicAMP and the rate of secretion. This suggests that there is a late acting G_i-like GTP-binding protein which can inhibit exocytosis by a cAMP independent pathway. Since such inhibition is mediated via cell surface receptors, it would appear likely that this GTP-binding protein is located at the plasma membrane. The non-hydrolysable analogues of GTP do not support inhibition, presumably because they support the activation response so strongly.

1.6.4 Exocrine secretory systems

1.6.4.1 Pancreatic acini

There is strong evidence for the involvement of G_P in the activation of exocytosis from pancreatic acini, but it has only recently become clear that a late acting GTP-binding protein is also involved in the control of secretion. Patch clamp experiments have shown that acetylcholine induces capacitance changes indicative of exocytotic activity in pancreatic acinar cells. These capacitance changes are abolished when 1-2 mM EGTA or GDP-ß-S is included in the pipette solution, and can be potentiated by GTP-γ-S. This supports a role for G_P in linking cell surface receptors to a rise in intracellular Ca^{2+}, leading to exocytosis [Maruyama, 1988]. Furthermore, addition of GTP-γ-S [Edwardson et al., 1990; Padfield et al., 1991; Kitagawa et al., 1990] or PMA [Kitagawa et al., 1990] (phorbol ester) to SL-O permeabilised acinar cells leads to an increase in the extent and sensitivity of exocytosis to Ca^{2+} in a manner which is strongly dependent on the presence of ATP. The fact that GTP-γ-S and phorbol esters both enhance the sensitivity of the system to Ca^{2+} suggests that they are both working via the same mechanism. Although not directly proven, PKC is the prime candidate for this common mechanism, which would explain the requirement for ATP in both the PMA and GTP-γ-S induced responses. This is therefore further evidence that GTP-γ-S acts through the PLC pathway by activating G_P. However, activation of G_P and the PLC pathway by GTP-γ-S does not appear to be the whole story.

If GTP-γ-S were working exclusively through PKC to enhance the sensitivity to Ca^{2+}, then addition of GTP-γ-S to phorbol ester treated cells should have no further effect. This is not the case. Maximal doses of GTP-γ-S and phorbol ester provided together are cumulative in their enhancement of secretion [Kitagawa et al., 1990] and this suggests that the guanine nucleotide acts at a second site in addition to G_P. Also it has been found that GDP and 2`-deoxyGDP can inhibit GTP-γ-S stimulated secretion under conditions in which IP_3 production is inhibited (pCa9, PLC activity suppressed) [Padfield et al., 1991]. Both these findings point to the involvement of a late acting GTP-binding protein in regulated secretion from the pancreas.

There are several monomeric (21.5 - 29kDa) GTP-binding proteins located on the cytosolic face of the zymogen granule membranes, and any or all of

these could constitute a component of the exocytotic mechanism [Padfield & Jamieson, 1991]. A synthetic peptide (Rab3AL) of the putative effector domain of Rab3a has been shown to stimulate amylase secretion, in an ATP dependent manner [Padfield et al., 1992]. An explanation for this result may be that the peptide acts to block the interaction of endogenous Rab3a protein (or a close relative) with its GAP protein, which would be an endogenous inhibitor of secretion. This has been suggested since the peptide represents the effector domain from mutant Rab3a proteins which fail to undergo GTPase stimulation when bound to GAP. However, since this peptide blocks the Rab1 mediated transfer of vesicles through the Golgi stacks [Plutner et al., 1990], its specificity does not appear to be that high. The other monomeric GTP-binding proteins may represent vestigial species related to the formation, maturation and maintenance of the secretory vesicles. The results from these various approaches make a strong case for thinking that in pancreatic acinar cells, fusion of secretory granules with the plasma membrane is mediated by a GTP-binding protein acting at a stage subsequent to the action of Ca^{2+}. It remains to be seen if this granule GTP-binding protein is in any way related to Rab3a, or if Rab3a peptide can stimulate in vitro granule fusion.

One other line of evidence points to a late acting GTP-binding protein in amylase secretion. In a cell-free system, the fusion of isolated zymogen granules to pancreatic plasma membranes can be stimulated by GTP and its non-hydrolysable analogues or $[ALF_4]^-$ [Nadin et al., 1989]. In these experiments, fusion was measured by the release of amylase and by fluorescence dequenching of the membrane probe octadecylrhodamine B-chloride. Such fusion requires neither Ca^{2+}, cytosol proteins, nor ATP. Since the granules contain potent active lipases it might be questioned whether the membranes retain their native lipid composition [Meldolesi et al., 1971], and high levels of certain lipid metabolites can stimulate non-specific fusion and lysis [Poole et al., 1970]. Thus there remain questions over the interpretation of this data.

1.6.5 Blood cells

1.6.5.1 Platelets

Ca^{2+} dependent secretion of all three types of platelet granules (alpha, dense and lysosomal) from HVD permeabilised platelets can be

positively modulated by the addition of GTP-γ-S [Haslam & Davidson, 1984a; Knight & Scrutton, 1986; Athayde & Scrutton, 1990; Coorssen et al., 1990]. For alpha and dense granules it is the affinity for Ca^{2+} which is increased, whereas for lysosomal granules it is the actual extent of secretion which is increased. These responses can be mimicked by the addition of phorbol ester or agonists which activate the PLC pathway such as thrombin [Haslam & Davidson, 1984b; Knight et al., 1984; Coorssen et al., 1990]. The GTP-binding protein involved in this reaction is almost certainly G_P, leading to activation of PKC via the PLC pathway. This is supported by the fact that the thrombin receptor is a (novel) member of the trimeric GTP-binding protein receptor family [Vu et al., 1991], and thrombin mediated increase in Ca^{2+} affinity is entirely GTP dependent [Haslam & Davidson, 1984a; Knight & Scrutton, 1986].

In contrast to the very strong case that can be made for the role of G_P, there are only hints that a late acting GTP-binding protein may play a part in the control of secretion from platelets. If sufficiently high concentrations of guanine nucleotide are used (100 μM GTP-γ-S), then it is possible to induce secretion from all three types of secretory granule under conditions in which the concentration of Ca^{2+} is suppressed (below pCa9) to the point at which there is unlikely to be any activation of phospholipase C [Athayde & Scrutton, 1990; Coorssen et al., 1990]. This suppression of Ca^{2+} probably rules out the activation of PLA_2 as well, which has been suggested to regulate exocytosis in platelets [Smith et al., 1973]. Mastoparan (a wasp venom peptide that can stimulate guanine nucleotide exchange on trimeric GTP-binding proteins [Higashijima et al., 1990; Higashijima et al., 1988; Weingarten et al., 1990]) has also been shown to stimulate exocytosis of alpha and dense granules from intact platelets [Wheeler-Jones et al., 1992; Ozaki et al., 1990]. Mastoparan did not stimulate PLC (measured by IP_3 production and $[Ca^{2+}]_i$) and its effects were found to be resistant to PKC inhibitors [Wheeler-Jones et al., 1992]. Encapsulation of GDP-ß-S into reversibly HVD permeabilised platelets depressed mastoparan induced exocytosis. These findings would also appear to point to a late acting GTP-binding protein, possibly of the trimeric family, located on the plasma membrane.

1.7 Myeloid cells

The cells of the granulocytic myeloid family provide several examples of cells in which secretion is regulated by a late acting GTP-binding protein (named G_E) and Ca^{2+}. This includes permeabilised neutrophils [Cockcroft, 1991], HL60 cells [Stutchfield & Cockcroft, 1988], eosinophils [Cromwell et al., 1991; Nusse et al., 1990] and mast cells [Howell et al., 1987]. For all of these, exocytosis can proceed in the absence of ATP, but when ATP is present it enhances the sensitivity to both effectors.

1.7.1 Neutrophils

The concept of a late acting GTP-binding protein regulating exocytosis at a point distal to PLC (and by inference G_P) was first put forward from data obtained with Sendai virus permeabilised neutrophils [Gomperts et al., 1986; Barrowman et al., 1986]. Exocytotic fusion of azurophilic granules (measured by ß-glucuronidase release) could be elicited by a combination of both guanine nucleotide and micromolar Ca^{2+}. Ca^{2+} on its own was a poor stimulus, whilst GTP-γ-S could elicit high levels of secretion when Ca^{2+} was strongly suppressed. Because Ca^{2+} and low concentrations of guanine nucleotide act in synergy and pretreatment of the cells with phorbol ester inhibits high levels of Ca^{2+} induced secretion, it is unlikely that secretion is stimulated by the guanine nucleotides activating PKC via G_P and PLC. This conclusion is further strengthened by the fact that the extent of GTP-γ-S-induced secretion increased as the concentration of Ca^{2+} was suppressed towards and even below pCa10. Under these conditions activation of both PLC and PLA_2 (also suggested as an activator of neutrophil secretion [Walsh et al., 1983]) could more or less be ruled out. More recent experiments on human neutrophils or HL60 cells permeabilised with SL-O have confirmed these results, and have shown that secretion can be stimulated without generation of the products of PLC [Stutchfield & Cockcroft, 1988] or PLA_2 [Cockcroft, 1991] activation.

Evidence from patch pipette permeabilised human neutrophils is in close agreement with the data achieved with SL-O permeabilised cells. Inclusion of GTP-γ-S and ATP in the pipette solution is sufficient to induce degranulation even when the intracellular medium is also supplemented with EGTA to suppress the level of Ca^{2+}) [Nusse & Lindau, 1988].

Further evidence in favour of a second site of action for GTP in the stimulus-secretion sequence of rabbit neutrophils came from the finding that GDP and GDP-ß-S are inhibitory to secretion induced by Ca^{2+} [Gomperts et al., 1986]. Since ATP was a requirement for the Ca^{2+} induced secretion it is possible that the Ca^{2+} response was in fact entirely dependent on GTP. This idea is backed up by later findings on SL-O permeabilised neutrophils which showed that they only undergo exocytosis in response to Ca^{2+} in the presence of ATP [Cockcroft, 1991].

As activation of GTP-binding proteins linked to PLC or PLA_2 is clearly unnecessary for exocytosis to occur, this evidence represents a strong case for a late acting GTP-binding protein controlling exocytosis in neutrophils, which may even be mediating the Ca^{2+} and ATP dependent secretory response.

As well as secreting bacteriocidal substances, neutrophils also possess another killing mechanism. This is the respiratory burst and there is now good evidence for a late acting GTP-binding protein in its regulation [Lu & Grinstein, 1990; Abo et al., 1991; Knaus et al., 1991]. The GTP-binding protein involved appears to be rac1, a member of the monomeric GTP-binding protein family, and it is possible that it regulates assembly of the proteins at the plasma membrane via the cytoskeleton.

1.7.2 Eosinophils

Eosinophils show remarkably similar responses to neutrophils, except that a combination of ATP and Ca^{2+} cannot stimulate secretion. SL-O permeabilised cells secrete maximally in response to GTP-γ-S and Ca^{2+}, though they also exhibit a substantial (50%) amount of Ca^{2+} independent secretion [Cromwell et al., 1991; Nusse et al., 1990]. Interestingly, unlike GTP-γ-S, GTP cannot stimulate secretion on its own, and this may explain why ATP and Ca^{2+} cannot elicit exocytosis, if ATP acts to generate GTP as seems likely in neutrophils [Gomperts et al., 1986] and mast cells [Lillie & Gomperts, 1992a]. The presence of ATP is not necessary, but the secretory response is increased in magnitude in its presence, and the sensitivity to the two effectors is increased. Patch clamp experiments on both guinea pig peritoneal [Nusse et al., 1990; Lindau et al., 1992] and horse blood eosinophils [Scepek et al., 1991] also show that in the

presence of ATP, GTP-γ-S alone is able to induce exocytosis, and the presence of Ca^{2+} increases the rate of granule fusion.

1.8 Mast cells

Of the granulocytic myeloid cells, and indeed in the control of exocytosis by any secretory cell, the role of GTP-binding proteins has been studied in most detail in the mast cell. Access to the cytosol of these cells can be gained in a number of ways, each giving a complementary view of the control of exocytosis. Permeabilised cells can be used to gain two different views of exocytosis by measuring completed events (in dose response experiments) or the timecourse of secretion (in kinetic experiments).

Rather than using a permeabilisation agent on bulk cell suspensions, access to the cytosol of individual cells can be gained using either microinjection or the patch pipette in the whole cell mode. Both these approaches have yielded data in close agreement with the permeabilised cells, and in addition the patch pipette allows very fine detail of the kinetics of individual granule fusions. The similarities and differences between the results obtained from these various methods allows more to be learned about the regulation of secretion by both GTP-binding proteins and Ca^{2+}.

1.8.1 SL-O permeabilised Cells: Ca^{2+} and guanine nucleotide, the dual effector system

With the SL-O permeabilised preparation it is possible to control the cytosolic concentration of Ca^{2+}, guanine nucleotides, ATP (after metabolic inhibition), Mg^{2+} and the composition of the permeabilisation buffer. After permeabilisation with SL-O, cytosolic contents are rapidly lost [Howell & Gomperts, 1987; Gomperts et al., 1987] but the secretory machinery remains intact for several minutes [Howell et al., 1989]. From this finding it would appear that none of the essential elements for exocytosis are soluble cytosolic proteins. In order to stimulate exocytosis in a simple NaCl based buffer both Ca^{2+} and guanine nucleotide are required [Howell et al., 1987; Koopmann & Jackson, 1990]. These were first termed the "essential effectors" as both are necessary, and together they are sufficient to trigger release of up to 100% of the secretory granule contents. Non-hydrolysable analogues of GTP (GTP-γ-S, GppNHp,

GppCH$_2$p) offer the best support to secretion, followed by unmodified nucleoside triphosphates in the order ITP>XTP>GTP [Howell et al., 1987; Churcher et al., 1990a] The tetra fluroaluminate ion also supports secretion [Sorimachi et al., 1988], so it is clear that any ligand capable of activating a trimeric GTP-binding protein is also capable of stimulating secretion.

The GTP-binding protein which activates exocytosis is quite distinct from G_P, the activator of phospholipase-C [Cockcroft & Gomperts, 1985], as exocytosis can be stimulated under conditions when PLC is inhibited by neomycin [Cockcroft et al., 1987; Aridor & Sagi-Eisenberg, 1991]. PLA_2 is another GTP-binding protein regulated plasma membrane enzyme that has been suggested as a stimulus to secretion in mast cells [Peters et al., 1985; Schleimer et al., 1986]. Application of exogenous phospholipase A_2 can stimulate secretion from intact mast cells [Chi et al., 1982], although one possible explanation of this would be the ability of PLA_2 to cause cell lysis by altering the lipid composition of the membrane, generating fusogenic lipids [Poole et al., 1970]. Certainly the combined stimulus for exocytosis in permeabilised mast cells, Ca^{2+}-*plus*-guanine nucleotide, should also activate PLA_2 [Burch et al., 1986; Axelrod et al., 1988; Bokoch & Gilman, 1984], and in permeabilised mast cells the two GTP-binding protein mediated functions (secretion and PLA_2 activation) have proved hard to separate. In permeabilised mast cells considerably higher concentrations of both effectors are needed to activate PLA_2 as opposed to secretion [Churcher et al., 1990a], and in the related RBL-2H3 cell-line exocytosis can proceed without discernible release of arachidonic acid in intact cells after treatment with dexamethasone [Collado-Escobar et al., 1989]. It therefore seems unlikely that GTP-binding protein activation of PLA_2 or PLC is the prime stimulus to secretion in mast cells, and the distinct GTP-binding protein involved in the control of regulated secretion has been termed G_E [Gomperts et al., 1986].

1.8.2 Microinjected cells

Work on microinjected cells has yielded results remarkably similar to those obtained in SL-O permeabilised cells. Exocytosis (assessed by counting the proportion of degranulated cells) depends on both the provision of extracellular Ca^{2+} and on the concentration of microinjected GTP-γ-S [Tatham & Gomperts, 1991]. Injection of Ca^{2+} alone is without effect

[Tatham & Gomperts, 1991; Tasaka et al., 1970] though there is one report of a positive outcome, following iontophoresis of Ca^{2+} [Kanno et al., 1973]. Significantly, the cells remain responsive to microinjection of GTP-γ-S even when extensively depleted of ATP (to about 1% of normal levels) following prolonged incubation with metabolic inhibitors, to the point of becoming absolutely insensitive to stimulation by polybasic agonists (compound 48/80). After metabolic depletion (the standard condition for SL-O permeabilised experiments), the requirement for the guanine nucleotide merely shifts to about 10 fold higher concentration (probably through a decrease in the level of phosphorylation).

1.8.3 Patch pipette and Chloride buffers

1.8.3.1 Deleterious effect of Cl^-

At the biochemical level, patch pipette work on mast cells differs in one important respect from the main experience of work with permeabilised cells in suspension. This is that it requires the use of pipette (intracellular) solution based on K-glutamate (see below), because the use of KCl causes marked morphological changes in the cells within a few minutes [Almers & Neher, 1987]. When Cl^- was used the cells took on a grainy appearance and became very sensitive to hydrostatic pressure, collapsing under suction and swelling with slight pressure. Both phenomena were associated with significant capacitance changes (indicating changes in membrane surface area). It was concluded that high concentrations of intracellular Cl^- lead to a rapid disintegration of the cytoskeleton. It has also been suggested that the modulation of exocytosis by Cl^- results from Cl^--dependent changes of the granule membrane potential. However measurements of individual mast cell granules membrane potentials at the time of fusion indicate a widely scattered range of potentials (between 11 and 160 mV) [Breckenridge & Almers, 1987]. This is therefore unlikely to explain the effect of Cl^- on exocytosis or affect the ability of a granule to undergo exocytosis.

Despite these inhibitory actions of Cl^-, and the decreased reproducibilty of degranulation, 20μM GTP-γ-S on its own (ie maintaining intracellular calcium at about 50 nM) is able to stimulate exocytosis i.e. a persistent stimulus to a GTP-binding protein alone is sufficient to induce exocytosis. Under these circumstances, degranulation is markedly delayed,

taking 8 - 20 minutes before it commences, after which the degranulation is rapid. In contrast to the effects of Cl^-, when glutamate is used as the main intracellular anion the morphology and integrity of the cells is retained for at least 15 min [Almers & Neher, 1987].

1.8.4 Glutamate electrolyte solutions

1.8.4.1 SL-O permeabilised cells

Simply by changing the major anion in the permeabilisation buffer from Cl^- to glutamate (or other amino acids eg aspartate), several differences in the control of secretion become apparent [Churcher & Gomperts, 1990]. The rationale for exploring the effects of glutamate is a historical one. The earliest work on permeabilised chromaffin cells [Knight & Baker, 1982] demonstrated that Cl^- based buffers inhibited secretion compared with glutamate based buffers. For this reason glutamate has been used as the major intracellular anion in the majority of work on permeabilised cells. This was never possible in permeabilised mast cells as the fluorescent assay for histamine (released at nM concentrations from the granules) is also sensitive to glutamate (present at ~140mM in buffers), thus making the use of buffers formulated with glutamate impractical. In contrast to the findings in chromaffin cells, anions such as Cl^- were able to support secretion in mast cells, and this became the anion of choice. With the development of a fluorescent assay for the granule marker enzyme hexosaminidase it became possible to test the effects of glutamate.

1.8.4.2 Stimulation of exocytosis by GTP-γ-S alone

In the absence of ATP, both Ca^{2+} and a guanine nucleotide are required to stimulate maximal exocytosis, just as they are in Cl^-, but GTP-γ-S on its own can also elicit a low (15%) level of release. In the presence of ATP, once again both effectors are still needed to induce total release of hexosaminidase, but now, either Ca^{2+} or GTP-γ-S alone can stimulate secretion. Ca^{2+} (with ATP) can stimulate 60% secretion, whilst GTP-γ-S (with ATP) can elicit 30%. Since the response to Ca^{2+} is totally dependent on the provision of ATP, one possible explanation would be that ATP is generating GTP to synergise with the Ca^{2+}. This possibility was originally addressed by using 1mM GDP-ß-S, but this failed to inhibit the Ca^{2+} and ATP response. Whilst this might be taken as an indication that no

GTP-binding protein is involved in this pathway, it must be noted that the same treatment also failed to inhibit Ca^{2+} and GTP-γ-S stimulated secretion under the same conditions [Churcher & Gomperts, 1990].

1.8.4.3 Cl^-, glutamate and the Hoffmeister series

Several possible explanations of the effects of varying anions on the secretory mechanism have been put forward. In the patch pipette the most likely explanation is that Cl^- disrupts the cytoskeleton far more than glutamate [Almers & Neher, 1987]. This would fit with the widely held view that the position of the anion in the Hoffmeister series determines its ability to be a cell-friendly intracellular electrolyte. The Hoffmeister series ranks anions in decreasing order of chaotropic effects. Glutamate ranks lower than Cl^- as it less chaotropic, and therefore presumed to disrupt the organisation of intracellular proteins less. However in SL-O permeabilised mast cells, secretion is supported by a range of anions in an order which does not fit the Hoffmeister series. Furthermore, contrary to the findings in patch pipette studies, SL-O permeabilised mast cells remain able to secrete for far longer in Cl^- than glutamate based buffers.

1.8.4.4 Anion binding by GTP-binding proteins

Another, possibly more plausible, explanation comes from work on the direct effect of anions on trimeric GTP-binding proteins. A number of specific effects of anions on GTP-binding protein functions have been described [Higashijima et al., 1987b; 4749]. Thus, both Cl^- and Br^- increase the affinity of $G_o\alpha$ for GTP-γ-S and GTP, while SO_4^{2-} decreases affinity and F^-, I^-, and NO_3^- have little effect. Glutamate has not been tested. The enhancing effect of Cl^- is expressed at concentrations in the range 3-20mM (ie probably below the normal range of intracellular Cl^- concentrations in myeloid cells [Ince et al., 1987]) and a specific binding site on the GTP-binding protein has been inferred. Cl^- also appears to suppress the GTPase activity of G_o. These phenomena are not confined to G_o and may well explain the enhancing effect of chloride and azide on the stimulation of adenylyl cyclase by hormones, fluoride and GTP-analogues [Johnson et al., 1975; Svoboda & Christophe, 1978]. The effects of Cl^- on the GTP-binding protein regulated atrial muscarinic K^+ channel appear to be similar to those seen for G_o. Cl^- based buffers increase channel openings in response to acetyl choline by decreasing the GTPase activity

of G_K and also enhance basal turn-on of the channel by GTP in the absence of agonist [Nakajima et al., 1992]. In mast cells addition of Cl^- inhibits GTP-γ-S dependent (Ca^{2+} independent) secretion in glutamate buffers. Considering that Cl^- increases the affinity of GTP-γ-S binding to G_O, this is almost the opposite effect to that which would be anticipated if G_E were a trimeric GTP-binding protein. However, bearing in mind the strong sequence homologies that exist between trimeric α-subunits and the monomeric GTP-binding proteins, a direct anion effect at the level of G_E remains a possibility.

1.8.5 Patch pipette and glutamate buffers

For the reasons stated above glutamate has almost universally been used as the main intra-cellular anion in patch-clamp experiments, with Cl^- not more than 25mM. The results obtained with such cell preparations are in broad agreement with those found in SL-O permeabilised cells in glutamate buffers. In the presence of ATP either GTP-γ-S [Lindau & Nusse, 1987; Neher, 1988a] or Ca^{2+} (at high concentrations [Penner & Neher, 1988]) can stimulate exocytotic release, as measured by an increase in membrane capacitance.

1.8.5.1 GTP-γ-S induced secretion

For GTP-γ-S induced secretion, as long as the nucleotide concentration is between 20-400μM, the extent of the capacitance increase is always about four fold, indicating complete degranulation. The concentration of GTP-γ-S appears only to affect the delay before secretion occurs and the speed of degranulation once initiated [Fernandez et al., 1987]. The lower the concentration of GTP-γ-S, the longer the delay before granule fusion begins [Fernandez et al., 1987]. Such GTP-γ-S dependent secretion is generally seen with an extracellular Ca^{2+} concentration of 2mM, but even when intracellular Ca^{2+} is reduced to 60nM by inclusion of EGTA in the pipette, half the cells still degranulate completely [Neher, 1988a; Lindau & Nusse, 1987]. This has been confirmed in cells loaded with the Ca^{2+} indicator Fura-2, where exocytosis can occur at pCa8 or below [Neher & Penner, 1988; Nusse & Lindau, 1990]. From this it would appear that in whole cell patch experiments no increase in Ca^{2+} concentration above resting levels is required for secretion to occur, and a clear role for a late acting GTP-binding protein is evident. These data would benefit from the

application of modern Ca^{2+} imaging equipment which would allow resolution of the concentration of Ca^{2+} directly below the plasma membrane where fusion actually occurs, rather than a spatially averaged concentration.

1.8.5.2 Ca^{2+} induced secretion

When Ca^{2+} is allowed to fluctuate, or is deliberately raised, the onset of GTP-γ-S triggered secretion is much more rapid [Lindau & Nusse, 1987]. This has been investigated in great detail by simultaneous recordings of $[Ca^{2+}]_i$ (with Fura-2) and exocytosis (by membrane capacitance changes) [Neher, 1988a; Neher & Penner, 1988]. It thus appears that the length of the onset delay and subsequent rate of exocytosis are dependent on the Ca^{2+} concentration. In support of this idea is the finding that when the buffering of Ca^{2+} is weak, then application of antigen, compound 48/80 or introduction of GTP-γ-S into cells results in large and rapid Ca^{2+} transients [Almers & Neher, 1985; Neher, 1988b]. Although the Ca^{2+} transients are clearly not necessary [Neher & Almers, 1986; Neher, 1988b], there is a striking coincidence between the occurrence of a calcium transient and the onset of exocytosis. Occasionally second Ca^{2+} transients occur, and these also lead to an increase in the rate of granule fusion. Thus either Ca^{2+} at high concentrations, or GTP-γ-S can stimulate secretion from mast cells in the presence of ATP. No experiments have been reported in the absence of ATP. When Ca^{2+} and GTP-γ-S are provided secretion occurs after shorter delays and at a faster rate. In this respect Ca^{2+} appears to act as a modulator of GTP-γ-S induced secretion, and presumably therefore modulates the activity of GTP-binding protein late in the exocytotic control pathway.

1.9 Summary

In summary, there is evidence (mainly in the form of modulation or stimulation of secretory responses by guanine nucleotides) for GTP-binding proteins situated late in the regulated exocytotic pathway of many cells. In the majority of systems the only clues as to the identity of the GTP-binding proteins involved come from comparisons of the ability of GTP and its non-hydrolysable analogues to stimulate secretion. Where GTP provides the best stimulus and non-hydrolysable analogues of GTP inhibit, a monomeric GTP-binding protein is implicated. Where non-hydrolysable

analogues (or AlF_4^-) are more potent than GTP, a trimeric GTP-binding protein is implicated. For those cells where specific peptides of GTP-binding proteins can affect secretion, these appear to be of the monomeric family (eg Rab3a), and a role for monomeric GTP-binding proteins at some point in the regulated secretory pathway would seem likely. Identification and isolation of GTP-binding proteins controlling regulated secretion has not yet been achieved.

Chapter 2

Methods

2.1 Calcium Buffers

The calcium buffers were prepared by mixing equimolar solutions (100mM nominal concentration) of EGTA or HEDTA with their calcium salts in proportions calculated with a computer program based on the algorthithm of Perrin and Sayce [Perrin & Sayce, 1967] and using the binding constants listed in Table 2.1. The calcium buffers were diluted in appropriate electrolyte solutions so that the final chelator concentration was 3mM (dose response experiments) or 5mM (kinetic and labelling experiments). Note that since the Ca^{2+} was provided as the Cl^- salt, the contribution of the calcium buffers to the final concentration of Cl^- varies from 0.62mM to 1.24mM or from 0.13mM to 5.78mM as pCa varies in the range 7 - 5 for EGTA or HEDTA buffers respectively. A Ca^{2+} selective electrode was used to ensure that the actual Ca^{2+} concentration in the EGTA and HEDTA buffers was as close as possible. Both sets of buffers produced a linear scale of potential (slope 31mV/decade, at 20°C), with a fixed difference between the two eqivalent Ca^{2+} buffer sets of 0.125 of a pCa unit.

2.2 Mast Cell Preparation

Mast cells were obtained by peritoneal lavage of large (>500g) male Sprague Dawley rats. The mast cells were isolated from contaminating types by centrifugation at 247g through a cushion of Percoll (Pharmacia Ltd, Milton Keynes, Bucks, UK) as previously described [Tatham & Gomperts, 1990], washed twice by resuspension and centrifugation at 462g and finally resuspended in appropriate isoosmotic electrolyte solutions (ie formulated with NaCl, Na.glutamate, glycine or GABA).

2.3 Permeabilisation Buffers

These solutions were initially prepared as 1.36M stocks containing 0.2M PIPES (Sigma chemical co) (nominal 10x final concentration). The pH was adjusted to pH6.8 by further addition of PIPES or NaOH. Before use, these concentrated stocks were diluted with water to concentrations equivalent to 290mOsm measured on a WESCOR model 5000 vapour pressure osmometer (Wescor Inc. Logan, Ut, USA). Bovine serum albumin (Beohringer Mannheim, Fraction V) (1mg ml^{-1}) was added and the pH readjusted to 6.80. When required, Mg^{2+} was added from a 0.3M stock solution prepared by titrating analytical reagent grade MgO with PIPES.

2.4 Metabolic Inhibition

All the experiments in this thesis use metabolically inhibited cells. Before permeabilising the cells they were incubated for 5 minutes together with metabolic inhibitors (2-deoxyglucose (6mM) and antimycin-a (5μM), Sigma chemical Co.) in order to deplete the level of intracellular ATP. This allows the simplest possible conditions under which to perform experiments, and allows the controlled readdition of ATP to study its effects.

2.5 Mg^{2+} Depletion

The specification for ANALAR NaCl (BDH) states an upper limit of 0.002% for magnesium, which would give 6.5μM in a solution of 0.15M NaCl. Under the conditions of calcium buffering (in the kinetics experiments, 5mM EGTA at pH6.8) this would not be significantly reduced. In experiments where severe depletion of Mg^{2+} was required Aristar grade NaCl was used (containing 0.05 ppm contaminating Mg^{2+}). In order to reduce this further 5mM N-hydroxyethylethylene diaminetriacetic acid (HEDTA) was used in place of EGTA. This chelator binds Mg^{2+} with about the same affinity as Ca^{2+}. In addition, all glassware was washed with EDTA in order to eliminate all possible sources of contaminating Mg^{2+}. In order to accurately estimate the contaminating levels of Mg^{2+} (and Ca^{2+}) the concentrations of these ions in the concentrated NaCl and glutamate buffer solutions was measured, with added BSA, in an atomic absorption spectrophotometer (PYE Unicam). Ca^{2+} was measured at 422.7 nm and Mg^{2+} at 285 nm.

Table 2.1: Thermodynamic and stability constants used in the formulation of calcium buffers.

	EGTA	HEDTA
$\Delta H(H_1)$	-5.84	-9
$\Delta S(H_1)$	23.3	14
$\Delta H(H_2)$	-5.76	-2.4
$\Delta S(H_2)$	20.8	16
$\Delta H(Ca)$	-8.38	-6.5
$\Delta S(Ca)$	21.6	15
$\Delta H(Mg)$	5.18	3.4
$\Delta S(Mg)$	41.5	43
log K(H,L)	9.32	9.515
log K(H,HL)	8.72	5.23
log K(H,HHL)	2.76	2.62
log K(H,HHHL)	2.11	
log K(Ca,L)	10.63	7.86
log K(Mg,L)	5.42	7.00
log K(Ca,HL)	5.3	
log K(Mg,HL)	3.4	
log K_{app} (pH6.8, 37°C)		
Ca^{2+}	6.19	5.13
Mg^{2+}	1.6	4.27

Binding constants for 37°C were derived from the values of ΔH and ΔS [Anderegg, 1964; Wright et al., 1965; Moeller & Chu, 1966; Martell & Smith, 1974] listed above: values of pK_a have been converted (by addition of 0.11) to mixed constants for compatibility with the standard buffers used for calibration of glass electrodes [Martell & Smith, 1974].

The results indicated total Mg^{2+} and Ca^{2+} in the 20x ARISTAR NaCl to be unmeasurable ($<1\mu M$). In the 10x Glutamate solution there was $50\mu M$ Ca^{2+} and $8\mu M$ Mg^{2+}. The maximum contaminating levels of ions in each solution when diluted to working concentrations and buffered with 5mM EGTA or HEDTA are set out below in Table 2.2.

Table 2.2: Contaminating levels of Mg^{2+} and Ca^{2+} in permeabilisation buffers (5mM HEDTA or EGTA)

buffer	$[Ca^{2+}]$	$[Mg^{2+}]$
NaCl, EGTA	<0.01nM (pCa11)	<46nM
NaCl, HEDTA	<0.08nM (pCa12)	<0.53nM
Glutamate, EGTA	0.94nM (pCa9)	460nM
Glutamate, HEDTA	7.38nM (pCa8)	8.5nM

2.6 Standard Secretion Experiment Conditions

During the three years in which the work in this thesis has been carried out, the method for analysis of mast cell secretion has changed considerably (part of my work has been to develop these methods). Initially experiments were performed in plastic LP3 (2ml) tubes, involving manual transfer of tubes between racks and centrifuges. Now 96 well microtitre plates are used, considerably speeding up handling and reducing costs. The experiments described in Chapter 3 were exclusively performed using LP3 tubes. Those in Chapter 4 were performed entirely using the microtitre plate method. The method for setting up the two different formats is basically identical.

Purified, metabolically inhibited cells were treated with streptolysin-O (SL-O, 0.4iu ml^{-1}, permeabilising agent, Wellcome Diagnostics), 3mM Ca^{2+} buffer (EGTA or HEDTA as appropriate) to regulate Ca^{2+} in the range pCa7 to pCa5, and nucleotides as indicated (all nucleotides supplied by Boehringer Mannheim). Generally three 30 μl additions were made to each well or tube, one containing Ca^{2+} buffer and SL-O, one containing nucleotides and one containing the cells, giving a total volume of 90μl. After addition of the cells, the tubes or plates were incubated for 10 minutes at 37°C and then quenched by addition of 0.5ml (LP3 tubes) or 150μl (microtitre plate) of ice cold 0.15M NaCl (containing 10mM K.phosphate, pH7). The cells were sedimented by centrifugation at 462g and the supernatants were assayed for released N-acetyl-ß-D-glucosaminidase (hexosaminidase) as explained below.

In some of the experiments described here the strategy has been to permeabilise the cells under resting conditions and then apply the stimulus at set times afterwards (the *permeabilisation interval*). Cells were treated

with streptolysin-O and 0.2mM EGTA to maintain pCa7 and other additions as described. They were then transferred after the permeabilisation interval to tubes containing 3mM calcium buffer (to regulate Ca^{2+} in the range pCa7 - pCa5) and GTP-γ-S as indicated, and incubated for a further 10 minutes and assayed for release of hexosaminidase.

2.7 Kinetic Experiments

The change-over from plastic tubes to microtitre plates has also altered the methodology for kinetic experiments. For the experiments described in Chapters 3 and 5, plastic LP3 tubes were used. This involves alternate sampling (with a single pipette) of 6 reaction tubes into a specially arranged array of tubes containing quench solution (as previously described [Tatham & Gomperts, 1989]). This necessarily reduces the resolution of the experiment (hence the absence of time points at the beginning of some of the data sets). Chapter 4 contains data from experiments carried out using microtitre plates, which allows greater resolution of timecourses, as simultaneous sampling of 8 wells (with an 8-channel multipipette) is possible. A Teflon block drilled with eight wells (volume 3ml) spaced identically with the wells of a microtitreplate was used as the reaction vessel to sample from. Apart from these differences the basic set up of the experiments is identical.

2.7.1 NaCl based buffers

Cells were permeabilised with SL-O (0.4 iu/ml) and loaded with either guanine nucleotide or Ca^{2+} (as indicated). For those experiments in which guanine nucleotide was added first, Ca^{2+} was maintained at pCa7 with 0.2mM Ca^{2+} buffer (EGTA or HEDTA as appropriate). After one minute to allow entry of the guanine nucleotide or Ca^{2+}, the cells were transferred to tubes containing the other effector (guanine nucleotide or 5mM Ca^{2+} buffer as indicated) and other additions as indicated. Timed samples (70μl) were then removed (using a metronome as a timer) and quenched in 1.5ml ice cold phosphate buffered saline (pH 7). Cells were then sedimented at 462 g for 5min, and processed for released hexosaminidase as below. For further details see Tatham & Gomperts, 1989.

2.7.2 Glutamate based buffers

SL-O permeabilised cells lose secretory competence more rapidly in glutamate than in chloride based buffers, even in the presence of ATP (data not shown). Because of this, cells cannot be left open to equilibrate with one effector for 1 minute, and a different strategy was developed for kinetic experiments in glutamate buffers. Reactions were triggered by addition of SL-O (0.4iu/ml) to cells in the presence of Ca^{2+}, Mg^{2+} and guanine nucleotides as indicated. Samples (100μL) were withdrawn, using an 8-channel multipipette at timed intervals (again using a metronome) and quenched into 200μL of ice cold phosphate buffered saline (pH7) contained in a 96-well microtitreplate. Cells were then sedimented by centrifugation at 462g for 5 minutes and processed for released hexosaminidase as below.

2.8 Hexosaminidase Determination

During the work described in this thesis, the previous methods for incubations and analysis of secreted hexosaminidase (which employed plastic test tubes) have been adapted to the use of 96-well microtitre plates. The methods have been described in detail elsewhere [Tatham & Gomperts, 1990; Gomperts & Tatham, 1992].

2.8.1 Plastic test-tube method

Some of the experiments described in Chapters 3 and 5 used this method of analysis.

50μl of supernatant were sampled into a plastic test tube and incubated with 50μl substrate (1mM 4-methylumbelliferyl N-acetyl-ß-D-glucosaminide dissolved in 0.2M citrate buffer, pH4.5) for 1 hour at 37°C. 0% and 100% calibration samples were prepared from reagent blanks and cells lysed with 0.1% Triton X100 respectively. The tubes were then quenched with 1.5ml of 0.1M NaBicarbonate / 0.1M NaHydrogencarbonate solution. The fluoresence was then measured by hand on a Perkin-Elmer LS-5 (356 - 448nm), and recorded on a chart recorder for further evaluation.

2.8.2 96-well microtitre plate method

All the experiments in Chapter 4 and some in chapters 3 and 5 used the microtitre plate method of hexosaminidase analysis.

After centrifugation to pellet the cells, 50μl samples of supernatant were transferred to corresponding wells in black plastic 96-well microtitre plates. To establish a calibration scale, further sets of samples from reagent blanks and from cells lysed with Triton X100 (0.1%) were prepared. The reactions were initiated by addition of 50μl of substrate, and the plates were maintained at 37°C for about 90 minutes. Reactions were terminated by addition of 150μl of Tris (1M) and the fluorescence (365 - 450nm) was measured using an automatic fluorescence plate reader (Fluoroskan: Flow Laboratories (UK)) equipped with facilities for direct transfer of data for processing on a computer spread sheet.

2.9 Expression of Concentrations

A note on the expression of concentrations: in this work I have used serial dilutions to prepare logarhythmically graded concentrations of nucleotides which are expressed in the form of the exponent (eg $10^{-5.5}$M etc). Single concentrations, unrelated to the graded series are expressed conventionally in molar (or μmolar) units. pCa indicates $-\log_{10}[Ca^{2+}]$.

With the exception of kinetic experiments, all data points represent the means of duplicate determinations and all the experiments have been repeated on at least five separate occasions. Representative experiments are shown in each figure.

2.10 Periodate labelling method

Mast cells or rat hepatocytes were metabolically inhibited and treated with 2mM Diisopropyl Fluorophosphate to inhibit serine proteases. The experiment was carried out in a 96 well plate, with approximately 250,000 cells per well. The cells were permeabilised with SL-O (0.4iu/ml) in the presence of α-^{32}P-GTP, 5mM EGTA or HEDTA buffer and other nucleotides as indicated. Cells were incubated at 37°C in a specially constructed incubation chamber for 1-5 minutes and then spun down at 462g at 4°C for 1 minute. The supernatant was then removed and 100μL of 2mM Sodium Periodate in BSA free NaCl or NaGlutamate based buffer was

added and the cells incubated for a further 1 minute at 37°C. After this 5μl of 40mM Sodium Cyanoborohydride was added and the incubation continued for another 1 minute. At this point the microtitre plate was removed from the incubator and 5μl of 50mM sodium borohydride added. The cell suspension was then removed from the microtitre plate and diluted into 1ml of ice cold pH7 PIPES buffered saline in eppendorf tubes. These were then spun at 19,000g for 10 mins to pellet the cells. The supernatant was removed and 25μL of sample buffer added (containing PMSF and protease inhibitors). The samples were sonicated for 5 seconds to destroy any DNA, boiled for 5 mins to denature the proteins and finally spun at 9000g for 1 minute to sediment any debris.

2.11 SDS-PAG Electrophoresis

Biorad minigel apparatus was used to run poly-acrylamide gels, according to the method of Laemmli, 1970. The stacking gel was 3.5% acrylamide and the main gel 12.5% acrylamide. The samples and molecular weight standards (Biorad Low Molecular weight range) were loaded with a microsyringe. The running buffer contained 192mM Glycine, 25mM TRIS and 0.1% SDS. The gels were run with a Pharmacia Multidrive XL programmed to ramp the voltage from 50-150 V over 20 minutes (to move the samples through the stacking gel) and then to run at a constant voltage of 150V for 1 hour for the main gel.

2.12 Western blotting

A Pharmacia Novablot apparatus was used for western blotting. Transfer buffer contained 39mM Glycine, 48mM TRIS, 0.0375% SDS and 10% Methanol. The stacking gels were removed from the SDS-PA Gels and the orientation marked on them. The gels were then washed in transfer buffer for 5 minutes. 8 transfer buffer saturated pieces of Whatman 3mm chromatography paper were then layered on the anode and rolled to exclude air bubbles. A methanol wetted sheet of Immobilon P (Millopore) was used as the transfer membrane, and was placed on top of the paper. The washed gels were carefully placed on the Immobilon P, and covered with 1 sheet of saturated paper. This was again rolled to exclude air. Finally 7 more saturated pieces of paper were stacked on top and rolled once more. The cathode was then placed in position and the apparatus run with a Pharmacia Multidrive XL at 100mA constant current per gel

($2mAcm^{-2}$) for 1 hour. Both the Immobilon P and the gels were then stained for protein content with Coomasie Brilliant Blue (R250) to check protein transfer. All but high molecular weight proteins (<190 KDa) were transferred in 1 hour. The Immobilon was then dried with Bastien's fan heater until I couldn't hold it.

2.13 Autoradiography

Direct autoradiography was performed using Hyperfilm ß-Max (Amersham) in cassettes with intensifying screens. Exposures were generally overnight, and developing was done by hand using Kodak chemicals.

2.14 Densitometry

Densitometry was carried out using a Hoeffer 300 transmittance / reflectance densitometer, set for transmittance reading. The densitometer was zeroed for the autoradiograph being scanned and then scanned at 6.5 $cmmin^{-1}$. The analogue output from the densitometer was converted into digital with a Hoeffer A/D converter, and the data stored on a Macintosh SE30 computer. Hoeffer software was then used to process the data, compare the various scans and output the final illustrations to a laser printer.

Chapter 3

Mg^{2+} and Nucleotides as Modulators of the Exocytotic Reaction

3.1 Introduction

The presence of ATP is not required for secretion triggered by Ca^{2+} and GTP-γ-S in either NaCl or zwitterionic solutions [Howell et al., 1987; Koopmann & Jackson, 1990; Churcher & Gomperts, 1990]. This implies that a phosphorylation reaction does not comprise an essential step in the terminal stages of exocytosis from mast cells. When ATP is provided to SL-O permeabilised mast cells in NaCl based buffer at least four separate effects can be discerned:

1. In the presence of ATP the sensitivity to both Ca^{2+} and guanine nucleotide are enhanced [Howell et al., 1987; Gomperts et al., 1987].

2. Responsiveness to delayed stimulation is maintained following permeabilisation [Howell et al., 1989].

3. ATP restores responsiveness to cells rendered refractory to stimulation by prior permeabilisation [Howell et al., 1989; Churcher et al., 1990b; Gomperts et al., 1990].

4. The onset of exocytosis is retarded [Gomperts & Tatham, 1988; Tatham & Gomperts, 1989; Churcher et al., 1990a].

In all these cases ATP must be regarded as a modulator, not an effector of exocytosis. The role of ATP in secretory systems is generally assumed to be that of a phosphoryl donor in a kinase mediated protein phosphorylation. There are however two other alternative roles ATP might play:-

1. As a phosphoryl donor in a nucleotide transphosphorylation, of particular interest would be if GDP was phosphorylated to GTP which could activate GTP-binding proteins.

2. As an allosteric regulator of protein function, which would not involve hydrolysis of the ATP. eg ATP^{4-} acts as an allosteric inhibitor of K^+ channels in islet cells [Ashcroft & Kakei, 1989].

Based on the effects of various pharmacological treatments (eg with phorbol esters [Howell et al., 1989] and stearylmethylglycerol [Howell et al., 1989], and in permeabilised cells with a protein kinase C-pseudo-substrate peptide (19-31) inhibitor [Churcher et al., 1990b] and neomycin [Howell et al., 1989]) the ability of ATP to enhance effector sensitivity and to restore sensitivity to cells rendered refractory by previous permeabilisation appears to be via phosphorylations mediated by protein kinase C.

Many non-hydrolysable analogues of GTP [Howell et al., 1987; Churcher et al., 1990a] and also other nucleoside triphosphates which can act as ligands for GTP-binding proteins (eg ITP and XTP [Bilezikian & Aurbach, 1974]) are capable of synergising with Ca^{2+} to activate the secretory response in the absence of ATP [Howell et al., 1987]. UTP and CTP are unable to induce secretion. Unlike the non-hydrolysable analogues of GTP (ie GTP-γ-S, GppNHp and $GppCH_2p$), there is the possibility that the activating nucleoside triphosphates (ie ITP and XTP) may additionally act as donors in phosphorylation reactions catalysed by protein kinases. The purpose of the experiments described here was to explore the effects of a panel of nucleoside triphosphates (ATP, ATP-γ-S, AppNHp, ITP, XTP, GTP, UTP and CTP), and of Mg^{2+} as effectors and modulators of exocytosis in mast cells.

It is shown that three of the four modulatory responses of ATP are mediated by phosphorylation, probably via Protein Kinase C. The ability of GTP, XTP and ITP to synergise with Ca^{2+} in the stimulation of secretion is modulated by Mg^{2+}, whereas the response to GTP-γ-S is not. The enhancement in sensitivity for the two effectors is dependent on the presence of Mg^{2+} and is supported by non-adenine nucleotides. These nucleotides are not able to act as phosphoryl donors for protein kinases, but are substrates for nucleoside diphosphate kinase in the generation of ATP from ADP. The ability of ATP to induce onset delays is unique in that

it occurs in the absence of Mg^{2+}, and is therefore unlikely to involve a phosphorylation step.

3.2 Results

3.2.1 Non-guanine nucleotides can stimulate secretion

It has previously been shown that ITP, GTP and XTP are capable of synergising with Ca^{2+} in the stimulation of secretion from permeabilised mast cells [Howell et al., 1987]. When Mg^{2+} is removed, as in Figures 3.1 and 3.2, the sensitivity of the secretory response to GTP-γ-S is clearly unchanged. In contrast, secretion in response to GTP shows a pronounced increase in both sensitivity and maximum magnitude of secretion. This is likely to reflect the Mg^{2+} dependence of GTP hydrolysis on a GTP-binding protein, possibly G_E. Under the same conditions the ability of XTP to stimulate secretion is abolished and the sensitivity to ITP is reduced. The effects of Mg^{2+} on these two nucleotides could be explained in a number of ways. Mg^{2+} is an important co-factor in nucleotide binding to GTP-binding proteins, and the lack of Mg^{2+} could affect the binding of these nucleotides to a GTP-binding protein (although this would clearly differ from the effect seen with GTP). Alternatively, because these are unmodified nucleoside triphosphates there are also two other possibilities. One is that they could act as phosphoryl donors in reactions catalysed by protein kinases and the other is that they could be substrates for nucleoside diphosphate kinase to generate ATP from ADP. Both of these possibilites would also be Mg^{2+} dependent processes.

If either of these were the case, they might be expected to modify the secretory response in a manner similar to ATP. Figures 3.3 and 3.4 illustrate the effect of ITP, at various concentrations, on the dependence on Ca^{2+} (figure 3.3) and GTP-γ-S (figure 3.4) of secretion from mast cells permeabilised with streptolysin-O. As the concentration of ITP was elevated in the range 10^{-5} - $10^{-4.25}$M there was a systematic enhancement in the sensitivity to both Ca^{2+} and GTP-γ-S while there was little effect on the maximal extent of secretion. At concentrations higher than 10^{-4}M, ITP itself synergised with Ca^{2+} and induced secretion in the absence of GTP-γ-S (figure 3.4).

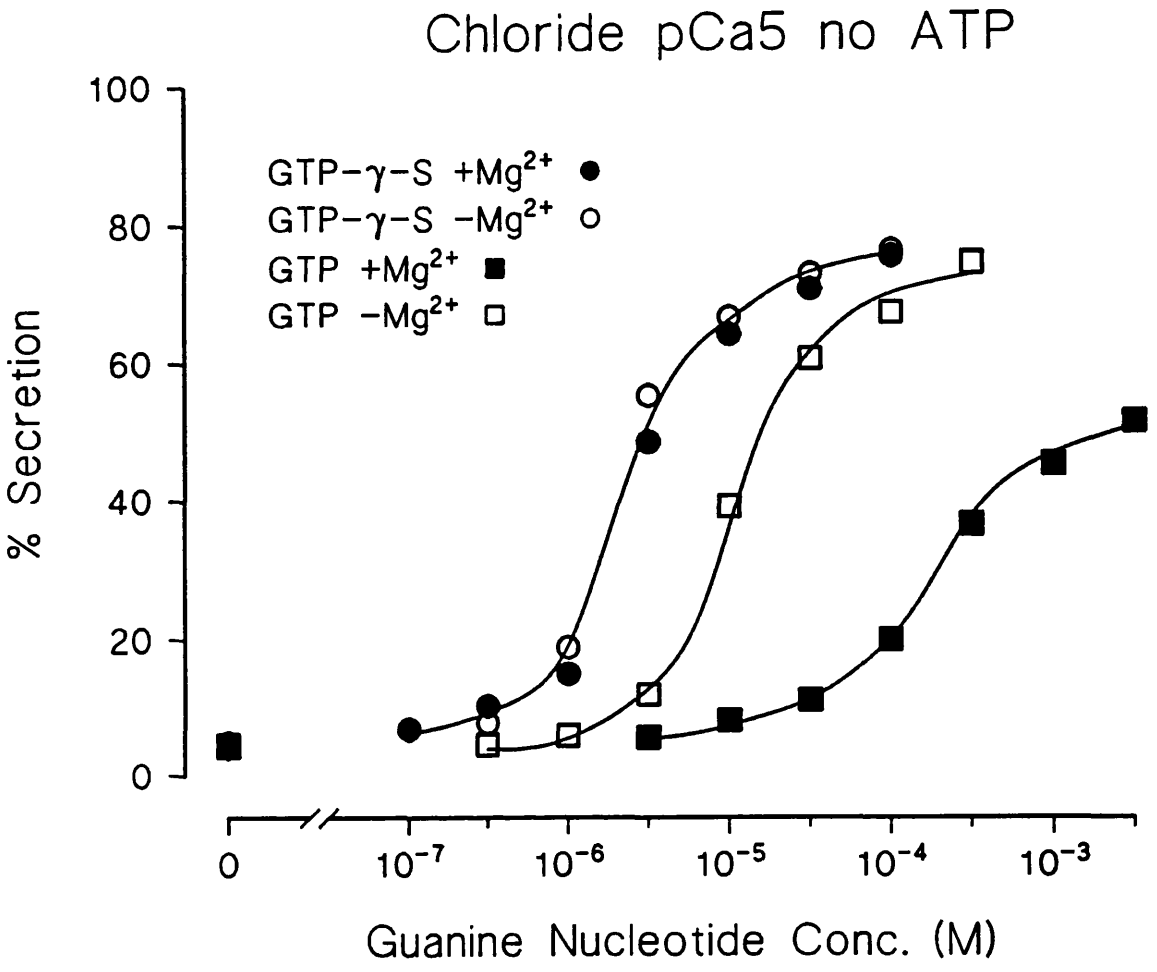


Figure 3.1. The effect of removal of Mg^{2+} on secretion triggered by Ca^{2+} and either GTP or GTP-γ-S from permeabilised mast cells.

Mast cells, suspended in buffered NaCl and treated with metabolic inhibitors, were permeabilised with SL-O in the presence of Ca^{2+} (buffered with 3mM EGTA at pCa5) and either GTP (■,□) or GTP-γ-S (●,○) at a range of concentrations as indicated. Filled symbols indicate EGTA buffering and the presence of Mg^{2+} (2mM), open symbols HEDTA buffering and the absence of Mg^{2+} ($<10^{-9}$M).

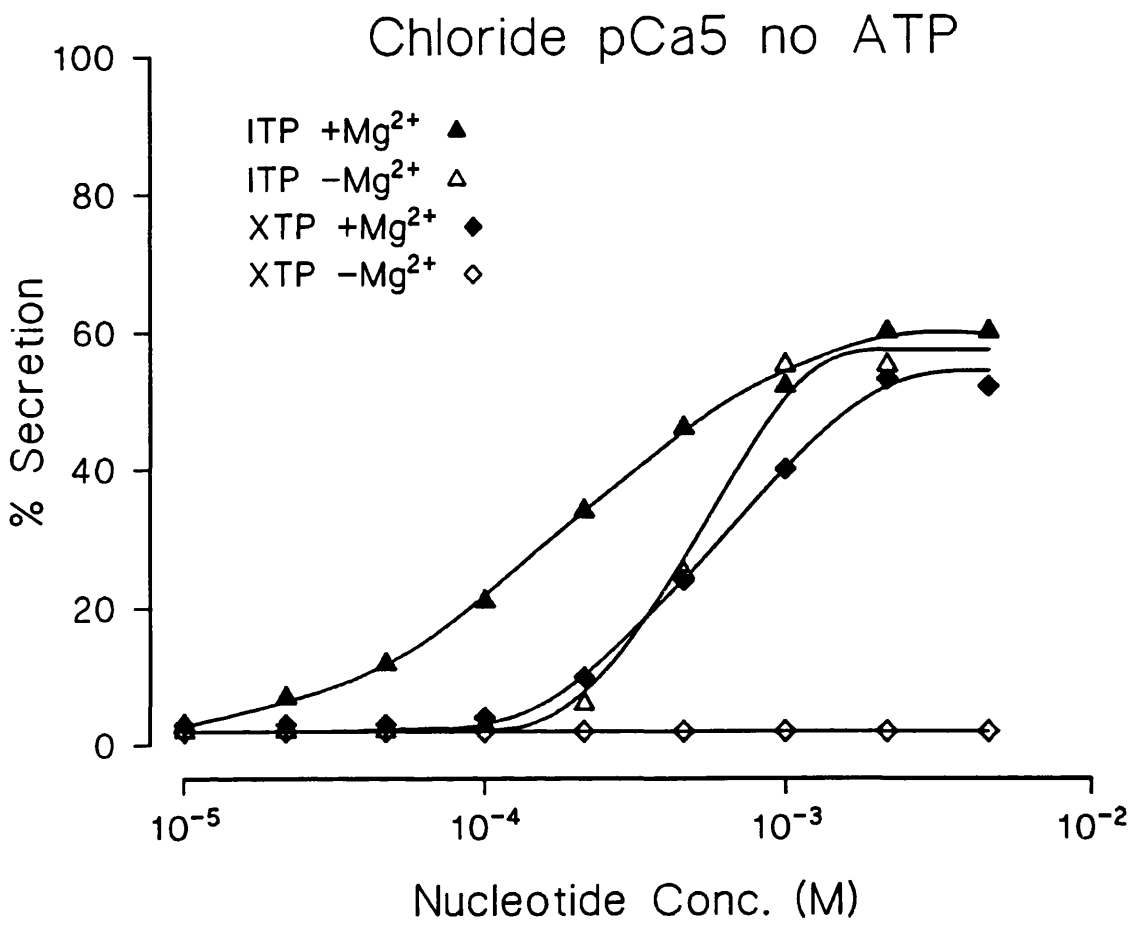


Figure 3.2. The effect of removal of Mg^{2+} on secretion triggered by Ca^{2+} and either XTP or ITP from permeabilised mast cells.

Mast cells, suspended in buffered NaCl and treated with metabolic inhibitors, were permeabilised with SL-O in the presence of Ca^{2+} (buffered with 3mM EGTA at pCa5) and either ITP (▲,△) or XTP (◆,◇) at a range of concentrations as indicated. Filled symbols indicate EGTA buffering and the presence of Mg^{2+} (2mM), open symbols HEDTA buffering and the absence of Mg^{2+} ($<10^{-9}$M).

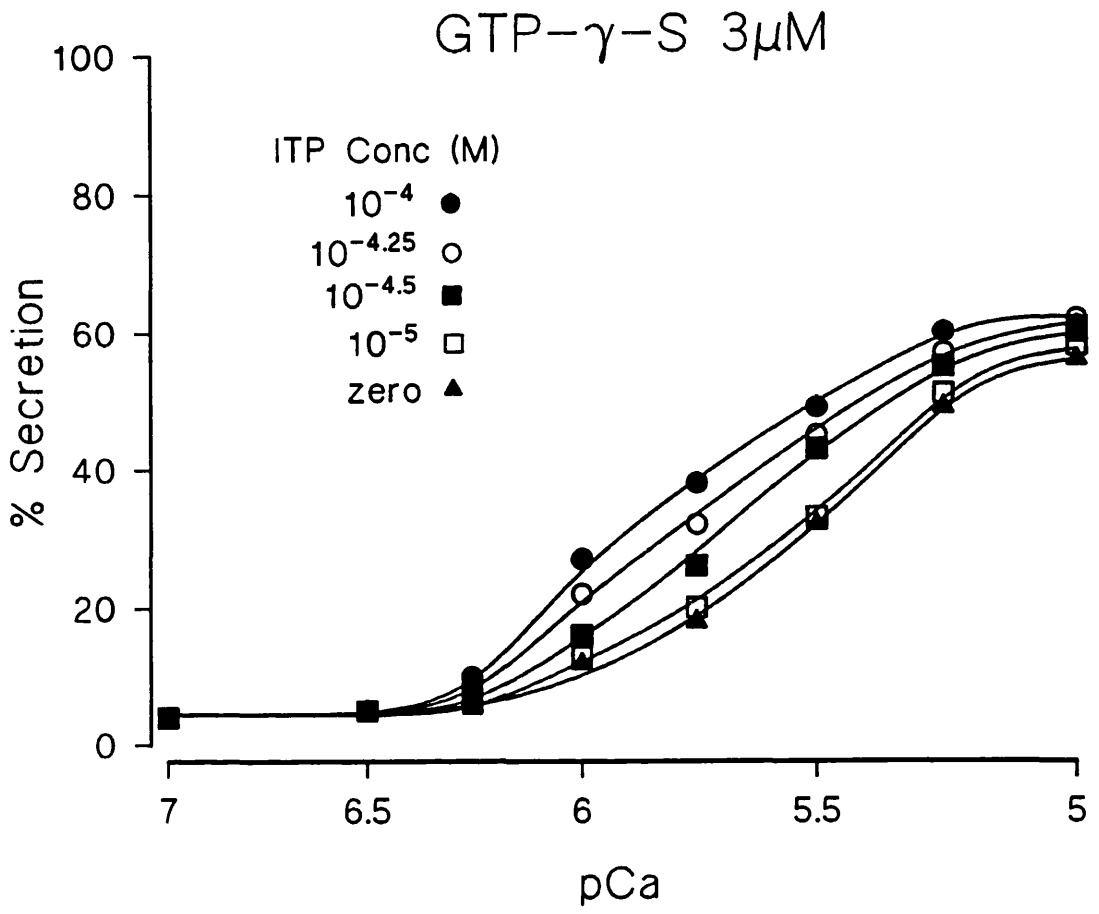


Figure 3.3. The effect of a range of concentrations of ITP on the dependence of exocytosis from permeabilised mast cells on Ca^{2+}.

Mast cells, suspended in buffered NaCl and treated with metabolic inhibitors, were permeabilised with SL-O in the presence of Ca^{2+} (buffered with 3mM EGTA as indicated) and 3μM GTP-γ-S, and in presence of various concentrations of ITP: ▲, zero; □, 10^{-5}M; ■, $10^{-4.5}$M; ○, $10^{-4.25}$M; ●, 10^{-4}M.

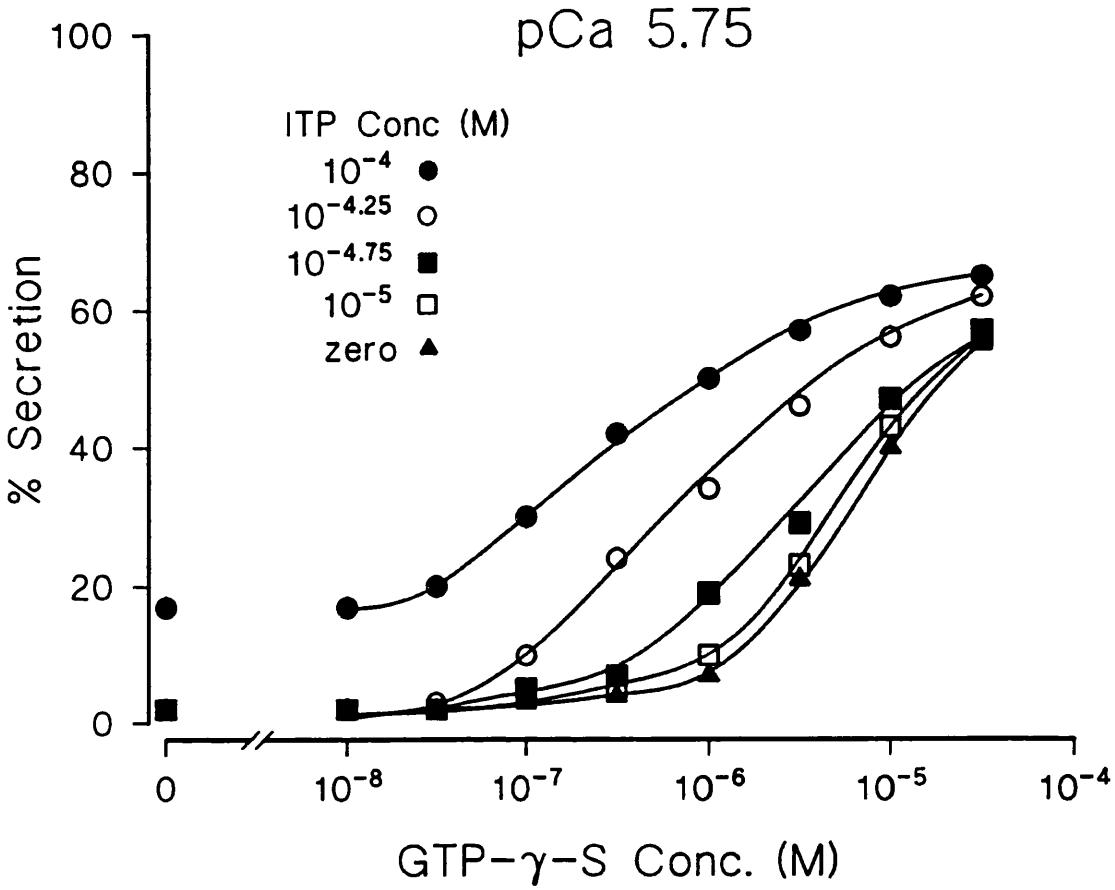


Figure 3.4. The effect of a range of concentrations of ITP on the dependence of exocytosis from permeabilised mast cells on GTP-γ-S.

Mast cells, suspended in buffered NaCl and treated with metabolic inhibitors, were permeabilised with SL-O in the presence of Ca^{2+} (pCa5.75, buffered with 3mM EGTA) and GTP-γ-S as indicated, and in presence of various concentrations of ITP: ▲, zero; □, 10^{-5}M; ■, $10^{-4.75}$M; ○, $10^{-4.25}$M; ●, 10^{-4}M.

In a similar way, XTP also enhances the sensitivity to Ca^{2+} and GTP-γ-S and at higher concentrations (250 μM) it also synergises with Ca^{2+} to induce secretion in the absence of GTP-γ-S (results not shown). In subsequent experiments, in order to ensure that secretion was not stimulated by those nucleotides capable of acting as ligands for GTP-binding proteins (ITP, XTP and GTP), these were always used at the sub-stimulatory concentrations established by these initial experiments (see Table 3.1).

Table 3.1: The working concentrations of the panel of Nucleotides used to modulate exocytosis from permeabilised mast cells.

Nucleotide	ATP or ATP-γ-S	ITP	UTP	CTP	XTP	GTP	AppNHp
Minimal Stimulatory Conc.	n/e	100μM	n/e	n/e	250μM	100μM	n/e
Conc. Used	100μM & 1mM	50μM	1mM	1mM	200μM	75μM	1mM

Minimal Stimulatory Concentration is the lowest concentration at which the nucleotide is able to stimulate secretion with Ca^{2+} in the absence of ATP. n/e indicates no effect at 1mM.

3.2.2 Various nucleotides can substitute for ATP in enhancing effector sensitivity

Figure 3.5 illustrates and summarises the effect of a range of nucleotides and non-hydrolysable analogues of ATP on the sensitivity to GTP-γ-S. ITP (50 μM), XTP (200 μM), UTP (1 mM), CTP (1 mM), ATP (100 μM) and ATP-γ-S (1 mM) all enhance the sensitivity to GTP-γ-S to approximately the same extent. In contrast, GTP (75 μM) and AppNHp (1 mM) do not enhance the sensitivity to GTP-γ-S. GTP cannot be tested at concentrations in excess of 100 μM as it then synergises with Ca^{2+} to induce secretion in a manner similar to ITP and XTP (see Table 3.1). Although Mg^{2+} is not an obligatory requirement for secretion, the enhancement in effector sensitivity by nucleoside triphosphates only occurred when Mg^{2+} was also provided.

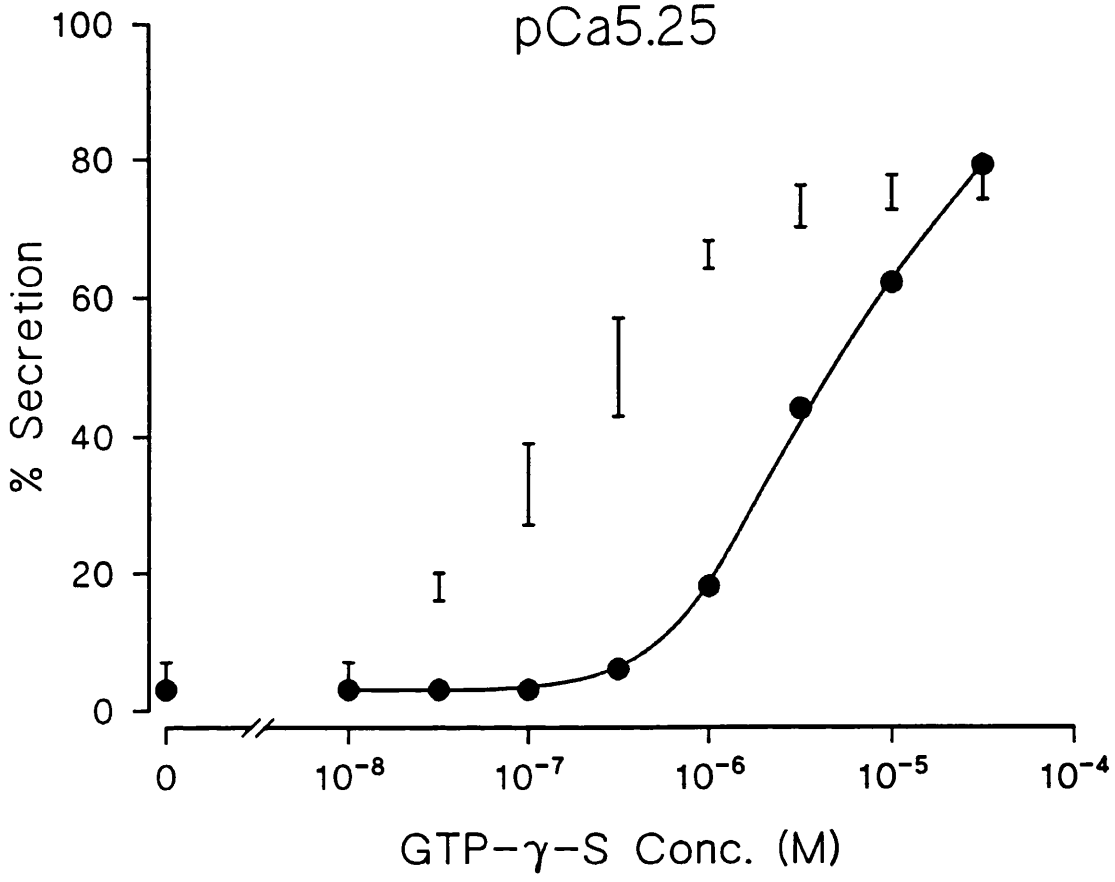


Figure 3.5. The effect of various nucleoside triphosphates and non-hydrolysable analogues of ATP on the sensitivity for GTP-γ-S in the exocytotic reaction of permeabilised mast cells.

Mast cells suspended in buffered NaCl and treated with metabolic inhibitors, were permeabilised with SL-O in the presence of 3mM EGTA buffer (to maintain pCa5.25) and GTP-γ-S as indicated, and in the absence or presence of ATP, AppNHp, ATP-γ-S, ITP, XTP, GTP, UTP or CTP, at the concentrations listed in table 3.1. Data points (filled circles) indicate dependence on GTP-γ-S in the absence of added nucleotide. Addition of AppNHp or GTP (75μM) produced results indistinguishable from these. The zone indicated by the bars indicates the range of shifts in the sensitivity for GTP-γ-S caused by all the other nucleotides tested.

Previous experiments [Cockcroft et al., 1987; Howell et al., 1989] have indicated that the enhancement in effector sensitivity caused by ATP is probably mediated by a phosphorylation reaction catalysed by protein kinase C. The experiment illustrated in figure 3.6 shows that the enhancement due to either ATP, ITP, XTP or UTP is abolished when the cells are treated with AMG.C_{16}, a specific inhibitor of protein kinase C at the concentrations used [van Blitterswijk et al., 1987; Howell et al., 1989; Kramer et al., 1989]. Note that ATP and UTP can affect the dependence on the guanine nucleotide only through an enhancement of its sensitivity (see figure 3.5) while XTP and ITP are additionally capable of synergising with Ca^{2+} in the induction of exocytosis.

The question arises here of whether the kinase catalysing phosphorylation is truly non-specific towards nucleotides as suggested by these results, or whether ATP is actually the donor following its regeneration in the permeabilised cells by transphosphorylation between non-adenine nucleoside triphosphates and the residual ADP. I addressed this question in the experiment illustrated in figure 3.7. Here I tested the ability of UTP to enhance effector affinity when applied to the cells 20 seconds after permeabilisation, by which time cellular ADP can fairly be presumed to have become infinitely diluted (based on diffusion calculations nucleotides will leave SL-O permeabilised cells in less than 10 seconds [Lindau & Gomperts, 1991]. Under these conditions, UTP is incapable of enhancing the sensitivity for GTP-γ-S. However, when supplemented with ADP, UTP is once again capable of enhancing effector sensitivity. When CDP is substituted for ADP, UTP can no longer enhance the sensitivity for GTP-γ-S. This suggests that the combination UTP-plus-ADP enhances sensitivity through the formation of ATP which is then the phosphoryl donor in a kinase catalysed reaction.

3.2.3 ATP can restore and maintain responsiveness in permeabilised cells

When the dual stimulus (Ca^{2+}-plus-GTP-γ-S) is applied to the mast cells at times after permeabilisation (a permeabilistion interval) the responsiveness of the exocytotic mechanism declines to zero within 5 minutes [Howell et al., 1989; Churcher et al., 1990b]. However, if ATP is provided at the

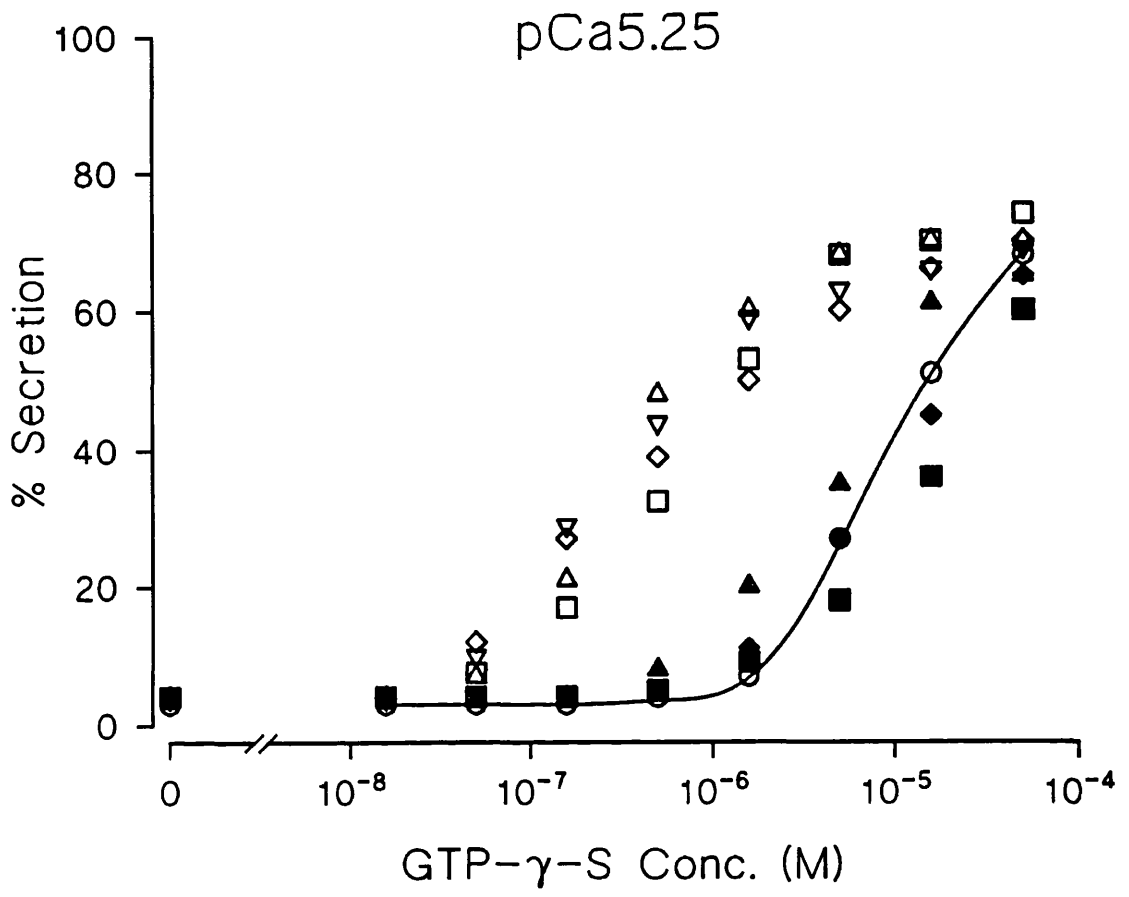


Figure 3.6. Protein kinase C inhibitor AMG.C_{16} reverses the shift in sensitivity induced by nucleoside triphosphates.

Mast cells suspended in buffered NaCl were incubated with metabolic inhibitors for 4 minutes, and then for a further minute with 30μM AMG.C_{16} (closed symbols), or DMSO (open symbols). They were then permeabilised with SL-O in the presence of 3mM EGTA buffer (to maintain pCa5.25) and GTP-γ-S as indicated, and in the presence of either ATP (□), ITP (◇), XTP (▽) , UTP (△) or control (○) at the concentrations listed in table 3.1. The continuous line joins the points representing secretion due to GTP-γ-S in the absence of nucleoside triphosphate.

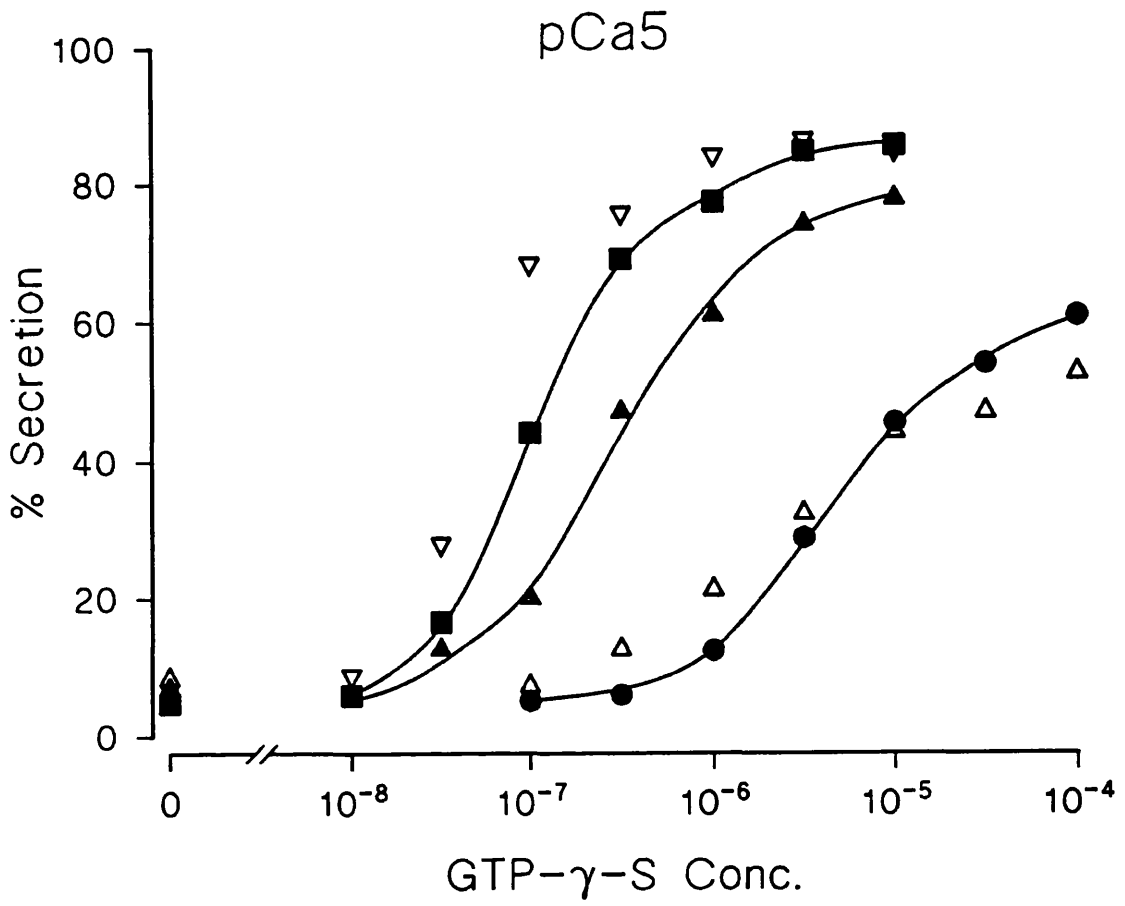


Figure 3.7. 20 seconds prepermeabilisation abrogates the ability of UTP to enhance sensitivity to GTP-γ-S.

Mast cells were treated with metabolic inhibitors together with 0.2mM calcium buffer, pCa7 for 5 minutes. They were then permeabilised by SL-O and stimulated either simultaneously, or after 20 seconds, by addition of GTP-γ-S (at concentrations indicated), 3mM calcium buffer pCa5, and nucleotides as indicated. No additional nucleotide, with or without prepermeabilisation (●): these data were superimposable; ATP, 1mM, with or without prepermeabilisation (■): these data were superimposable; UTP, 0.5mM, no prepermeabilisation (▲), 20 seconds prepermeabilisation (△); UTP plus ADP (250μM), 20 seconds prepermeabilistion (▽). In this experiment I also tested the effect of adding ATP + ADP and UTP + CDP after 20 seconds prepermeabilisation; in neither case did the supplementary nucleoside diphosphate cause any further change in the sensitivity to GTP-γ-S (these data have been omitted for clarity).

time of permeabilisation then maximum responsiveness (ie to Ca^{2+} and GTP-γ-S, both at 10^{-5}M) is fully maintained for 3 minutes and then declines gradually over 20 minutes.

The experiment illustrated in Figure 3.8 was designed to test the ability of various nucleotides to maintain responsiveness of permeabilised cells to late stimulation. In this experiment I measured the dependence of secretion on Ca^{2+} (pCa5.25) and a range of concentrations of GTP-γ-S, added either at the time of permeabilisation or after an interval of 3 minutes. As shown earlier (Howell et al., 1987, and this chapter: figure 3.5), in cells stimulated at the time of permeabilisation, ATP enhances the sensitivity to GTP-γ-S (almost 100 fold in the experiment illustrated). Following a 3 minute permeabilisation interval, the maximum response was fully maintained in the presence of ATP and there was only a small decrease in sensitivity to the guanine nucleotide. In contrast, responsiveness to all concentrations of GTP-γ-S was lost if the cells were permeabilised in the absence of either ATP or Mg^{2+}. None of the other nucleotides (including those which are capable of enhancing the sensitivity to effectors: see figure 3.5) were able to substitute for Mg.ATP in maintaining responsiveness following prior permeabilisation.

It has previously been shown that the responsiveness of cells rendered refractory by permeabilisation can be restored by addition of ATP together with Ca^{2+} and GTP-γ-S [Howell et al., 1989; Churcher et al., 1990b]. Figure 3.9 illustrates the dependence of secretion on the concentration of GTP-γ-S for cells stimulated 2, 3 and 4 minutes after permeabilisation by addition of Ca^{2+} (pCa5.25) and a range of GTP-γ-S, together with the panel of nucleotides listed in Table 3.1. Of these, only ATP is capable of restoring exocytotic responsiveness and this is dependent on the presence of Mg^{2+}. Note that following recovery there is a substantial component of secretion which is now manifest in the absence of guanine nucleotide: it has previously been demonstrated that this is dependent on Ca^{2+} (in the range pCa6 - 5) and on ATP [Churcher et al., 1990b]. The possibility remains that this is also due to a transphosphorylation reaction, regenerating GTP from GDP (presumably bound, considering the time interval between permeabilisation and additon of effectors).

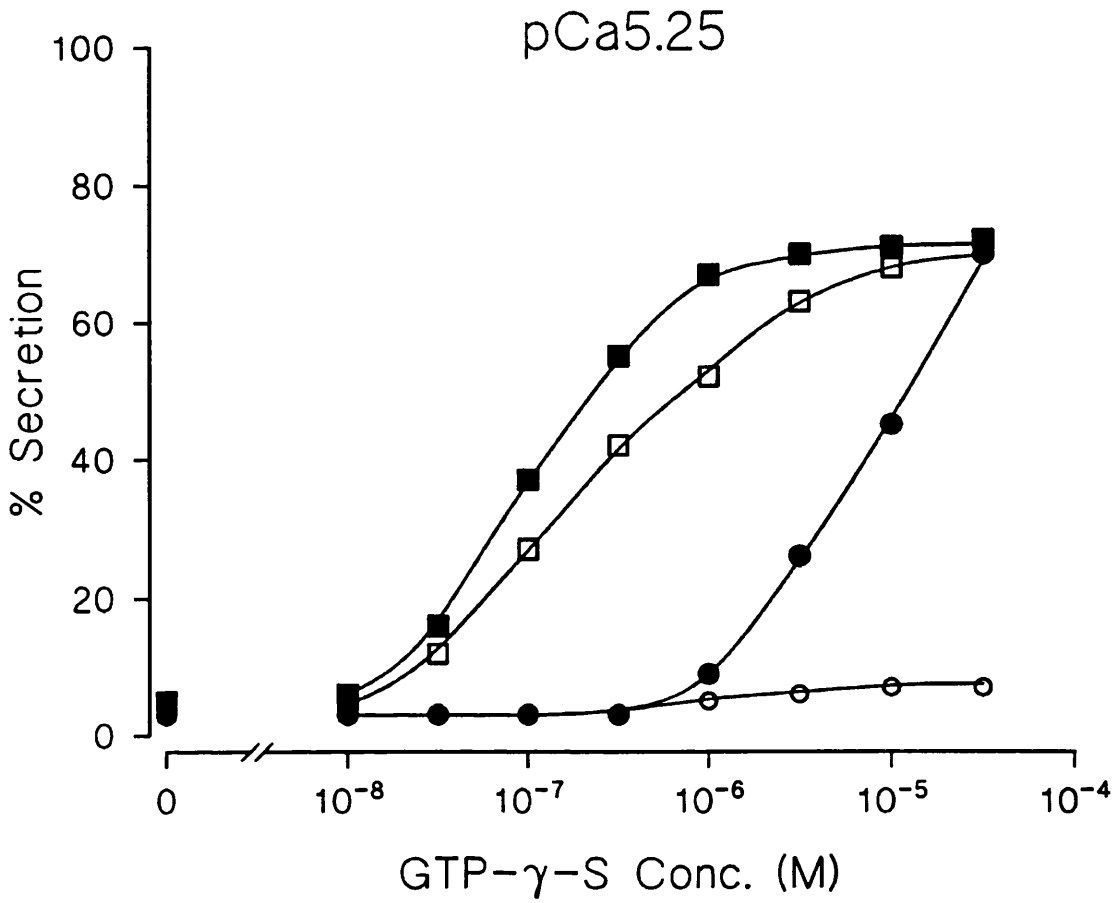


Figure 3.8. The ability of various nucleotides to maintain responsiveness of permeabilised cells to late stimulation by GTP-γ-S.

Mast cells in NaCl solution were treated with metabolic inhibitors and then permeabilised with SL-O in the presence of nucleotides (see table 3.1). They were either stimulated to secrete by immediate transfer to 3mM EGTA buffer (pCa5.25) together with GTP-γ-S (closed symbols: ■, ATP; ●, AppNHp, GTP or none). Alternatively, the permeabilised cells were incubated for 3 minutes before stimulation (open symbols: □, ATP; ○, all other nucleotides (see table 3.1).

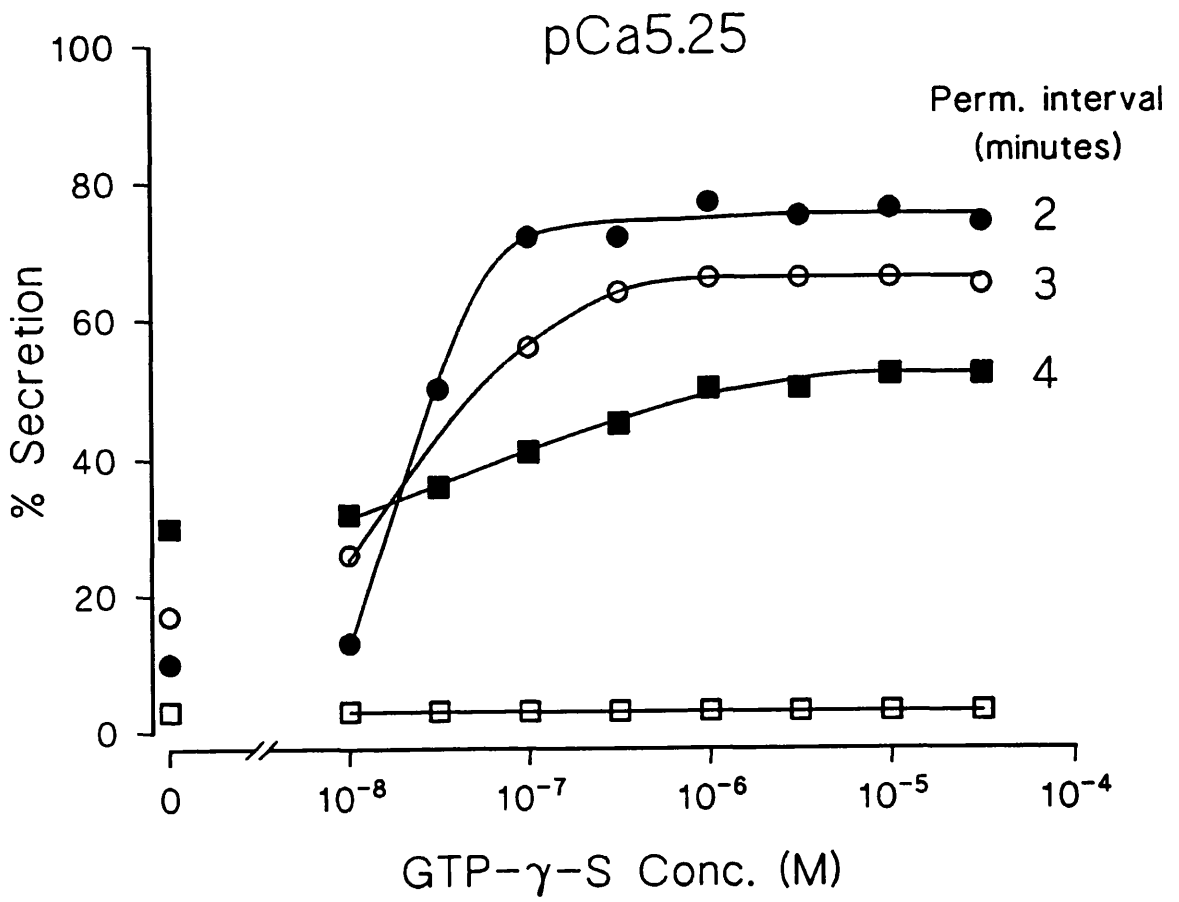


Figure 3.9. The effect of various nucleotides on the GTP-γ-S concentration dependence of secretion in pre-permeabilised cells.

Mast cells in NaCl solution were treated with metabolic inhibitors and then permeabilised with SL-O in the presence of 0.2 mM EGTA buffer (to set pCa7). After 2 (●), 3 (○) or 4 (■) minutes they were triggered by addition of 3mM EGTA buffer to set pCa5.25 and GTP-γ-S as indicated together with 100μM ATP. Apart from ATP, no other nucleotide from the test panel was able to reconstitute the exocytotic mechanism (open squares: 3 minute time point illustrated).

3.2.4 Effects of ITP and ATP on the timecourse of secretion

The effects of ITP and ATP on the timecourse of the exocytotic reaction are shown in figure 3.10. As previously reported [Gomperts & Tatham, 1988; Tatham & Gomperts, 1989], ATP and ATP-γ-S delay the onset of exocytosis. These two nucleotides increase the rate and the extent of secretion at any given concentration of GTP-γ-S. ITP (also XTP, UTP and CTP: data not shown) acts similarly to ATP in this latter respect, but it fails to induce onset delays. GTP (at substimulatory concentrations), AppNHp and ADPßS have no perceptible effects on the kinetics of secretion. The ability of nucleotides to increase the rate and extent of secretion requires the presence of Mg^{2+} and is probably a reflection of the enhancement in the affinities of the two essential effectors described above. In contrast, and unlike the other modulatory effects of ATP on exocytosis, the induction of onset delays by ATP and ATP-γ-S occurs in the absence of Mg^{2+} (figure 3.11).

3.3 Discussion

Exocytosis can be elicited by application of Ca^{2+}-plus-GTP-γ-S to mast cells, neutrophils and eosinophils permeabilised in NaCl solutions in the absence of ATP [Howell et al., 1987; Stutchfield & Cockcroft, 1988; Nusse et al., 1990]. However, when mast cells are permeabilised in the presence of ATP the process is modulated in at least four definable ways.

1. the sensitivity to both of the effectors (Ca^{2+} and a guanine nucleotide) are enhanced [Howell et al., 1987; Gomperts & Tatham, 1988].

2. the period during which the cells remain responsive to addition of essential effectors after permeabilisation is prolonged [Howell et al., 1989].

3. late addition of ATP restores responsiveness to cells rendered refractory by permeabilisation initially in its absence [Howell et al., 1989; Churcher et al., 1990b].

4. onset of secretion following addition of the two essential effectors is delayed [Tatham & Gomperts, 1989; Gomperts & Tatham, 1988; Churcher et al., 1990a].

Figure 3.10. The effects of ITP and ATP on the kinetics of the exocytotic reaction.

Mast cells in NaCl solution were treated with metabolic inhibitors and then permeabilised with SL-O in the presence of 0.2 mM EGTA buffer (to set pCa7) and GTP-γ-S as indicated: (a) in the absence of modulating nucleotide, (b) in the presence of ITP (50μM), or (c) in the presence of ATP (1mM). After 1 min incubation, 5mM EGTA buffer was added to set pCa5, and sampling was commenced. GTP-γ-S concentration: ●, 10^{-7}M; ○, $10^{-6.67}$M; ■, $10^{-6.33}$M; □, 10^{-6}M; ▲, $10^{-5.67}$M; △, $10^{-5.33}$M.

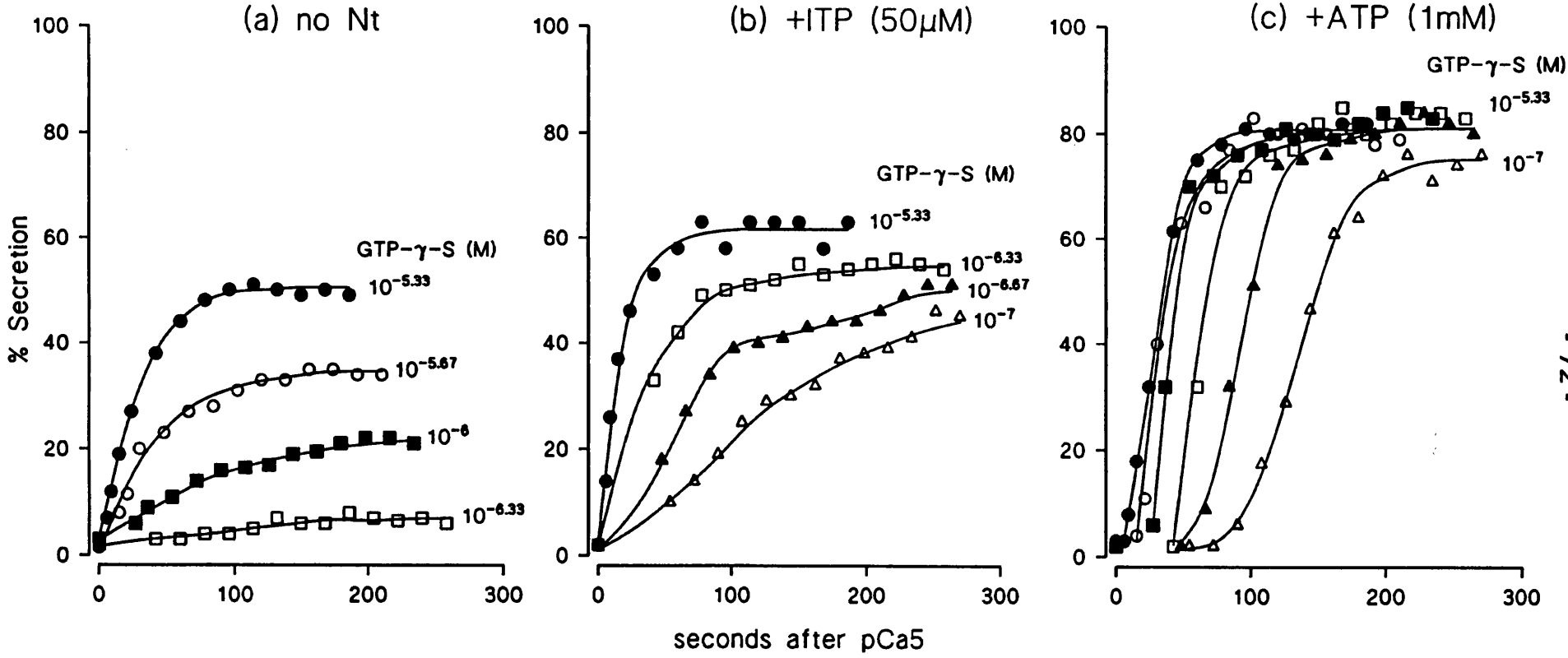
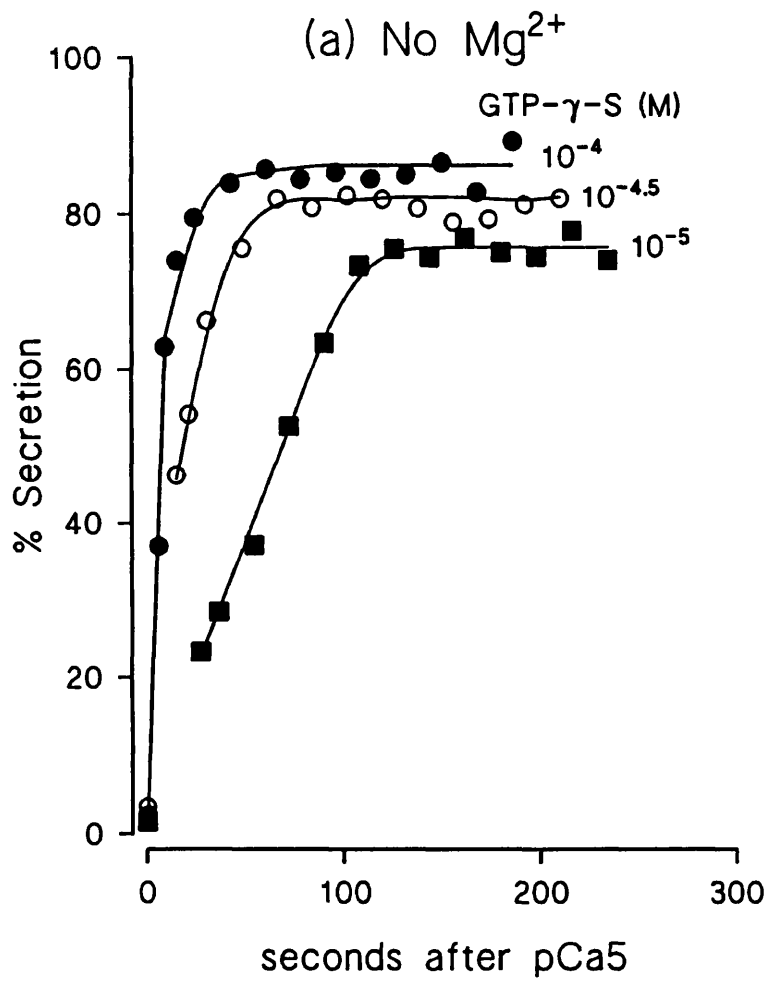
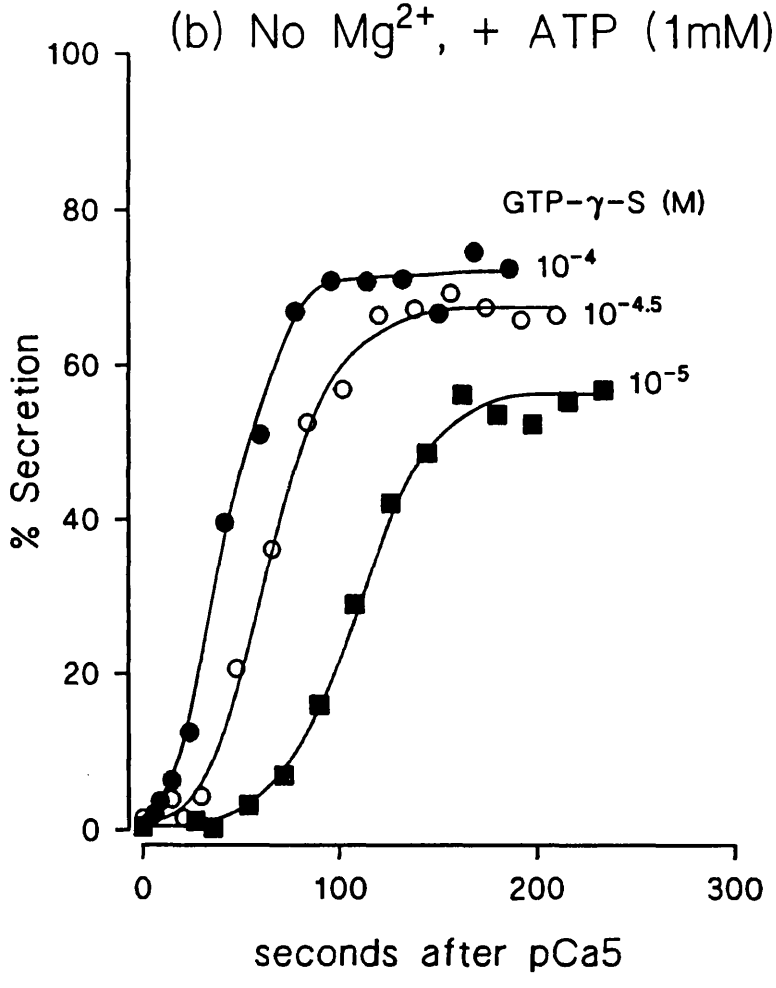

(a) no Nt
(b) +ITP (50μM)
(c) +ATP (1mM)
% Secretion
GTP-γ-S (M)
10^-5.33
10^-5.67
10^-6
10^-6.33
10^-6.67
10^-7
seconds after pCa5
0
20
40
60
80
100
0
100
200
300

Figure 3.11. Induction of onset delays by ATP occurs in the absence of Mg^{2+}.

The stimulus is applied under low sensitivity conditions in this experiment (due to the absence of Mg^{2+} even when ATP is provided) so a high concentration range of GTP-γ-S was employed, in the absence (a) or presence (b) of ATP (1mM). Ca^{2+} was buffered at pCa5 using HEDTA buffers to reduce free [Mg^{2+}]. Conditions otherwise as for figure 3.10. GTP-γ-S concentration: ■, 10^{-5}M; ○, $10^{-4.5}$M; ●, 10^{-4}M.


(a) No Mg^{2+}
GTP-γ-S (M)
10^{-4}
$10^{-4.5}$
10^{-5}
% Secretion
0
20
40
60
80
100
0
100
200
300
seconds after pCa5


(b) No Mg^{2+}, + ATP (1mM)
GTP-γ-S (M)
10^{-4}
$10^{-4.5}$
10^{-5}
% Secretion
0
20
40
60
80
100
0
100
200
300
seconds after pCa5

Three of these modulatory effects are likely to be due to phosphorylation reactions which could either be induced by the essential effectors of exocytosis themselves (Ca^{2+} together with a guanine nucleotide), or by the maintenance of a phosphorylation state. The fourth modulatory effect of ATP, the induction of onset delays, occurs in the absence of Mg^{2+}, and is therefore unlikely to be mediated by a phosphorylation reaction. In this chapter I have investigated the ability of various nucleotides to replace ATP as a modulator of the exocytotic process.

3.3.1 Enhancement of Sensitivity

The enhancement of the sensitivity to the two essential effectors (Ca^{2+} and GTP-γ-S) by ATP, has been characterised as a protein kinase C mediated reaction by the inhibitory action of neomycin (which inhibits phospholipase C, and hence diglyceride production [Cockcroft et al., 1987; Aridor et al., 1990]) and also the stimulatory effects of phorbol esters [Cockcroft et al., 1987]. I have now demonstrated that the PKC inhibitor AMG.C_{16} [van Blitterswijk et al., 1987; Howell et al., 1989; Kramer et al., 1989] is capable of suppressing the sensitivity shift caused by ATP, UTP, XTP or ITP. It therefore seems that all the nucleotides capable of enhancing the sensitivity (ie all members of the test panel with the exception of AppNHp and GTP) can act, either directly or indirectly, as substrates for PKC. The rate and extent of secretion due to any concentration of GTP-γ-S are also enhanced by these same nucleotides and this suggests that these may be manifestations of the same reaction. This is borne out by the results of kinetic experiments (figure 3.10a & b) in which it can be seen that an equivalent rate and extent of secretion is achieved at a concentration of GTP-γ-S 10-fold lower when ITP is also provided. The previous finding that PMA increases the rate of secretion (implicating PKC as the effector) [Tatham & Gomperts, 1989], strengthens the link between the increase in sensitivity and rate of release.

In principle, it might be possible to differentiate separate classes and subclasses (ie isoforms) of the various phosphorylating enzymes (kinases) by reference to their rank orders of preference for nucleoside triphosphates. Much attention has been paid to the specificities of the soluble kinases such as phosphofructokinase (muscle enzyme: ATP, CTP, UTP, ITP and GTP all act as donors [Uyeda & Racker, 1965; Bloxham & Lardy, 1973]), 3-phosphoglycerate kinase [Scopes, 1973] and glycerol

kinase [Thorner & Paulus, 1973]. Less attention appears to have been paid to the nucleotide specificity of the various phosphoprotein kinases. Although cold nucleoside triphosphates fail to inhibit ^{32}P-phosphorylation of histone substrates by brain protein kinase C [Wise et al., 1982] (an experiment I also tried with Dr Peter Parker with the same result) there is an early report that GTP can act as a donor in the reaction catalysed by brain phosvitin kinase [Rodnight & Lavin, 1964] (other nucleoside triphosphates not tested).

Unlike the other modulatory effects under discussion, non-adenine nucleotides (including ITP, XTP, CTP and UTP) are also capable of causing an enhancement of effector affinity. I therefore considered whether this might be due to these nucleotides acting directly as phosphoryl donors, or indirectly as a result of transphorylation to form ATP. One consequence of the procedure, which involves pretreatment of the cells with metabolic inhibitors, must be to elevate the concentration of ADP from its low "resting" level, up into the millimolar range. Thus, for cells stimulated at the time of application of the permeabilising agent, the effectors, together with any supporting nucleoside triphosphate, will be entering the cells at the same time as the accumulated ADP is leaking out. Under these conditions, enhancement of effector sensitivity appears to be non-specific between various purine and pyrimidine nucleoside triphosphates. If the stimulus is applied 20 seconds after permeabilisation (to allow the ADP to become infinitely diluted) then only ATP is capable of enhancing sensitivity. From this I conclude that non-adenine nucleotide triphosphates are capable of enhancing affinity by virtue of their propensity to act as donors in the nucleoside diphosphokinase reaction. Support for this conclusion is found in the observation that following a 20 second period of permeabilisation, UTP is once again capable of causing an enhancement of effector sensitivity if it is supplemented with ADP.

3.3.2 Maintenance and Restoration of Secretory Competence

Only ATP (as its Mg^{2+} salt) is capable of maintaining and restoring responsiveness in the minutes following permeabilisation. Previous results indicate that these functions are mediated by protein kinase C [Howell et al., 1989; Churcher et al., 1990b]. It is therefore likely that they are both consequences of the maintenance or restoration of an obligatory phosphorylation state which regulates the sensitivity for the two essential

effectors. The other non-adenosine triphosphates which support a shift in effector sensitivity cannot maintain or restore permeabilised cells since their mechanism of action relies on the presence of ADP, which is rapidly lost from the cells following permeabilisation. The progressive loss of responsiveness which occurs when the cells are permeabilised in the absence of Mg.ATP is thus most likely a reflection of a systematic shift of effector affinity due to dephosphorylation and which (in the short term) leaves the exocytotic apparatus unscathed. Note that ATP-γ-S is additionally capable of enhancing affinity.

3.3.3 Onset Delays

The observation that ATP and ATP-γ-S, but not AppNHp, induce delays in the onset of exocytosis was the basis for the earlier suggestion [Tatham & Gomperts, 1989; Gomperts & Tatham, 1988] that this might also be a manifestation of a phosphorylation reaction (or due to the maintenance of a phosphorylation state). However, the finding that ATP can induce delays even in the absence of Mg^{2+} now renders this explanation rather implausible. I have considered the possibility that the residual amounts of contaminating Mg^{2+} present in the electrolyte solutions might be sufficient to promote kinase activity even though it is evident that the other ATP-induced modulatory functions require Mg^{2+} in the millimolar range of concentrations. Under conditions in which the concentration of free Mg^{2+} was suppressed to below 10^{-8}M, the ability of ATP to induce delays remained unimpaired. This being so, a phosphorylation reaction is unlikely to be involved in the induction of onset delays by ATP and ATP-γ-S. Since the induction of delays occurs in the effective absence of Mg^{2+}, the antagonistic form of ATP is likely to be one of the anionic species, either ATP^{3-} or ATP^{4-}. This may explain why the non-adenine nucleoside triphosphates fail to induce delays despite their ability to generate ATP from ADP. The time in which the concentration of ADP remains high enough to allow transphosphorylation by the other nucleotides is obviously very short (<20 seconds, calculations suggest nucleotide diffusion times into and out of SL-O permeabilised cells will be of the order of 10 seconds [Lindau & Gomperts, 1991]), and in this time only a relatively small amount of ATP could be generated (which, as shown, is all that is needed to support a sensitivity shift). Since the proportion of ATP^{3-} or ATP^{4-} at any given concentration of Mg.ATP is small, the transphosphorylating nucleotides are unable to induce delays in the onset of secretion. The

transphosphorylation reaction also appears to have a requirement for the presence of Mg^{2+} (data not shown and [Parks & Agarwal, 1973]) and so I cannot test the ability of the non-adenine nucleotides to induce delays in its absence.

Anionic species of ATP have been shown to act as allosteric regulators of a number of intracellular activities, including the inhibition of K^+ channels in islet cells [Ashcroft & Kakei, 1989], some forms of phosphofructokinase (eg in red blood cells [Otto et al., 1990] and malarial parasites [Buckwitz et al., 1990a; Buckwitz et al., 1990b]) and also in formation of plasma membrane pores, possibly by GAP junctions [Beyer & Steinberg, 1991]. Thus there exists the possibility that ATP inhibits the onset of exocytosis by acting as an allosteric regulator of an enabling enzyme for exocytosis.

Chapter 4

Guanine Nucleotides are Essential but Ca^{2+} is Dispensible in Mast Cell Exocytosis

4.1 Introduction

Mast cells permeabilised by streptolysin-O in isotonic solutions based on the amino acid glutamate secrete up to 100% of their contained histamine and lysosomal enzymes when provided with both Ca^{2+} and a guanine nucleotide [Churcher & Gomperts, 1990]. A similar result is obtained when cells are permeabilised in simple salt solutions [Howell et al., 1987]. Non-hydrolysable analogues of GTP provide the most potent stimulus to exocytosis, and these are effective when applied at micromolar concentrations. It thus appears that exocytosis in both NaCl and glutamate based buffers is best stimulated by those nucleotides which can maintain the activation of a GTP-binding protein.

In permeabilised cells suspended in NaCl, the provision of ATP (as the Mg^{2+} salt) leads to an increase in the sensitivity to both Ca^{2+} and guanine nucleotide. As discussed in Chapter 3, this is probably due to a phosphorylation reaction catalysed by protein kinase C [Howell et al., 1987; Lillie et al., 1991]. Other nucleoside triphosphates including ITP, UTP, XTP, CTP are also capable of enhancing effector affinity, but do so indirectly by transphosphorylation of residual ADP to form ATP. GTP appears to be incapable of enhancing effector affinity, either directly or indirectly [Lillie et al., 1991].

The effect of providing ATP to cells permeabilised in iso-osmotic solutions of glutamate (either the D- or L-isomer) or aspartate is quite different to that which occurs in simple salt solutions. In the presence of ATP, secretion can be elicited by either Ca^{2+} or a guanine nucleotide acting alone, as *single effectors* [Churcher & Gomperts, 1990]. In view of the

ability of other nucleoside triphosphates to mimic the increase in affinity caused by ATP in simple salt solutions, the capability of these other nucleotides to support *single effector* secretion in glutamate buffers is examined in this work.

It has previously been reported [Churcher & Gomperts, 1990] that ATP supports *single effector* exocytosis (ie mediated by either Ca^{2+} or GTP-γ-S alone) from cells permeabilised in glutamate due to phosphorylations mediated by protein kinase C. This was interpreted as the operation of two parallel pathways (ie the one mediated by Ca^{2+}, the other by guanine nucleotide) leading to vesicle fusion. In the experiments to be described, the function of ATP has been re-examined in the light of the observations of the previous chapter. These indicated that an alternative role of nucleoside triphosphates in the modulation of secretion from permeabilised cells is in the transphosphorylation of other nucleoside diphosphates [Lillie et al., 1991]. The results indicate that such secretion induced by *single effectors* results from the ability of a nucleoside diphosphate kinase to generate GTP from GDP using ATP (or other nucleoside triphosphates) as a phosphoryl donor. The glutamate environment enhances the sensitivity of the secretory mechanism for guanine nucleotides and may also inhibit the hydrolysis of GTP by a GTP-binding protein (G_E) which regulates exocytotic membrane fusion. For mast cells stimulated immediately upon permeabilisation, the guanine nucleotide is essential for exocytosis. Ca^{2+}, which regulates the sensitivity of the system for GTP is, in the limit, dispensable.

4.2 Results

4.2.1 Glutamate increases sensitivity to guanine nucleotides

When mast cells are permeabilised in glutamate rather than NaCl based buffers, the effective affinity for guanine nucleotides in the stimulation of exocytosis is considerably increased, even in the presence of Mg^{2+}. By comparing figures 4.1 and 4.2 (closed symbols), it is apparent that the EC_{50} for GTP has shifted from $10^{-3.75}$ M to $10^{-4.75}$ M, and that the EC_{50} for GTP-γ-S has shifted from $\sim 10^{-5.5}$M to $\sim 10^{-7}$M, simply by altering the composition of the permeabilisation buffer. These experiments were carried out in the presence of fixed Ca^{2+} (pCa5) following treatment with

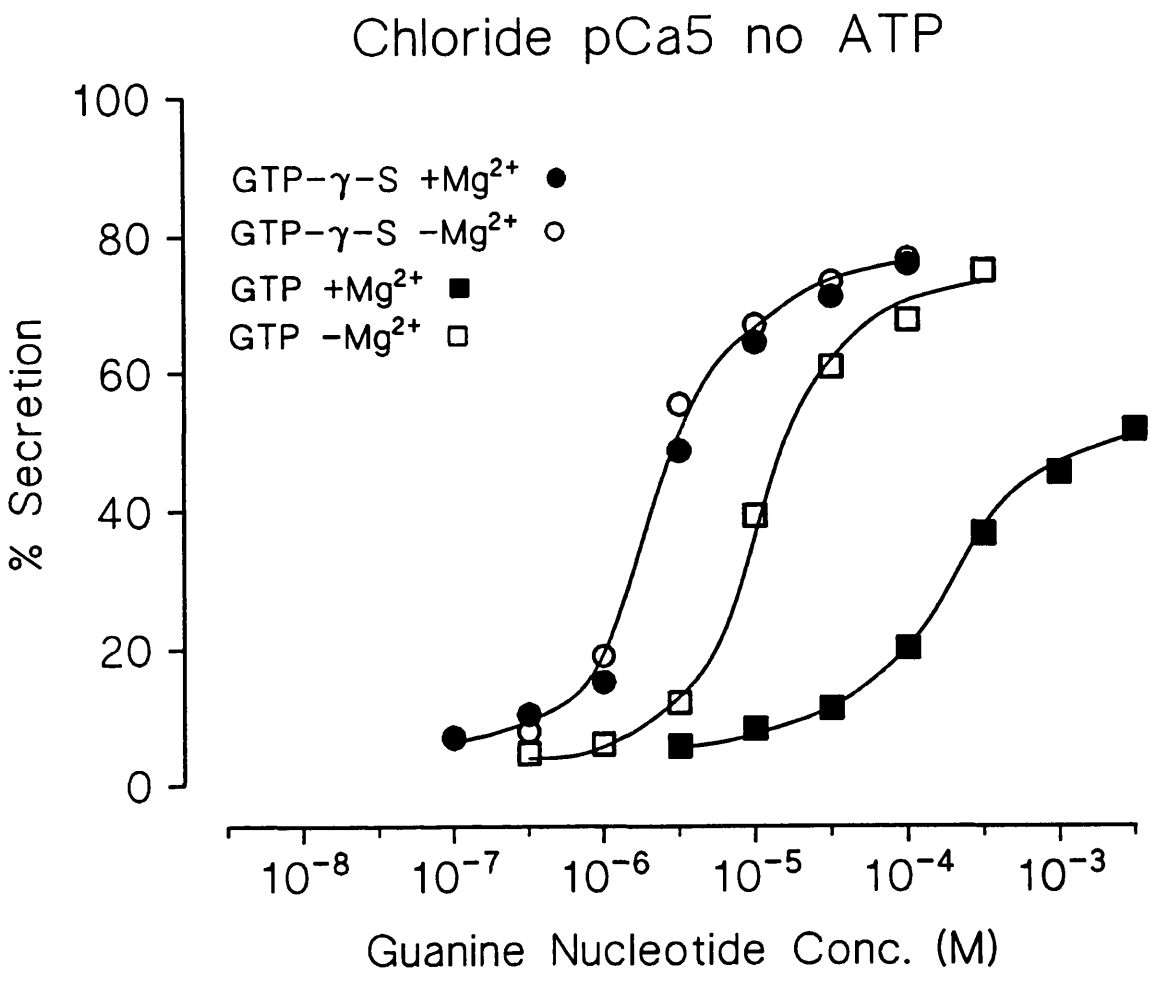


Figure 4.1. Exclusion of Mg^{2+} enhances the ability of GTP to induce exocytosis from permeabilised rat mast cells in chloride buffer.

Mast cells, suspended in buffered NaCl and pretreated with metabolic inhibitors, were incubated with SL-O and Ca^{2+} (buffered at pCa5) and guanine nucleotides as indicated, in the presence and absence of Mg^{2+}. At the end of 10 minutes the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase. Guanine Nucleotide: ■,□ GTP; ●,○ GTP-γ-S. Filled symbols indicate the presence of Mg^{2+} (1mM, Ca^{2+} buffered by 5mM EGTA); open symbols, Mg^{2+} absent (Ca^{2+} buffered by HEDTA 5mM, $[Mg^{2+}] < 10^{-9}$).

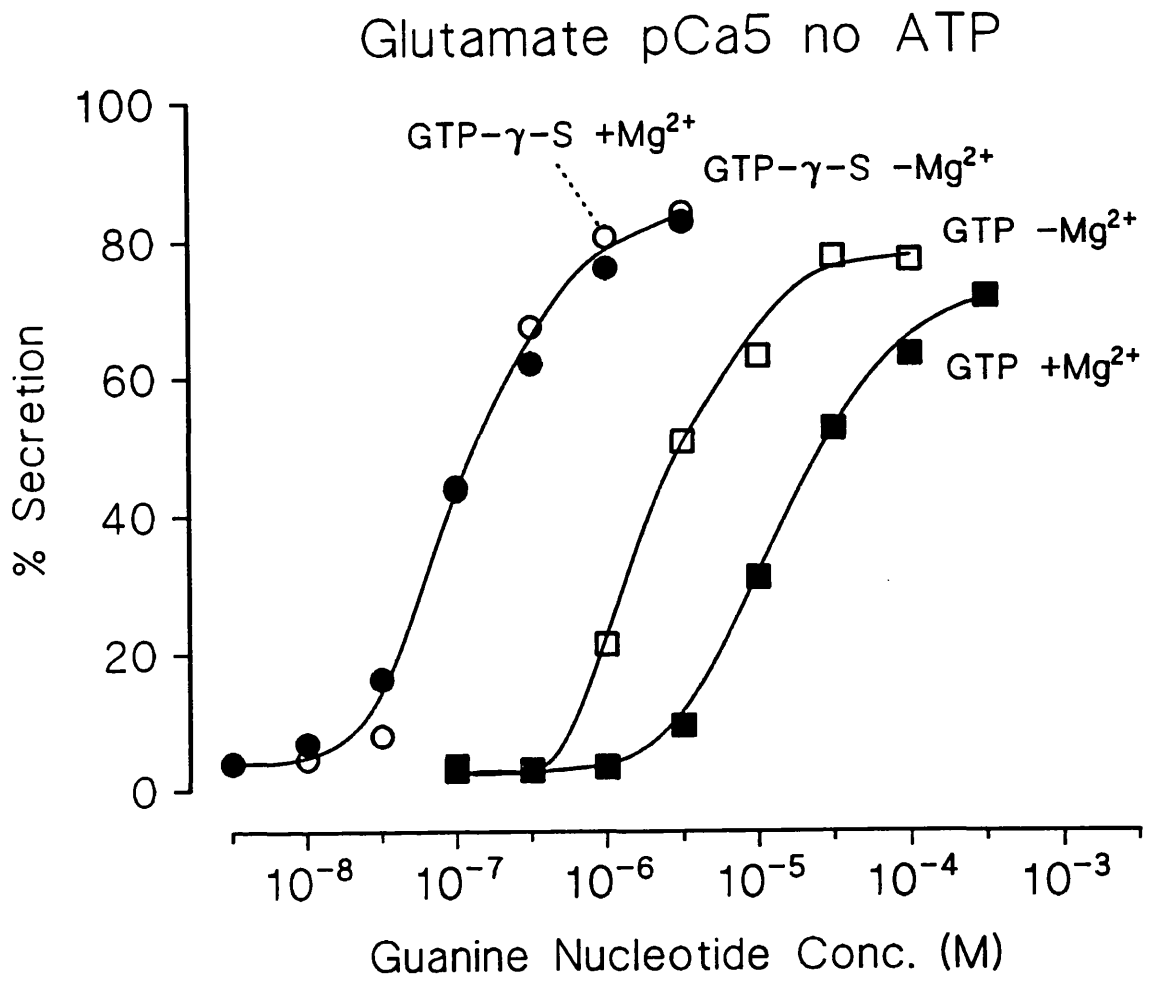


Figure 4.2. Exclusion of Mg^{2+} enhances the ability of GTP to induce exocytosis from permeabilised rat mast cells in glutamate buffer.

Mast cells, suspended in buffered glutamate and pretreated with metabolic inhibitors, were incubated with SL-O and Ca^{2+} (buffered at pCa5) and guanine nucleotides as indicated, in the presence and absence of Mg^{2+}. At the end of 10 minutes the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase. Guanine Nucleotide: ■,□ GTP; ●,○ GTP-γ-S. Filled symbols indicate the presence of Mg^{2+} (1mM, Ca^{2+} buffered by 5mM EGTA); open symbols, Mg^{2+} absent (Ca^{2+} buffered by HEDTA 5mM, $[Mg^{2+}] < 10^{-8}$).

metabolic inhibitors for 5 minutes to the point at which no secretion occurs in response to ionophores or agonistic ligands [Bennett et al., 1980; Howell et al., 1987]. When the Mg^{2+} is removed (figures 4.1 and 4.2, open symbols) there is no change in the sensitivity to GTP-γ-S in either buffer. The EC_{50}(GTP) in NaCl buffer shifts from approximately $10^{-3.75}$ M in the presence of Mg^{2+} to 10^{-5}M in its absence, and in glutamate buffer from $10^{-4.75}$M to $10^{-5.75}$M. In NaCl based buffers the sensitivity to GTP becomes fully comparable with that of GppNHp and exceeds that of $GppCH_2p$ in similar experiments ([Howell et al., 1987] carried out in the presence of Mg^{2+}). Exclusion of Mg^{2+} also increases the maximal level of secretion which can be elicited by GTP in NaCl based buffers. To ensure thorough depletion of Mg^{2+}, the chelator HEDTA was used to regulate pCa instead of the more conventional EGTA and under these conditions it is calculated that the concentration of free Mg^{2+} is less than 10^{-8}M (see Methods chapter). A plausible explanation of this result is that the omission of Mg^{2+} prevents the hydrolysis of GTP by GTP-binding proteins, and this ensures that the GTP-binding protein, G_E, which mediates exocytosis, becomes persistently activated. Glutamate based buffers also appear to be able to increase the sensitivity of the exocytotic machinery to guanine nucleotides, although the mechanism behind this effect is unclear. Since GTP is more effective in glutamate buffers even in the presence of Mg^{2+}, it is possible that part of the effect of glutamate is an inhibition of the GTPase activity.

4.2.2 Single effector secretion in glutamate buffers

Perhaps the most significant change that occurs in the control of secretion when amino acid based buffers are used is that either Ca^{2+} (in the "high" range pCa6-pCa5) or GTP-γ-S (at 100μM) can stimulate secretion in the presence of ATP [Churcher & Gomperts, 1990]. Such *single effector* induced secretion requires the presence of Mg^{2+}.ATP. In this work it was shown that both the D and L isomers of glutamate and aspartate can support (ATP-dependent) secretion stimulated by the *single effectors*. I have obtained similar results following permeabilisation of cells in isotonic glycine (figure 4.3) or γ-aminobutyrate (figure 4.4). It can be seen that whilst both amino acids support *single effector* secretion in the presence of ATP, the extent of Ca^{2+} or GTP-γ-S induced secretion is variable.

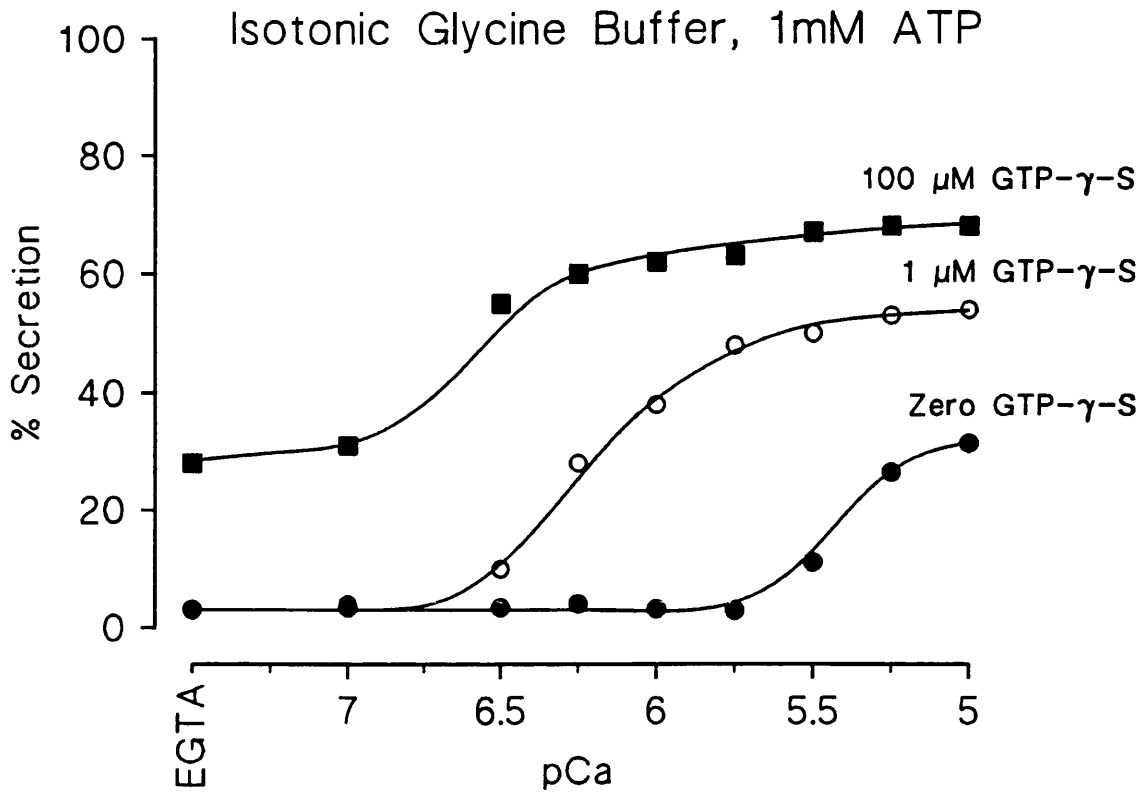


Figure 4.3. Glycine buffer is able to support ATP-dependent Ca^{2+} or GTP-γ-S induced single-effector secretion.

Mast cells, suspended in isotonic GABA based buffers and pretreated with metabolic inhibitors, were incubated with SL-O and 1mM Mg.ATP in the presence of Ca^{2+} (buffered with EGTA at concentrations indicated) and GTP-γ-S. At the end of 10 minutes the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase. GTP-γ-S, ●, zero; ○, 1μM; ■, 100μM.

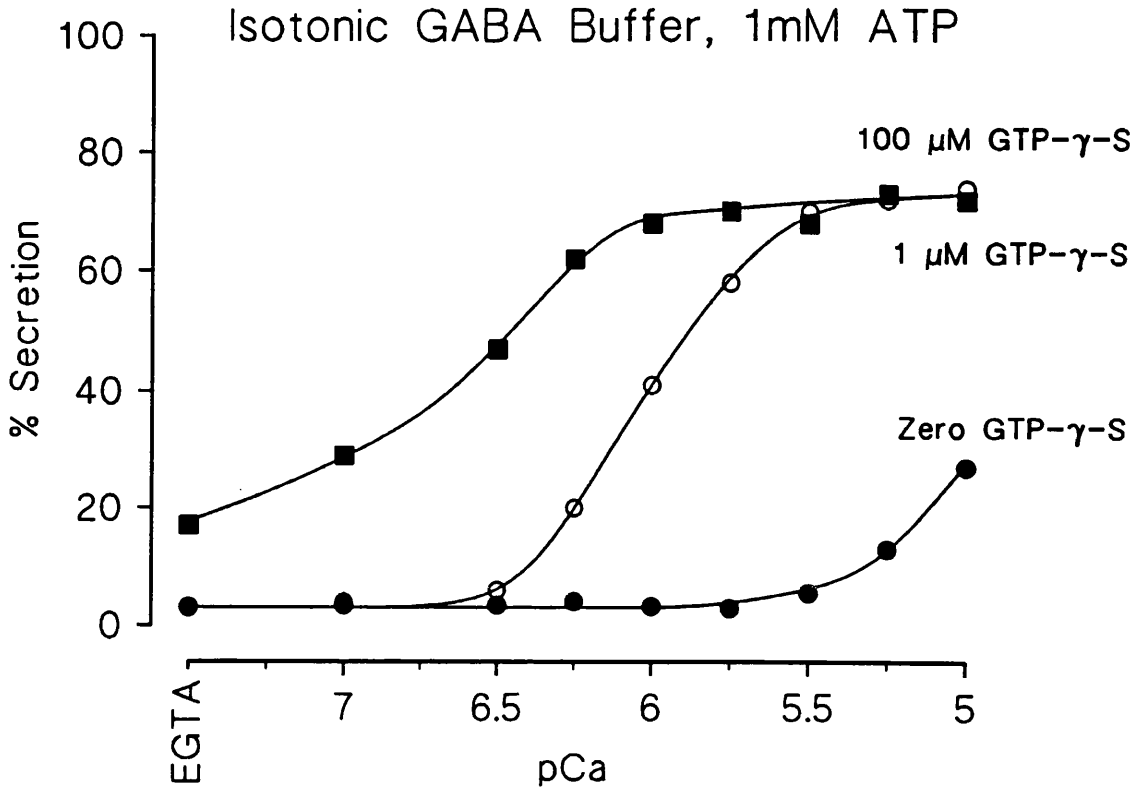


Figure 4.4. GABA based buffer is able to support ATP-dependent Ca^{2+} or GTP-γ-S induced single-effector secretion.

Mast cells, suspended in isotonic GABA based buffers and pretreated with metabolic inhibitors, were incubated with SL-O and 1mM Mg.ATP in the presence of Ca^{2+} (buffered with EGTA at concentrations indicated) and GTP-γ-S. At the end of 10 minutes the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase. GTP-γ-S, ●, zero; ○, 1μM; ■, 100μM.

Other nucleoside triphosphates such as UTP and CTP can substitute for ATP in the support of secretion mediated by Ca^{2+} (figure 4.5) but not by GTP-γ-S (figure 4.6) in the glutamate environment. Since the well known protein kinases all appear to require ATP [Lillie et al., 1991; and chapter 3] the finding that Ca^{2+} induced secretion can be supported by a range of both purine and pyrimidine nucleotides once again suggests the involvement of nucleoside diphosphate kinase.

The data illustrated in figure 4.7 supports both the involvement of a nucleoside diphosphate kinase and the key role of G_E in the regulation of exocytosis from permeabilised mast cells. The experiment tests the susceptibility of the Ca^{2+} induced (ATP dependent) response to modulation by GDP. Perhaps somewhat surprisingly, both the extent of secretion and the $EC_{50}(Ca^{2+})$ are suppressed by concentrations of GDP in excess of 100μM. At concentrations below this the effect of GDP is actually to enhance the extent of Ca^{2+} induced (ATP dependent) secretion. The fact that high concentrations of GDP can inhibit the Ca^{2+} and ATP response indicates that there is probably a GTP-binding protein involved, and that the GDP is competing with GTP at a GTP-binding site. That the addition of exogenous GDP at low concentrations can actually enhance the secretory response to Ca^{2+} and ATP, indicates that ATP probably acts as a phosphoryl donor for the generation of GTP from GDP. It appears that it is GDP (the substrate for generation of GTP from ATP) which limits the extent of secretion, probably because a high proportion of GDP is rapidly dissipated from the cells along with all other free aqueous solutes following permeabilisation. From this it can be concluded that GTP (and by implication a GTP-binding protein) remains an absolute requirement for secretion from permeabilised mast cells. The ability of Ca^{2+} to stimulate secretion in the absence of added guanine nucleotide becomes apparent only because of the increased sensitivity to GTP (formed by transphosphorylation of GDP) in the glutamate environment (see figure 4.2). In contrast to these results with GDP, a previous investigation using 1mM GDP-ß-S conspicuously failed to cause inhibition of Ca^{2+} and ATP induced secretion [Churcher & Gomperts, 1990]. However, it also failed to inhibit GTP-γ-S and Ca^{2+} induced exocytosis at the same concentration. It would therefore appear that GDP-ß-S is a poor competitor for GTP binding sites on GTP-binding proteins involved in exocytosis.

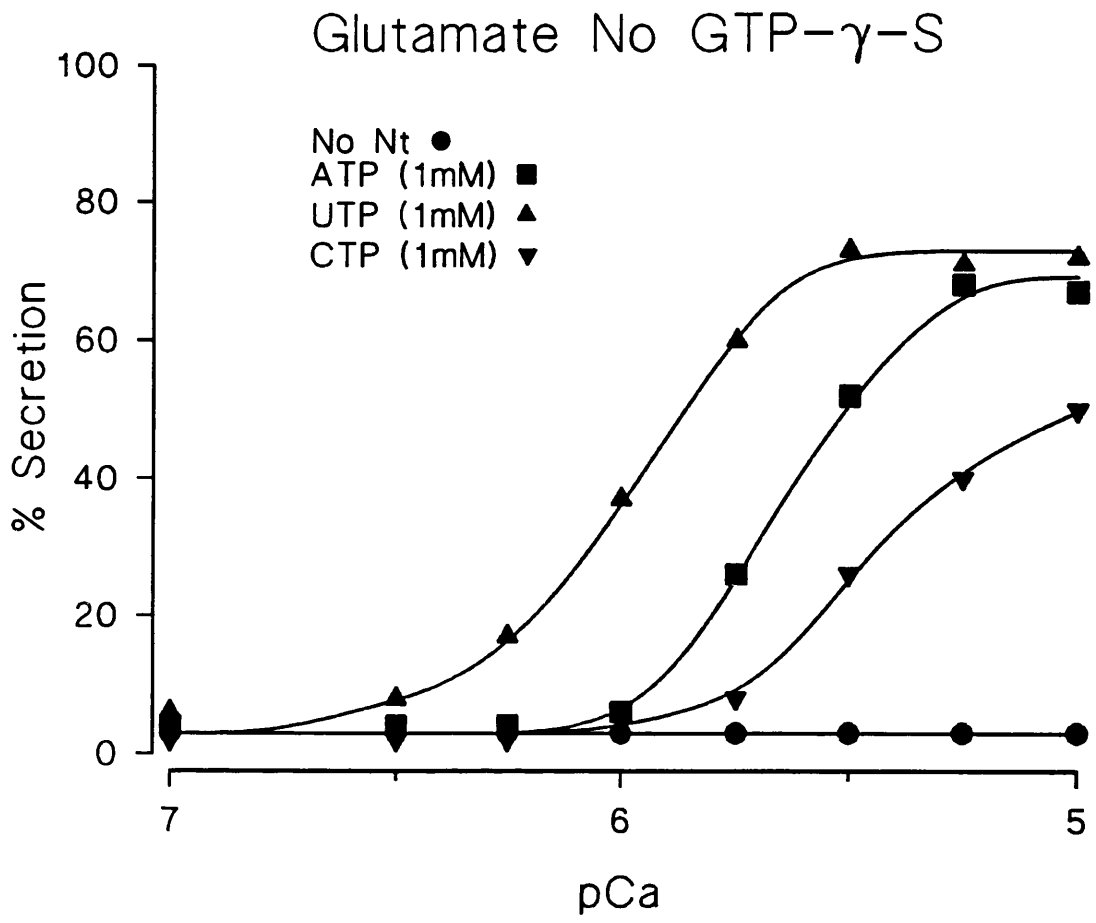


Figure 4.5. Dependence on nucleoside triphosphates of the Ca^{2+} alone stimulus to exocytosis from mast cells permeabilised in glutamate.

Mast cells, suspended in buffered isotonic Na.glutamate containing Mg^{2+} (2mM) and pretreated with metabolic inhibitors, were incubated with SL-O, nucleoside triphosphates (●, control; ■, ATP 1mM; ▲, UTP 1mM, ▼ CTP 1mM) and Ca^{2+} (buffered at concentrations indicated) in the absence of GTP-γ-S (100μM). At the end of 10 minutes the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase.

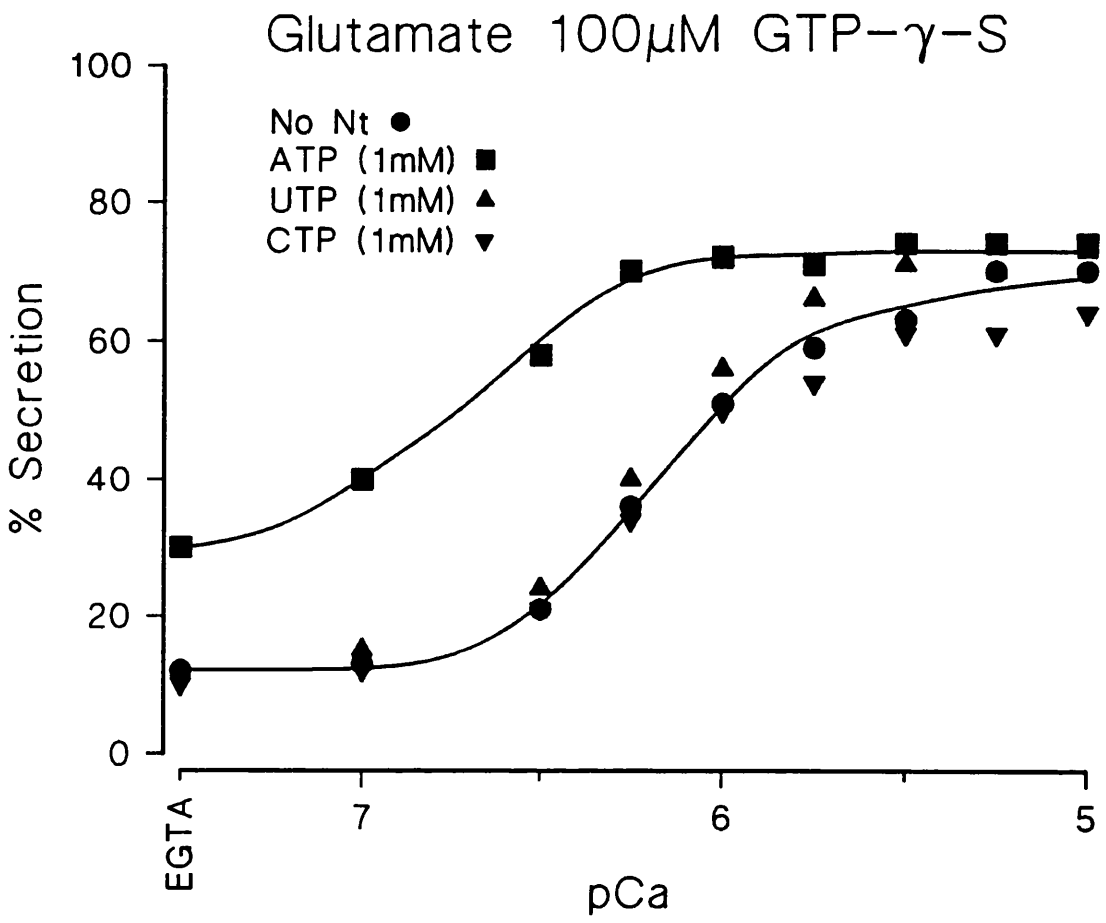


Figure 4.6. Dependence on nucleoside triphosphates of the GTP-γ-S alone stimulus to exocytosis from mast cells permeabilised in glutamate.

Mast cells, suspended in buffered isotonic Na.glutamate containing Mg^{2+} (2mM) and pretreated with metabolic inhibitors, were incubated with SL-O, nucleoside triphosphates (●, control; ■, ATP 1mM; ▲, UTP 1mM, ▼ CTP 1mM) and Ca^{2+} (buffered at concentrations indicated) in the presence of GTP-γ-S (100μM). At the end of 10 minutes the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase.

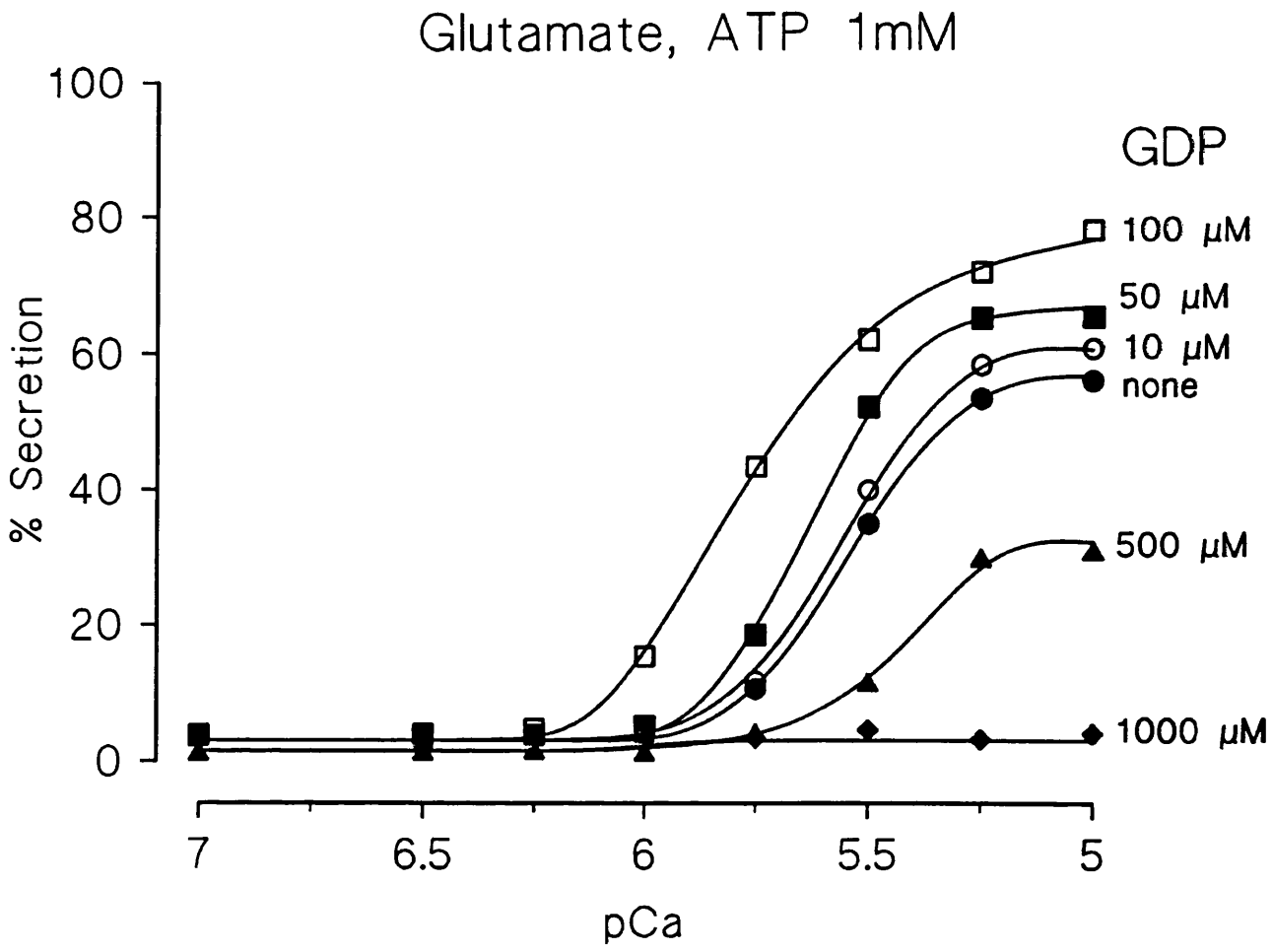


Figure 4.7. Ca^{2+} induced (ATP-dependent) secretion in glutamate based buffer is enhanced by low concentrations and inhibited by high concentrations of GDP.

Mast cells, suspended in isotonic glutamate based buffers and pretreated with metabolic inhibitors, were incubated with SL-O and 1mM Mg.ATP in the presence of Ca^{2+} (buffered at concentrations indicated) and GDP at various concentrations (as indicated). At the end of 10 minutes the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase. GDP concentration: ●, zero; ○, 10 μM; ■, 50μM; □, 100μM; ▲, 500μM; ◆, 1000μM.

In contrast with Ca^{2+}, GTP-γ-S can stimulate a true *single effector* secretion. In figure 4.8 this occurs in the absence of ATP and in the effective absence (10^{-9}M) of Ca^{2+}. Note that in the absence of Ca^{2+}, secretion is strongly dependent on the provision of Mg^{2+}, and this dependence is almost totally lost by raising Ca^{2+} from pCa9 to pCa7. GTP is unable to mimic GTP-γ-S in this *single effector* response in the total absence of Ca^{2+} (figure 4.9), as presumably the necessity for the presence of Mg^{2+} also permits the hydrolysis of the nucleotide. However it does support a similarly low level of secretion when Ca^{2+} is raised to pCa7 in the absence of Mg^{2+}.

4.2.3 Effects of Mg^{2+} and Ca^{2+} on the timecourse of GTP-γ-S induced secretion

Figure 4.10a and b represent timecourses of secretion due to combinations of GTP-γ-S (100μM) over a range of Ca^{2+} concentrations (pCa9 to pCa5) in the presence and absence of Mg^{2+} (1mM). Note that in these experiments the trigger was applied simultaneously with the permeabilising agent (SL-O) and so any delays preceding the onset of secretion must also register a time artefact of permeabilisation. The reason for adopting this procedure was that in the glutamate system (especially in the absence of Mg^{2+}) the permeabilised cells lose secretory competence much more rapidly than they do in Cl^- media, and so it is not possible to allow a time (1 minute) for equilibration with one effector before applying the second as a trigger. For this reason, more detailed kinetic studies have been performed in Cl^- based media [Lillie & Gomperts, 1992b; Lillie & Gomperts, 1992c; and see chapter 5]. Using HEDTA (5mM) to suppress the concentrations of both divalent cations (Ca^{2+} approx pCa8) a small extent of secretion commenced after an extended delay of about 150 secs, eventually achieving about 3%. In other more prolonged incubations (10 minute) it was found that such Ca^{2+} and Mg^{2+} deprived cells can achieve no more than about 5% secretion. When Mg^{2+} is supplied (1mM), and using EGTA as a buffer to achieve an even lower concentration of Ca^{2+} (5mM EGTA, - pCa9), the secretion commences earlier (within 50 seconds), and in this experiment it terminated after about 300 seconds at 10%. As Ca^{2+} is raised, secretion commences earlier and the enhancing effect of Mg^{2+} becomes progressively reduced so that it is without effect at pCa6 as shown in figure 4.10b. In addition, with the elevation of Ca^{2+} the

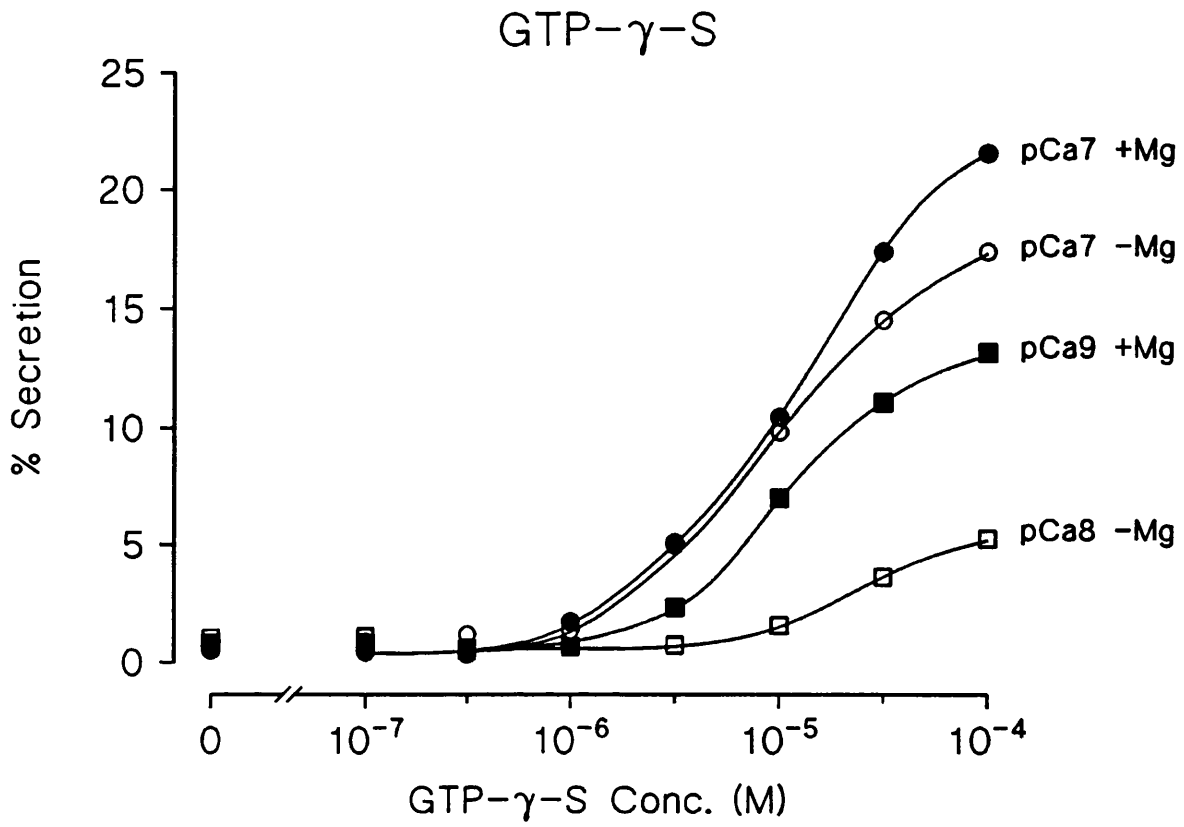


Figure 4.8. High concentrations of GTP-γ-S support secretion in glutamate buffer at low concentrations of Ca^{2+} and in the absence of ATP.

Mast cells, suspended in isotonic glutamate based buffer and pretreated with metabolic inhibitors, were incubated with SL-O and GTP-γ-S at the concentrations indicated in the presence of of pCa7 (●,5mM EGTA; ○,5mM HEDTA) or the virtual absence of Ca^{2+} (■,5mM EGTA pCa9; □,5mM HEDTA pCa8). EGTA was used in the presence of 1mM Mg^{2+}, supplied as Mg-PIPES (closed symbols) and HEDTA was used in the absence of Mg^{2+} (open symbols). At the end of 10 minutes the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase.

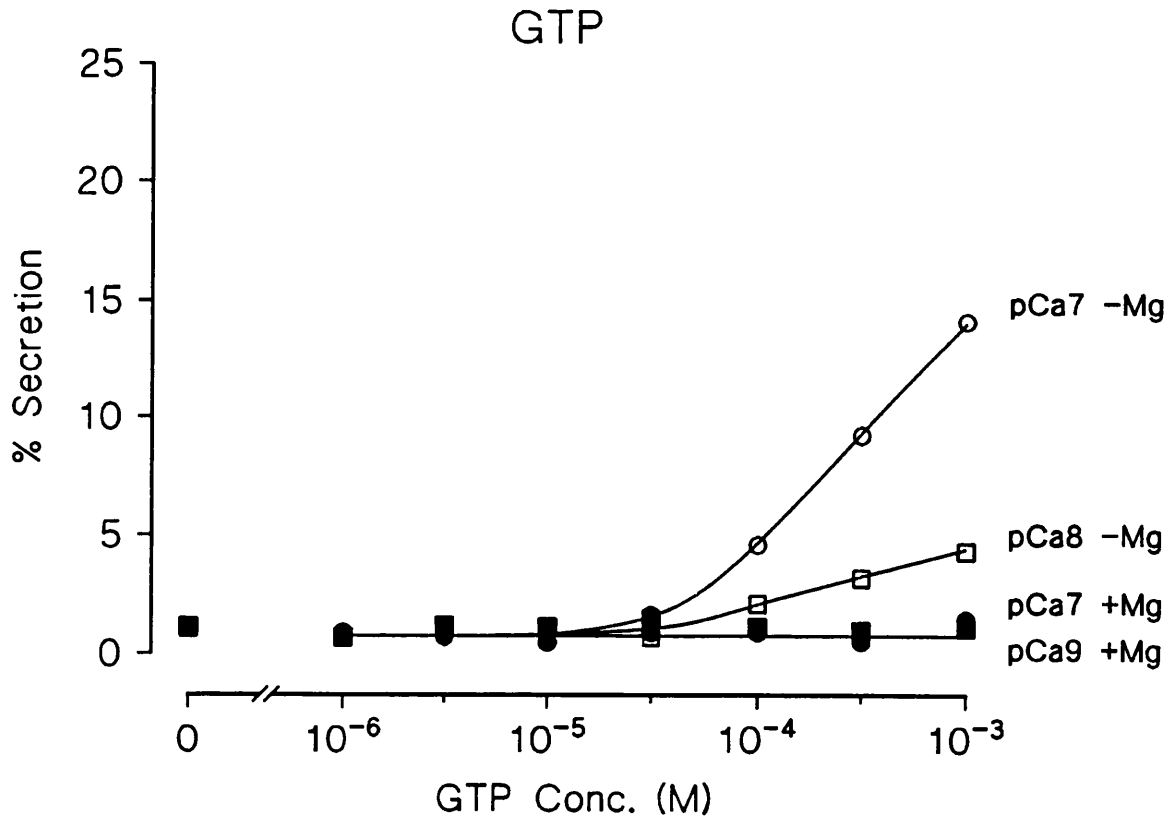


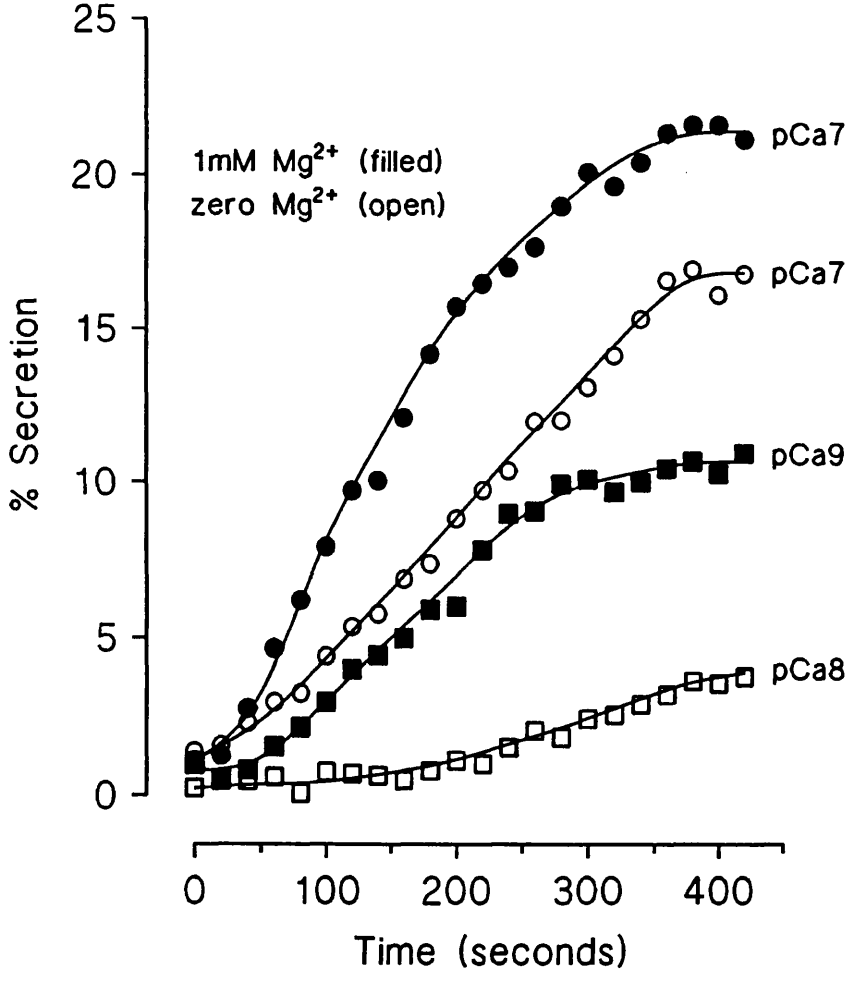
Figure 4.9. High concentrations of GTP support secretion in glutamate buffer at low concentrations of Ca^{2+} and in the absence of ATP.

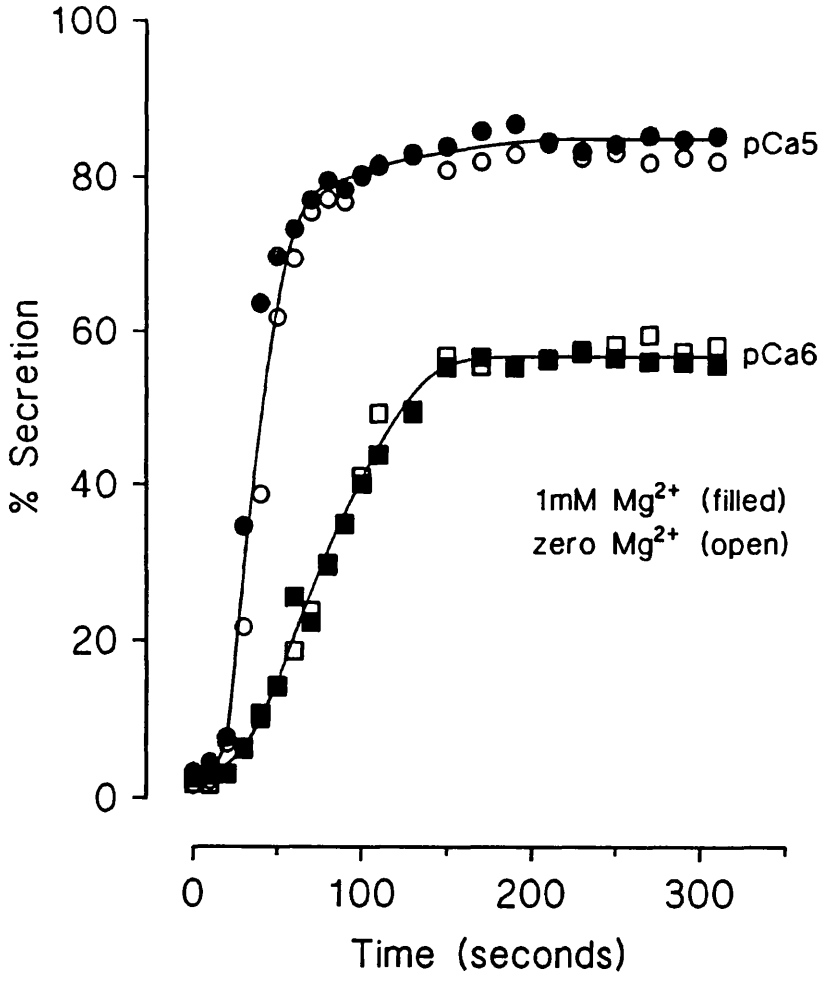
Mast cells, suspended in isotonic glutamate based buffer and pretreated with metabolic inhibitors, were incubated with SL-O and GTP at the concentrations indicated in the presence of of pCa7 (●,5mM EGTA ;○,5mM HEDTA) or the virtual absence of Ca^{2+} (■,5mM EGTA pCa9; □,5mM HEDTA pCa8). EGTA was used in the presence of 1mM Mg^{2+}, supplied as Mg-PIPES (closed symbols) and HEDTA was used in the absence of Mg^{2+} (open symbols). At the end of 10 minutes the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase.

Figure 4.10. Time course of exocytosis showing the effect of Ca^{2+} concentration on the dependence of secretion on Mg^{2+}.

Mast cells, suspended in glutamate based buffers and pretreated with metabolic inhibitors, were triggered to secrete by addition of SL-O together with 100μM GTP-γ-S and in the presence (EGTA Ca^{2+} buffers, closed sysmbols) or absence (HEDTA Ca^{2+} buffers, open symbols) of 1mM Mg^{2+}. pCa5 ●,○; pCa6 ■,□; pCa7 ●,○; HEDTA alone □; EGTA alone ■. Samples were removed at 10 second (pCa5 and 6) or 20 second (pCa7, HEDTA and EGTA) intervals and quenched in ice-cold isotonic NaCl. The cells were then sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase.

Glutamate, 100μM GTP−γ−S





secretion becomes faster and the delays preceding commencement are further decreased. I have been unable to determine whether the requirement for Mg^{2+} (in the absence of Ca^{2+}) should be regarded as being absolute. It is possible that the low level secretion (~5%) that occurs in the absence of Mg^{2+} (suppressed by HEDTA to $<10^{-9}$M) is a reflection of the higher free concentration of Ca^{2+} (pCa8) that remains when HEDTA rather than EGTA is used as the chelator. This small amount of Ca^{2+} might relieve the dependence on Mg^{2+} just sufficiently to allow the very low level of exocytosis that is observed. What is clear is that in the absence of Ca^{2+}, Mg^{2+} promotes GTP-γ-S-induced secretion strongly.

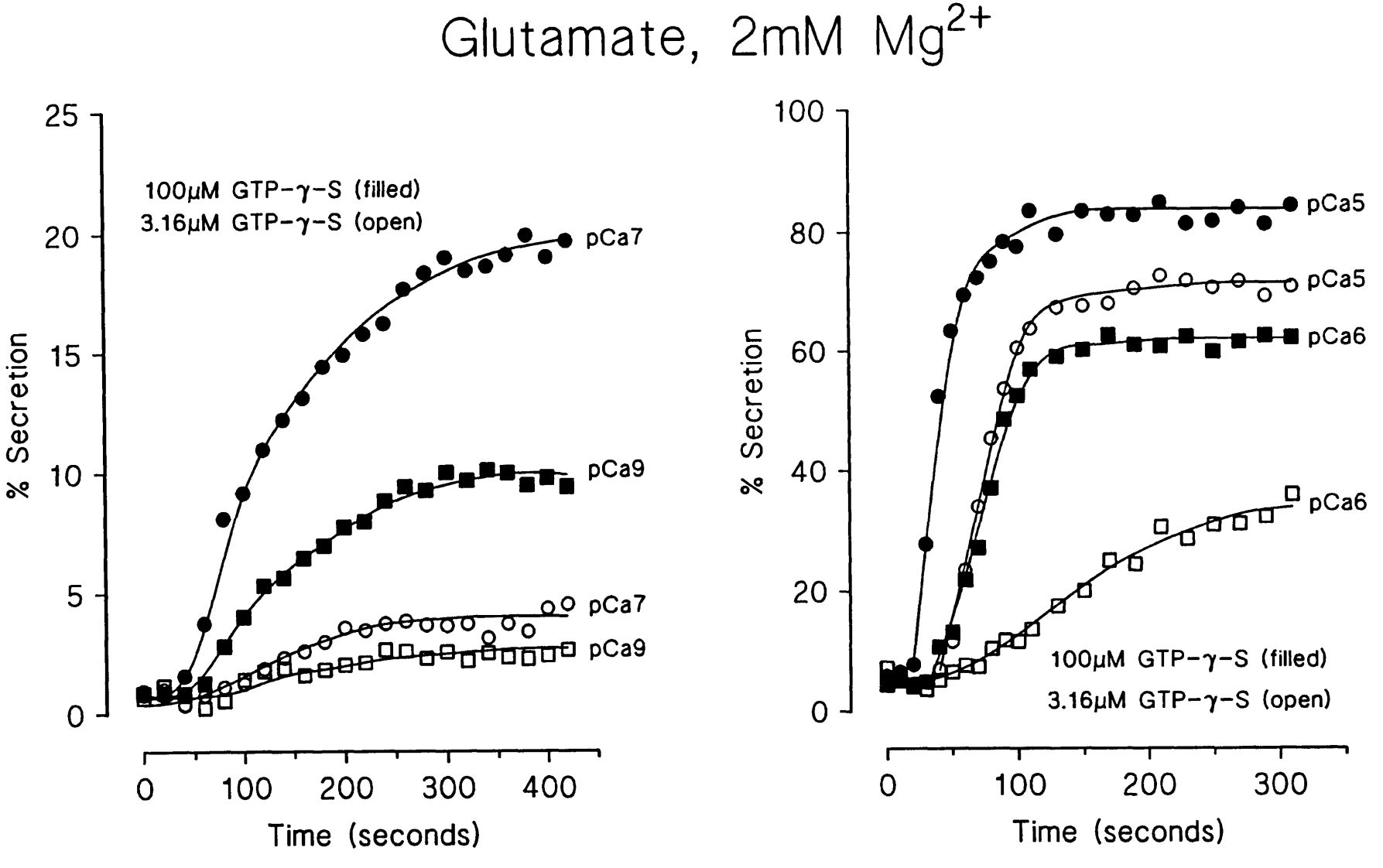
The absolute dependence of secretion on GTP-γ-S is maintained at all concentrations of Ca^{2+}, although as the concentration of Ca^{2+} is increased from pCa9 to pCa7 (figure 4.11a) and from pCa6 and pCa5 (figure 4.11b) the extent of secretion at any given concentration of GTP-γ-S becomes greater and the time taken for completion shorter. In this experiment, the concentration of Mg^{2+} was fixed at 1mM. Systematically raising the concentration of Ca^{2+} has the same effect as was seen in the experiment of figure 4.10, where the delay before the onset of secretion is reduced as the Ca^{2+} is increased. The effect of Ca^{2+} appears to be primarily in the control of the onset delay and the sensitivity to the guanine nucleotide.

4.3 Discussion

From the results presented here it is apparent that regulated exocytosis from rat mast cells permeabilised with streptolysin-O has an absolute requirement for a guanine nucleotide. This holds true regardless of whether the cells are permeabilised in simple uni/univalent (NaCl) or amino acid based buffers (glutamate, glycine, GABA etc). In glutamate however, a hydrolysis-resistant guanine nucleotide (GTP-γ-S, but not GTP) can stimulate a true *single-effector* secretion of low magnitude in the effective absence of Ca^{2+} (pCa9) (figures 4.8, 4.10a and 4.11a). This is strongly promoted by Mg^{2+}, but I have been unable to demonstrate that this is an absolute requirement.

Figure 4.11. Time course of exocytosis showing the effect of Ca^{2+} concentration on the dependence of secretion on GTP-γ-S.

Mast cells, suspended in glutamate based buffers and pretreated with metabolic inhibitors, were triggered to secrete by addition of SL-O together with EGTA Ca^{2+} buffer (pCa5 ●,○; pCa6 ■,□; pCa7 ●,○; or EGTA alone ■,□) and 1mM Mg^{2+} in the presence of 3.16μM (open symbols) or 100μM (closed symbols) GTP-γ-S. Samples were removed at 10 second (pCa5 and 6) or 20 second (pCa7, HEDTA and EGTA) intervals and quenched in ice-cold isotonic NaCl. The cells were then sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase (an identical result was obtained with 2mM Mg^{2+}).


Glutamate, 2mM Mg^{2+}
% Secretion
25
20
15
10
5
0
100μM GTP-γ-S (filled)
3.16μM GTP-γ-S (open)
pCa7
pCa9
pCa7
pCa9
0
100
200
300
400
Time (seconds)
% Secretion
100
80
60
40
20
0
pCa5
pCa5
pCa6
pCa6
100μM GTP-γ-S (filled)
3.16μM GTP-γ-S (open)
0
100
200
300
Time (seconds)

4.3.1 Patch clamp experiments on single mast cells

Previous investigations on single mast cells using the patch-clamp technique to monitor the increase in capacitance due to fusion of secretory granules have repeatedly shown that exocytosis can be achieved by infusion of GTP-γ-S together with EGTA (ie at a vanishingly low cytosol concentration of Ca^{2+}) [Fernandez et al., 1984; Neher, 1988a; Penner et al., 1987], or by Ca^{2+} (at concentrations greater than pCa6) in the absence of added guanine nucleotide [Penner & Neher, 1988]. All these results were achieved in the presence of ATP and with the use of an intracellular dialysing solution formulated with glutamate (ie conditions in which this work would predict a high affinity state for the guanine nucleotide). This is in close agreement with the results I have obtained in SL-O permeabilised mast cells.

Reconstitution experiments using nucleoside diphosphate kinase have indicated the possibility of direct phosphorylation of GDP bound to monomeric (ADP-ribosylating factor [Randazzo et al., 1991]), and (with less certainty [Kikkawa et al., 1991]) trimeric (G_s and G_o [Kikkawa et al., 1990]) GTP-binding proteins. There is also some evidence that in rat liver membranes, this enzyme and G_s are closely associated [Kimura & Shimada, 1990]. These observations, taken together with the results described in this chapter suggest that it is unlikely that ATP-dependent, Ca^{2+} induced exocytosis from either permeabilised or patch-clamped cells was ever truly independent of guanine nucleotide.

4.3.2 Guanine nucleotides as prime regulators of exocytosis from myeloid granulocytes

Further support for an important role for GTP in Ca^{2+} induced secretion from intact cells has previously been indicated by manipulations designed to cause selective depletion of guanine nucleotides. Mast cells (or mast cell-lines) treated with mycophenolic acid or ribavirin (inhibitors of inosine monophosphate dehydrogenase, and hence of the purine nucleoside salvage pathway [Yamada et al., 1988]) fail to respond not only to IgE-directed ligands, but also to Ca^{2+} ionophores [Marquardt et al., 1987; Wilson et al., 1988; Wilson et al., 1989; Mulkins et al., 1992].

My results also extend previous findings concerning the central role of guanine nucleotides in the stimulation of exocytosis from other myeloid

granulocytes. It has previously been reported that whereas GDP inhibits azurophilic granule exocytosis stimulated by Ca^{2+} from rabbit neutrophils permeabilised by treatment with *Sendai* virus, GTP-γ-S can induce exocytosis at concentrations of Ca^{2+} as low as 10^{-10}M [Barrowman et al., 1986]. From this it was inferred that there was an essential role for a GTP-binding protein, G_E [Gomperts et al., 1986]. GTP-γ-S also induces extensive Ca^{2+} independent secretion of hexosaminidase from guinea pig eosinophils permeabilised by SL-O in glutamate: in these cells, Ca^{2+} by itself is unable to induce secretion even when ATP is provided [Nusse et al., 1990].

4.3.3 GTP-binding proteins in constitutive secretion

These results stand in contrast to experience of the role of GTP in the constitutive interactions of the various pathways of vesicular traffic in endocytosis and exocytosis [Miller & Moore, 1991; Melançon et al., 1987; Salminen & Novick, 1987]. The GTP-binding proteins determining directionality and accuracy in the constitutive pathway of secretion in mammalian cells appear to be members of the *rab* family [Goud et al., 1990; Chavrier et al., 1990; Balch, 1990; Fischer von Mollard et al., 1990; Südhof & Jahn, 1991]. It is widely accepted that the hydrolysis of GTP, by allowing GTP-binding proteins (eg *SEC4*, *rab3a*) to cycle repeatedly between membrane-bound and free states, enables continuous delivery of products along a succession of compartments and finally to the eventual point of release [Walworth et al., 1989; Bourne, 1988; Bourne et al., 1990; Bacon et al., 1989; Fischer von Mollard et al., 1991; Novick et al., 1988; Miller & Moore, 1991]. Inhibition of hydrolysis (or the use of non-hydrolysable analogues of GTP) freezes the process of vesicle delivery. In the myeloid granulocytes, non-hydrolysable analogues provide the most potent stimulus to secretion, and under conditions where hydrolysis of GTP should be inhibited there is an enhancement of secretion.

4.3.4 Comparison of the effects of Mg^{2+} on secretion and the G_s-regulated adenylyl cyclase system

At a functional level at least, my observations, especially the various effects of Mg^{2+}, recall the activation of adenylyl cyclase. As with the stimulation of secretion from permeabilised mast cells, a low level

stimulation of adenylyl cyclase can be induced by non-hydrolysable analogues of GTP in the absence of an activated hormone receptor but in the presence of Mg^{2+} [Iyengar, 1981; Iyengar & Birnbaumer, 1981; Iyengar & Birnbaumer, 1982]. When an activating ligand for the receptor is supplied the extent and rate of cAMP production are markedly increased and the dependence on Mg^{2+} is diminished 1000 fold [Iyengar & Birnbaumer, 1982]. Similarly, in the activation of exocytosis, Ca^{2+} increases the rate and extent of secretion and more importantly brings about a systematic enhancement in the affinity for GTP-γ-S and Mg^{2+}. In these respects at least, G_E appears to operate in a similar manner to G_S in that it is able to stimulate its target (the fusion apparatus) on provision of a non-hydrolysable guanine nucleotide in the absence of a receptor-directed ligand (ie. Ca^{2+}) so long as Mg^{2+} is present. The kinetic pattern of activation of G_E (as measured by secretion in Cl^- based buffers), which is characterised by a Mg^{2+}-dependent onset delay, also has some similarity with G_S [see Chapter 5; Lillie & Gomperts, 1992b; Lillie & Gomperts, 1992c].

4.3.5 Anion effects on trimeric GTP-binding proteins

The receptor-linked GTP-binding proteins have also been shown to be sensitive to the anionic environment [Higashijima et al., 1987b]. However, for those that have been examined, the effects appear to be the opposite to those which have been inferred regarding the interaction of Cl^- on G_E. In the case of G_O, Cl^- inhibits the GTP hydrolysis, while enhancing the affinity for both GTP and GTP-γ-S due to an effect on the rate of dissociation. Similar mechanisms may underlie the enhancing effect of chloride and azide on the stimulation of adenylyl cyclase by hormones, fluoride and GTP-analogues [Johnson et al., 1975; Svoboda & Christophe, 1978]. Either way, the effect of Cl^- in all these systems (including exocytosis [Churcher & Gomperts, 1990]) is expressed at low concentrations ($<$20mM) and on the basis of investigations on G_O, a binding site for the Cl^- ion has been suggested [Higashijima et al., 1987b]. So far as I am aware, there are no published data concerning the effect of glutamate (or other amino acids) on the activation of adenylyl cyclase or isolated GTP-binding proteins, so a direct comparison of data is not possible.

4.3.6 Does Ca^{2+} control nucleotide exchange on G_E ?

Due to the similarity between the activation of G_s by glucagon and guanine nucleotides and the activation of G_E by Ca^{2+} and guanine nucleotides, I would like to propose that guanine nucleotide exchange on G_E is catalysed by Ca^{2+} following binding to a calcium binding protein (C_E) which has the characteristics of an intracellular *pseudo-receptor* (see figure 7.1 (p127) for a schematic representaion of this idea). When this is stimulated (by Ca^{2+}) then the requirement for Mg^{2+} is obviated. Not only does the stimulated *pseudo-receptor* preclude the requirement for Mg^{2+}, but it also increases the extent of the secretory response by at least ten-fold, enhancing the response to any given concentration of guanine nucleotide. Although the low molecular weight GTP-binding proteins related to *ras* are widely understood to be regulated by soluble exchange proteins (GnRPs [Bourne et al., 1990]) located in the cytosol, the signal transducing G-proteins of the heterotrimeric class are, in general, subject to regulation by cell surface receptors. An exception to this rule may be the growth associated protein (GAP-43) of neurite growth cones which can regulate the binding affinity for GTP-γ-S of G_o when these are reconstituted together *in vitro* [Strittmatter et al., 1990]. Another possible example may be the trimeric GTP-binding proteins involved in the control of vesicle trafficking [Burgoyne, 1992], which appear to be located on internal membranes (eg Golgi membrane [Stow et al., 1991]). It would seem likely that these are also regulated by some sort of intracellular receptor, to control the overall transport flux. If the interpretation of my data is correct, and C_E can eventually be shown to have the properties of a *pseudo-receptor* acting to catalyse nucleotide exchange on G_E, then this will represent another example of an intracellular protein controlling the activity of a trimeric GTP-binding protein.

Chapter 5

Mg^{2+} Modulates the Kinetics of Guanine Nucleotide and Ca^{2+} Induced Secretion

5.1 Introduction

By measuring the kinetics of secretion, as opposed to completed events, a very different view of the role of the effectors, Ca^{2+} and guanine nucleotides, in the regulation of exocytosis can be obtained [Gomperts & Tatham, 1988; Tatham & Gomperts, 1989]. The experiments described in this chapter were devised in order to achieve a description of the kinetics of exocytosis from permeabilised rat mast cells

This preparation represents the simplest that still allows a full exocytotic response to Ca^{2+} and guanine nucleotide. ATP was excluded. I have measured the rate of onset of exocytosis from permeabilised mast cells following the successive application of guanine nucleotides (GTP or GTP-γ-S) and then Ca^{2+} (or in the reverse order) in the presence and absence of Mg^{2+}. In contrast with the lack of effect of Mg^{2+} on the sensitivity of secretion to GTP-γ-S, Mg^{2+} profoundly alters the kinetics of GTP-γ-S triggered secretion. The results of Mg^{2+} deprivation suggest that the GTP-binding protein G_E shares several features in common with the trimeric GTP-binding proteins, and are not consistent with the role of Mg^{2+} in the function of monomeric GTP-binding proteins.

5.2 Results

5.2.1 Effect of Mg^{2+} on GTP triggered secretion

The experiment shown in figure 5.1 illustrates the timecourse of hexosaminidase secretion from rat mast cells which, following treatment

(5 minutes) with metabolic inhibitors, were permeabilised in the presence of Ca^{2+} (pCa5) and then triggered to release one minute later by addition of a range of concentrations of GTP in the absence (a) and presence (b) of Mg^{2+}. In agreement with earlier observations [chapters 3 + 4 and Lillie & Gomperts, 1992a] much higher concentrations of GTP are needed to elicit exocytosis when Mg^{2+} is provided and the maximal extent of secretion never exceeds 60%. In the absence of Mg^{2+}, and at high concentrations of GTP (10^{-3}M), exocytosis commences, and is already established at its maximum rate within 6 seconds (ie the time of removal of the first sample). At lower concentrations, which are nonetheless capable of inducing a near maximal extent of secretion, there are delays in the onset of exocytosis which become more prolonged as the concentration of GTP is further reduced. When the cells are stimulated by GTP in the presence of Mg^{2+} (figure 5.1b) an onset delay is clearly manifest even at 10^{-3} M. Again, these delays become more prolonged as the concentration of GTP is reduced.

5.2.2 Late addition of Mg^{2+} to GTP triggered cells

The effect of late delivery of Mg^{2+} to cells already stimulated to secrete is shown in figure 5.2. These cells were initially loaded with Ca^{2+} (pCa5) and then triggered to secrete by addition of a range of GTP concentrations (note: cf figure 5.1, the highest concentration of GTP in this experiment was 10^{-4}M). The left hand panel (figure 5.2a) represents the control experiment, and as before (figure 5.1a), this reveals the development of an extended onset delay as the concentration of the guanine nucleotide is reduced. In setting up the conditions for the experiment of figure 5.2b, the aim was to add Mg^{2+} (5mM) to the system at a time when experience dictated that exocytosis would be proceeding at its maximal rate. As can be seen, the effect of the addition of Mg^{2+} at this time is to terminate the progress of exocytosis. In subsequent experiments (not shown) I have found that the arrest of secretion (expressed as the divergence of the progress curves) occurs over a period of about 10 seconds following the late addition of Mg^{2+}. The time taken for secretion to cease remains constant regardless of the concentration of the stimulating GTP (in the range 10^{-3} - 10^{-5}M).

Figure 5.1. Time course of GTP-triggered exocytosis from mast cells permeabilised by SL-O in the presence and absence of Mg^{2+}.

Mast cells, suspended in buffered NaCl and pretreated with metabolic inhibitors were permeabilised in the presence of calcium buffer (a: HEDTA, 5mM; b: EGTA, 5mM) and in the absence (a) or presence (b) of Mg^{2+}, 2mM. After 1 minute, GTP was added at concentrations indicated and timed samples were withdrawn for analysis of secreted hexosaminidase. GTP concentration: ●, 10^{-3}M; ○, $10^{-3.5}$M; ■, 10^{-4}M; □, $10^{-4.5}$M; ▲, 10^{-5}M; △, $10^{-5.5}$M.

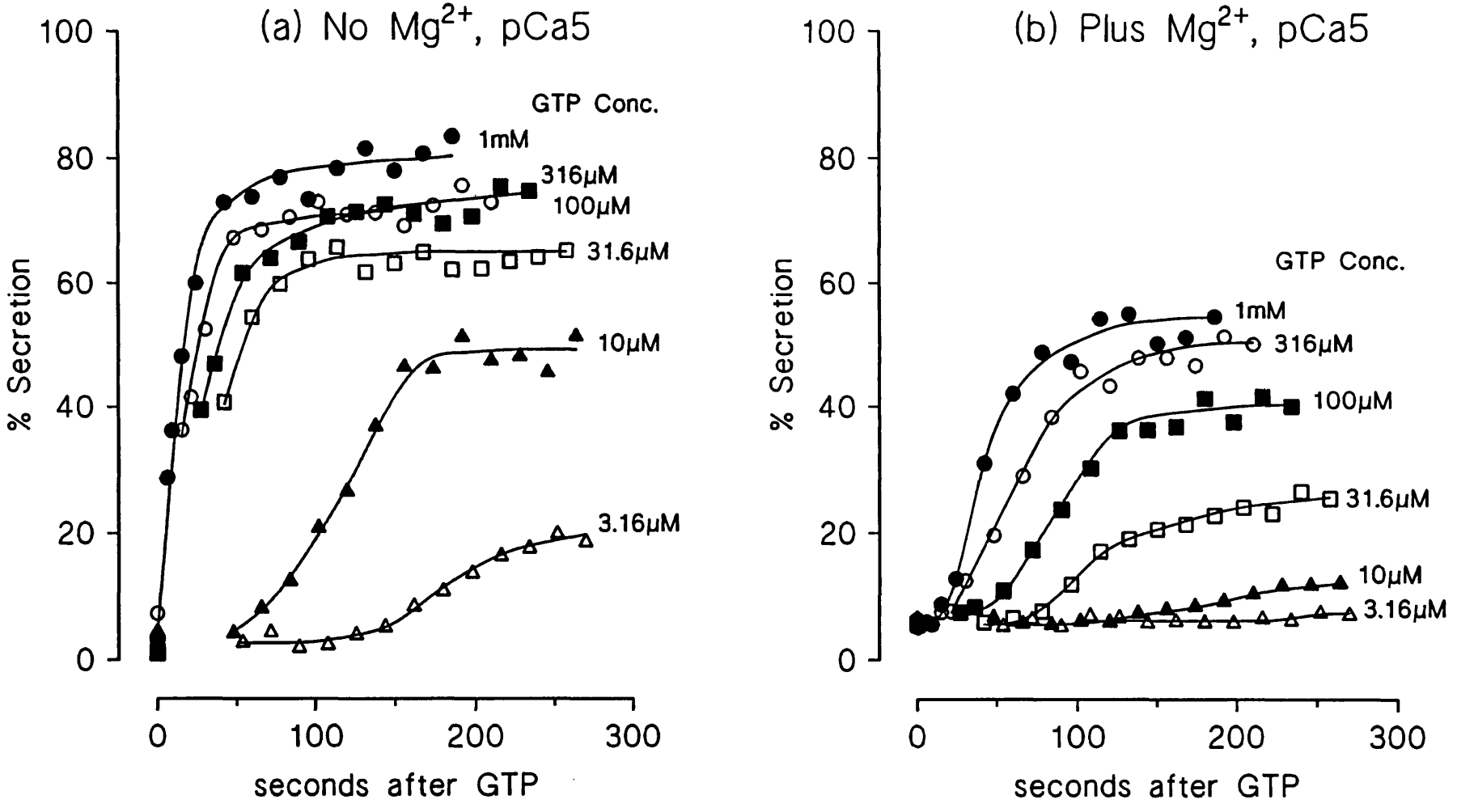
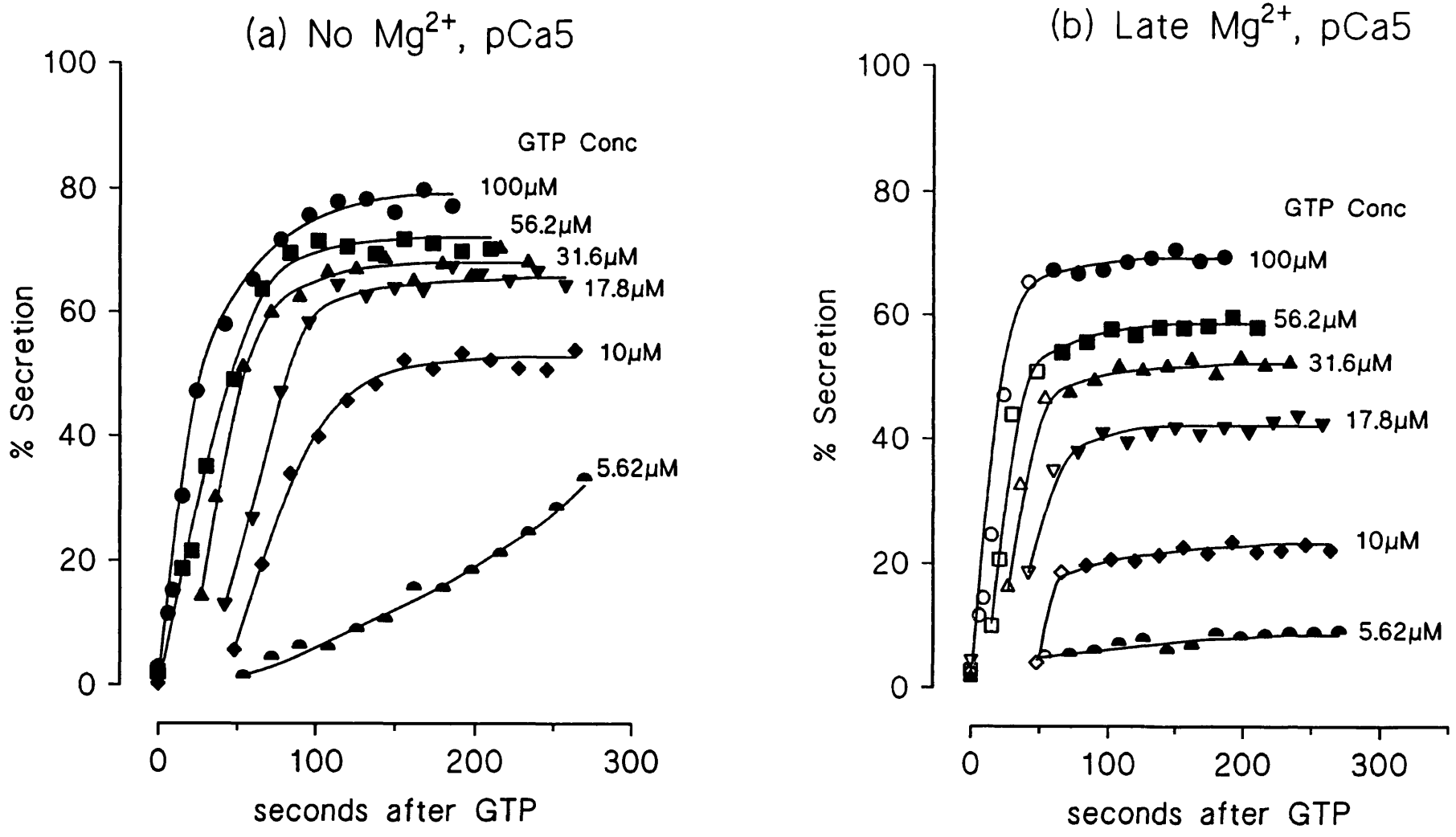

(a) No Mg2+, pCa5
(b) Plus Mg2+, pCa5
% Secretion
seconds after GTP
GTP Conc.
1mM
316μM
100μM
31.6μM
10μM
3.16μM
0
20
40
60
80
100
0
100
200
300

Figure 5.2. Effect of late addition of Mg^{2+} to cells undergoing exocytosis in response to a GTP trigger.

Mast cells, suspended in buffered NaCl and pretreated with metabolic inhibitors were permeabilised in the presence of calcium buffer (EGTA, 5mM, pCa5) and triggered one minute later by addition of GTP at concentrations indicated. Timed samples were withdrawn and processed as described in figure 5.1. In b) $MgCl_2$ (5mM) was then added to the secreting cells after about 1 minute (change from open to closed symbols) and sampling was continued.
GTP concentration: ●, 10^{-4}M; ■, $10^{-4.25}$M; ▲, $10^{-4.5}$M; ▼, $10^{-4.75}$M; ◆, 10^{-5}M; ◓, $10^{-5.25}$M.


(a) No Mg2+, pCa5
(b) Late Mg2+, pCa5
% Secretion
seconds after GTP
GTP Conc
100μM
56.2μM
31.6μM
17.8μM
10μM
5.62μM
0
20
40
60
80
100
0
100
200
300

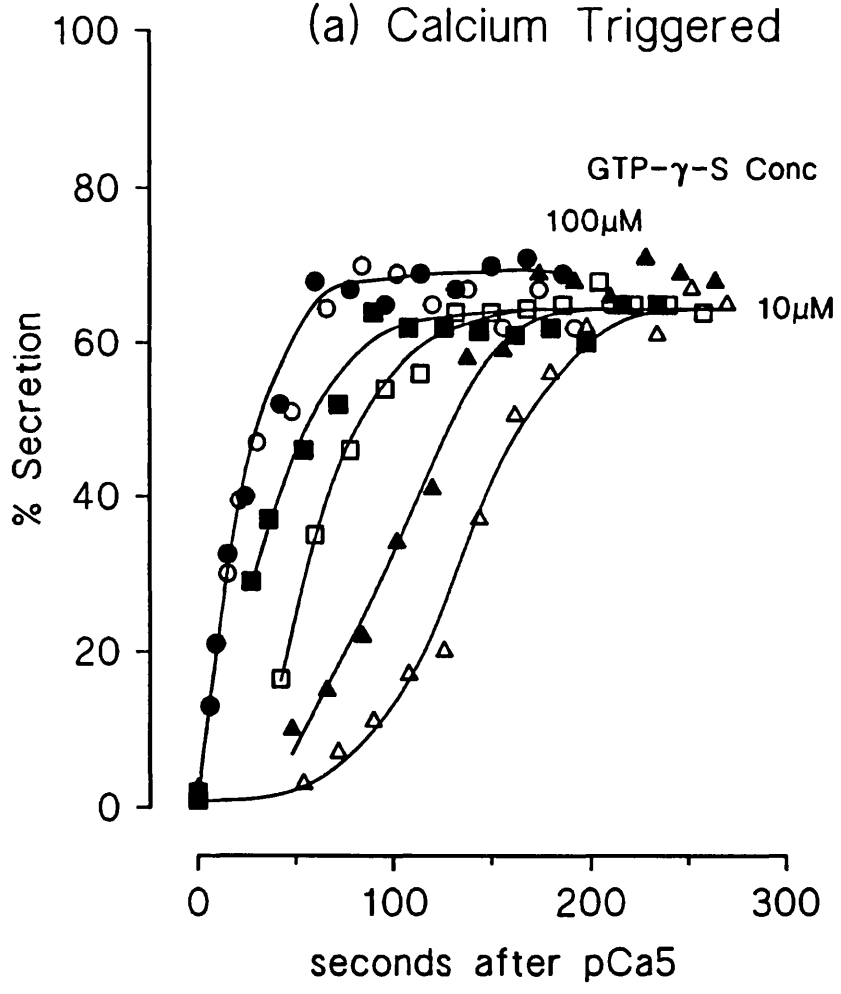
When GTP-γ-S (instead of GTP) is used as the stimulus, Mg^{2+} neither affects the sensitivity to the guanine nucleotide concentration [Lillie & Gomperts, 1992a], nor does it arrest ongoing secretion (not shown). It is therefore reasonable to conclude that the suppressive effects of Mg^{2+} on GTP-stimulated secretion arise as a result of GTP hydrolysis (figures 5.1b and 5.2b). Omission of Mg^{2+} in the present experiments implies a free concentration of Mg^{2+} of 10^{-9}M or less, which is probably below the level required to support the GTPase function of either trimeric [Chavrier et al., 1990; Brandt & Ross, 1986; Higashijima et al., 1987a] or monomeric [Frech et al., 1990; Kabcenell et al., 1990; Burstein & Macara, 1992] GTP-binding proteins. These results thus show that regulated exocytosis in the mast cell is well supported by GTP so long as precautions are taken to prevent its hydrolysis. Unlike the traffic of vesicles through the constitutive secretory pathway which is inhibited by non-hydrolysble analogues of GTP [Bourne, 1988; Miller & Moore, 1991], the stimulus to regulated secretion in mast cells is strongest under conditions in which the GTP-binding protein is maintained in an activated state. It is also worthy of note that in those monomeric GTP-binding proteins where it has been studied, GTP has a higher binding affinity than its non-hydrolysable analogues ($p21^{ras}$ [Feuerstein et al., 1989] and $p21^{RalA}$ [Frech et al., 1990]). Clearly, even under conditions when GTPase activity is inhibited, GTP never becomes a more effective stimulus to secretion than its non-hydrolysable analogues.

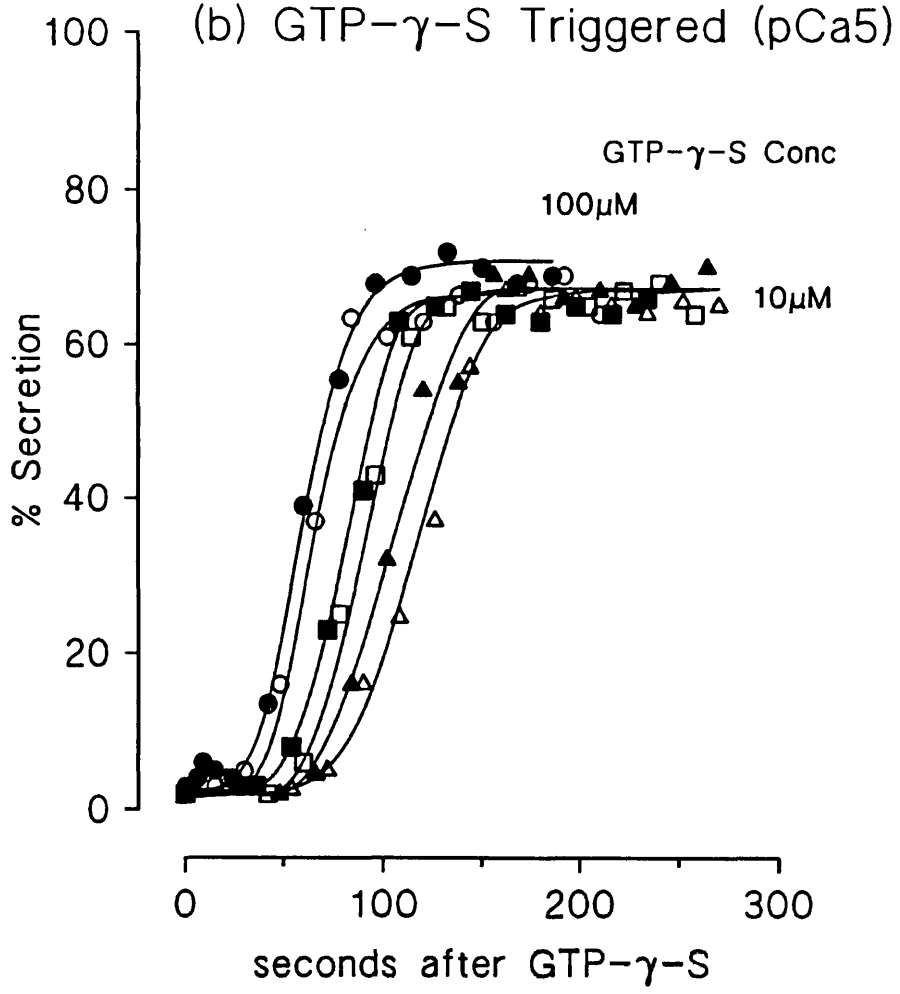
5.2.3 Mg^{2+} affects the kinetics of GTP-γ-S induced secretion

In contrast to the failure of Mg^{2+} to control the sensitivity to GTP-γ-S [Lillie & Gomperts, 1992a] and its inability to arrest secretion elicited by GTP-γ-S, Mg^{2+} does affect the kinetics of onset of exocytosis induced by GTP-γ-S. As shown in the experiment illustrated in figure 5.3, these delays are sensitive to the order in which Ca^{2+} and GTP-γ-S are supplied to the permeabilised cells. The cells were either (a) permeabilised in the presence of a range of concentrations of GTP-γ-S and then triggered by addition of Ca^{2+} (pCa5), or (b) permeabilised in in the presence of Ca^{2+} (pCa5) and then triggered with a range of GTP-γ-S. In this experiment, Mg^{2+} was omitted and Ca^{2+} was buffered using HEDTA. Note also that the concentrations of GTP-γ-S were stepped at 0.2 logarhythmic intervals. Delays preceding secretion are clearly manifest when GTP-γ-S is used to trigger exocytosis from cells previously loaded with Ca^{2+} (figure 5.3b).

Figure 5.3. Time course of Ca^{2+}-triggered and GTP-γ-S-triggered exocytosis from mast cells permeabilised by SL-O in the absence of Mg^{2+}.

Mast cells, suspended in buffered NaCl and pretreated with metabolic inhibitors were permeabilised in the absence of Mg^{2+} ($<10^{-9}$M) and the presence of a) GTP-γ-S at concentrations indicated or b) calcium buffer (HEDTA, 5mM, pCa5). One minute later exocytosis was triggered by addition of the complementary effector (a: Ca^{2+}, pCa5; or b: GTP-γ-S at concentrations indicated) and timed samples were withdrawn and processed for measurement of secreted hexosaminidase. GTP-γ-S concentration: ●, 10^{-4}M; ○, $10^{-4.2}$M; ■, $10^{-4.4}$M; □, $10^{-4.6}$M; ▲, $10^{-4.8}$M; △, 10^{-5}M.


(a) Calcium Triggered
100
80
60
40
20
0
% Secretion
GTP-γ-S Conc
100μM
10μM
0
100
200
300
seconds after pCa5


(b) GTP-γ-S Triggered (pCa5)
100
80
60
40
20
0
% Secretion
GTP-γ-S Conc
100μM
10μM
0
100
200
300
seconds after GTP-γ-S

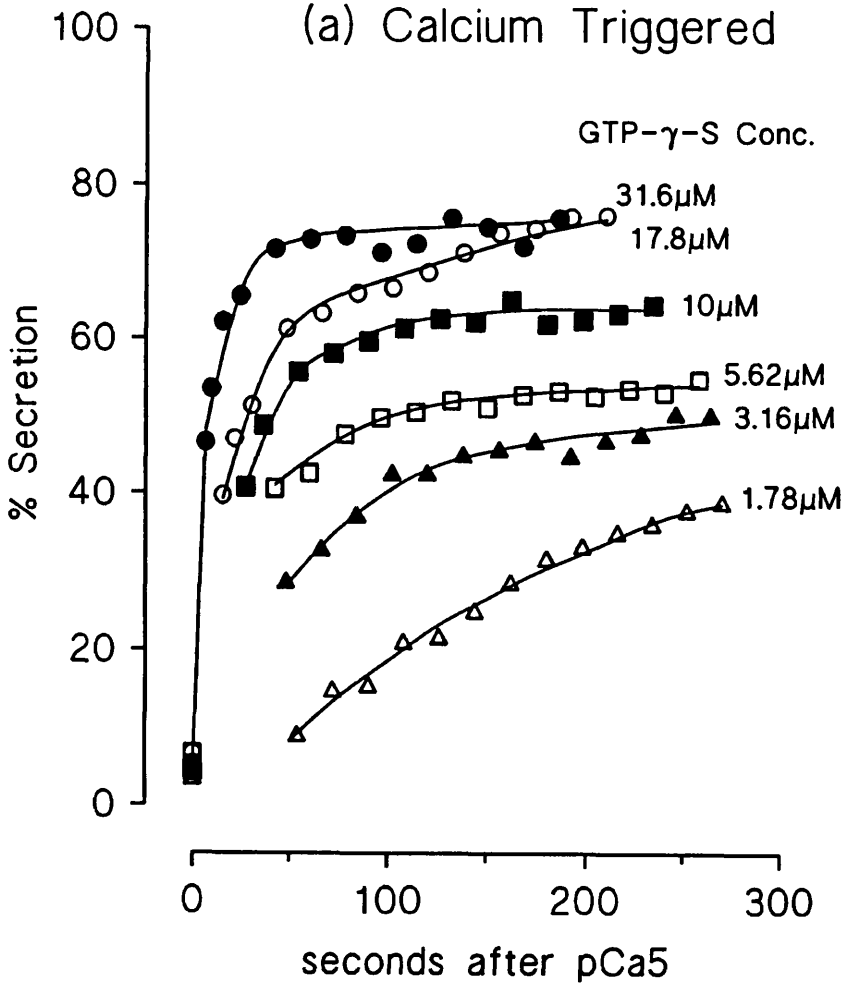
These delays appear to have two components, one dependent on the concentration of GTP-γ-S, the other independent. The GTP-γ-S concentration independent component is expressed as the failure of higher concentrations of GTP-γ-S (100μM - 1mM) to abolish the initial (30 second) delay. The GTP-γ-S concentration dependent delay is seen as the systematic extension of the Mg^{2+} dependent delay as the concentration of GTP-γ-S is reduced. The length of the initial (concentration independent) delay appears to be dependent on the identity of the guanine nucleotide. From figure 5.1a and figure 5.2a it is clear that there is no limiting delay in the absence of Mg^{2+}, but there is a concentration dependent extension of the delay as the concentration of GTP is lowered. I have also performed similar experiments with GppNHp as the triggering guanine nucleotide, and in this case there is a (Mg^{2+} dependent) limiting delay of 100 seconds before secretion commences (data not shown). It is therefore possible that this delay represents a post-binding event, such as activation, which occurs at different rates depending on the identity of the bound guanine nucleotide.

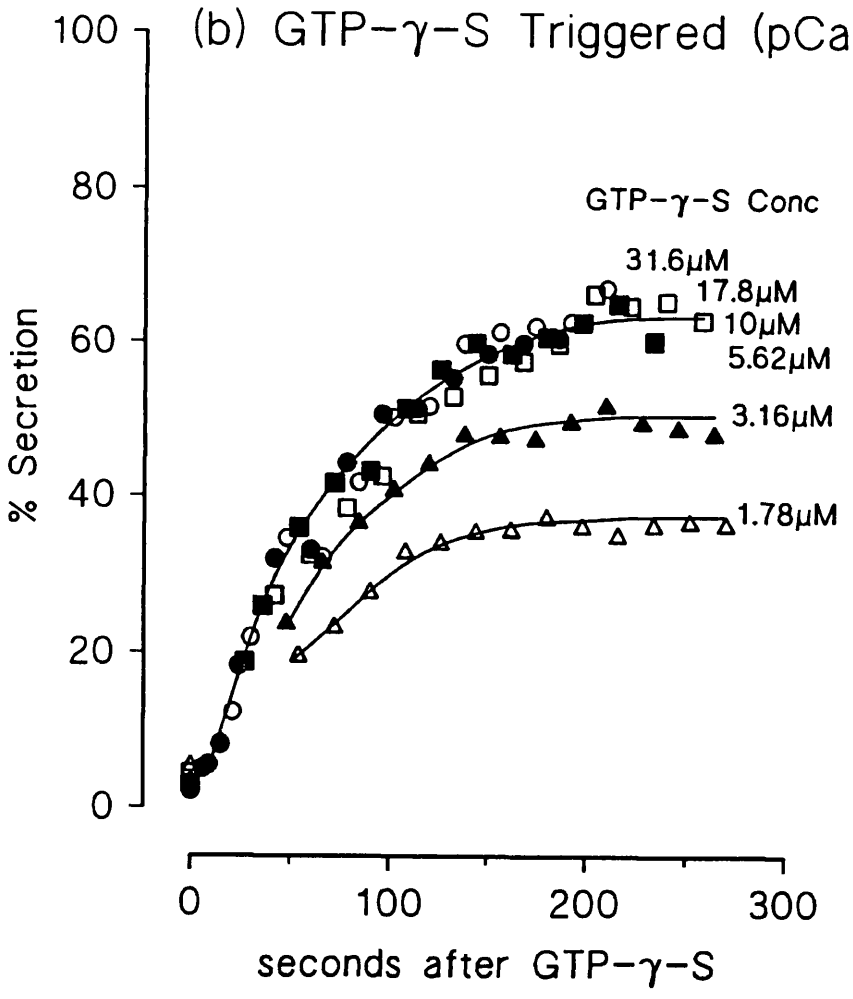
When Ca^{2+} is used as the trigger, and if the concentration of GTP-γ-S is sufficiently high (in this experiment, at concentrations at and above $10^{-4.4}$M), then the onset of exocytosis is abrupt, but if this is reduced even slightly ($10^{-4.6}$M), then onset delays once again become apparent.

The experiment illustrated in figure 5.4 is similar to that of figure 5.3 except that 2mM Mg^{2+} was provided (and EGTA was used to regulate pCa). In the presence of Mg^{2+}, the onset of secretion in cells loaded and triggered with either combination of Ca^{2+} and GTP-γ-S occurs without any substantial delay. There is a short onset delay when Ca^{2+} loaded cells are triggered with GTP-γ-S in the presence of Mg^{2+} (figure 5.4b), which may represent a diffusion dependent delay. Secretion from cells preloaded with Ca^{2+} (pCa5) and then triggered by addition of GTP-γ-S (figure 5.4b) occurs more slowly than when the cells are preloaded with GTP-γ-S and then triggered by addition of Ca^{2+} (figure 5.4a).

Figure 5.4. Time course of Ca^{2+}-triggered and GTP-γ-S-triggered exocytosis from mast cells permeabilised by SL-O in the presence of Mg^{2+}.

Mast cells, suspended in buffered NaCl and pretreated with metabolic inhibitors were permeabilised in the presence of 1mM Mg^{2+} and either a) GTP-γ-S at concentrations indicated or b) calcium buffer (EGTA, 5mM, pCa5). One minute later exocytosis was triggered by addition of the complementary effector (a: Ca^{2+}, pCa5; or b: GTP-γ-S at concentrations indicated) and timed samples were withdrawn and processed for measurement of secreted hexosaminidase. GTP-γ-S concentration: ●, $10^{-4.5}$M; ○, $10^{-4.75}$M; ■, 10^{-5}M; □, $10^{-5.25}$M; ▲, $10^{-5.5}$M; △, $10^{-5.75}$M.


(a) Calcium Triggered
GTP-γ-S Conc.
31.6μM
17.8μM
10μM
5.62μM
3.16μM
1.78μM
% Secretion
100
80
60
40
20
0
0
100
200
300
seconds after pCa5


(b) GTP-γ-S Triggered (pCa5)
GTP-γ-S Conc
31.6μM
17.8μM
10μM
5.62μM
3.16μM
1.78μM
% Secretion
100
80
60
40
20
0
0
100
200
300
seconds after GTP-γ-S

The delays in the onset of secretion in the absence of Mg^{2+} could reflect the time taken for GTP-γ-S to diffuse to its site of action or the time taken for GTP-γ-S to bind to G_E and then induce activation (eg. by conformational change or sub-unit dissociation). Either of these processes must have been overlooked in previous experiments in which GTP-γ-S was always allowed to equilibrate first, and Ca^{2+} applied as the trigger [Tatham & Gomperts, 1989]. I propose that because the delays are sensitive to Mg^{2+}, they are likely to reflect binding to and activation of a GTP-binding protein and not diffusion of the guanine nucleotide. Diffusion is almost certainly a small component, probably represented by the minimal delay in the presence of Mg^{2+} in GTP-γ-S triggered cells (figure 5.4b).

5.3 Discussion

The experiments described here were carried out in order to learn about the timecourse of secretion and the nature of the GTP-binding protein, G_E, which mediates exocytosis. The results are interpreted according to the established paradigms of the trimeric GTP-binding proteins involved in signal transduction (particularly G_S [Birnbaumer et al., 1985; Premont & Iyengar, 1990; Birnbaumer et al., 1990]) and the monomeric GTP-binding proteins related to the *ras* gene products [Hall, 1992a].

In particular I have studied the ability of Mg^{2+} to modulate the kinetics of the onset of secretion. In this work I have used Cl^- based buffers rather than the glutamate system from which the original observations pointing to the serial interaction between C_E and G_E was inferred [chapter 4 and Lillie & Gomperts, 1992a]. This is because cells permeabilised in iso-osmotic glutamate lose competence so rapidly (ie <1 minute in the absence of ATP) that it is necessary to provide both effectors simultaneously with the permeabilising agent (SL-O) in order to achieve a significant extent of secretion. It follows that the onset of secretion is masked by artefacts of permeabilisation and diffusion of both effectors to their sites of action. In Cl^- based media it is possible to permeabilise the cells and equilibrate the cytosol with the one effector and then to apply the second as a trigger. This reduces the variables controlling the onset of exocytosis.

5.3.1 Effects of Mg^{2+} on monomeric and trimeric GTP-binding proteins

It is well recognised that Mg^{2+} strongly affects the binding and activation of the trimeric GTP-binding proteins by guanine nucleotides [Iyengar & Birnbaumer, 1982; Iyengar, 1981; Higashijima et al., 1987a; Higashijima et al., 1987c; Higashijima et al., 1987d]. A salient effect of Mg^{2+} exclusion is that it allows GTP-γ-S to readily dissociate from α-subunits [Higashijima et al., 1987d]. When Mg^{2+} is provided, binding of GTP-γ-S becomes rapid and irreversible. Not only this, but high (1-100 mM, depending on the α-subunit) concentrations of Mg^{2+} stimulate activation of G_s [Brandt & Ross, 1985], G_i [Higashijima et al., 1987d] and G_o [Katada et al., 1986] by promoting GDP and ßγ-subunit dissociation. In contrast to this, the absence of Mg^{2+} actually increases the affinity of isolated p21 *ras* [Hall & Self, 1986] for GTP (relative to GDP) due to a marked increase in the off-rate of GDP [Feuerstein et al., 1987]. The absence of Mg^{2+} therefore promotes the activation of $p21^{ras}$, and this mechanism appears to be applicable to most monomeric GTP-binding proteins [Shoji et al., 1989; Kuroda et al., 1989; Wagner et al., 1987; Burstein & Macara, 1992; Frech et al., 1990], though not all [Kabcenell et al., 1990].

I believe that the results I have obtained, especially the modulatory effects of Mg^{2+} on the onset kinetics of exocytosis, can best be explained in terms of interactions with a GTP-binding protein having features in common with G_s [Birnbaumer et al., 1985; Premont & Iyengar, 1990]. However, it must be noted that modulation of secretion by Mg^{2+} is expressed at much lower concentrations than on the adenylyl cyclase system ($<$1mM vs 1 - 20mM). This may be due to Ca^{2+} acting through a *pseudo-receptor*, C_E, to accelerate guanine nucleotide exchange on G_E similar to the effect of providing glucagon to the adenylyl cyclase system of hepatocyte membranes [Birnbaumer et al., 1985].

5.3.2 Triggering with GTP-γ-S rather than Ca^{2+} reveals delays in the onset of exocytosis, which are Mg^{2+} sensitive

When Mg^{2+} is provided to the permeabilised mast cells there is a brief delay (~ 3 seconds) preceding the onset of GTP-γ-S triggered secretion (figure 5.4b). This delay probably registers the time taken for diffusion and for the activation of the GTP-binding protein after nucleotide binding. In

the absence of Mg^{2+} the activation by GTP-γ-S is retarded as indicated by extended delays at all concentrations (figure 5.3b). If one assumes that the diffusion component of the delay is unaffected by the presence or absence of Mg^{2+} then the Mg^{2+} sensitive delays are likely to be due to the increased time taken for binding and activation. In this respect at least, there appears to be a parallel between the activation of exocytosis by G_E and the activation of adenylyl cyclase by G_S [Iyengar & Birnbaumer, 1981; Iyengar, 1981]. The extension of the delay preceding the onset of GTP-γ-S-induced secretion in the absence of Mg^{2+} suggests that binding is now reversible.

Such onset delays appear to have two components, the one independent, the other dependent on the concentration of the guanine nucleotide (figure 5.3b). The concentration independent component is seen as the delay of about 30 seconds that occurs with 100μM GTP-γ-S, and cannot be shortened by addition of higher concentrations of GTP-γ-S (I have tested 1mM GTP-γ-S which has the same 30 second delay as 100μM GTP-γ-S). The concentration dependent component is clearly seen as the systematic extension of the delays as the concentration of GTP-γ-S is lowered. If one can assume that the binding of GTP-γ-S is reversible when Mg^{2+} is excluded (as for the trimeric GTP-binding proteins such as G_S and G_O [Higashijima et al., 1987d]) then the concentration-dependent component of the delays might register the binding reaction. The concentration-independent component, which is sensitive to the identity of the stimulating nucleotide (GTP < GTP-γ-S < GppNHp), could reflect the time taken for the GTP-binding protein to assume its active conformation or for dissociation of a trimeric complex. Either of these would take a fixed time once the guanine nucleotide has attached, regardless of its concentration and might be expected to be sensitive to the identity of the activating nucleotide [Iyengar & Birnbaumer, 1981].

5.3.3 Kinetics of exocytosis from Ca^{2+} triggered cells

For cells triggered by Ca^{2+} after equilibration with high concentrations of GTP-γ-S, exocytosis commences promptly both in the absence (figure 5.3a) and presence (figure 5.4a) of Mg^{2+}. This immediacy of response in the absence of Mg^{2+} is most likely due to the binding of GTP-γ-S which occurs during the 1 minute pre-incubation before the Ca^{2+} trigger is applied. However, for lower concentrations of GTP-γ-S (<10μM) and in

the absence of Mg^{2+} there are onset delays of similar duration to those which occur in cells initially equilibrated with Ca^{2+} and then triggered with the same concentration of GTP-γ-S (figure 5.3b). Under these conditions, the delay is not determined by the order of addition of the two effectors and probably reflects the failure of GTP-γ-S binding to reach a stimulatory level by the time the secondary trigger (Ca^{2+}) is applied. The addition of the Ca^{2+} trigger would then accelerate the rate of binding of GTP-γ-S to the GTP-binding protein if it is indeed acting as a *pseudo-receptor* (as previously suggested in chapter 4 and [Lillie & Gomperts, 1992a]). This would give similar results in terms of onset delays regardless of whether the cells were equilibrated with GTP-γ-S before the Ca^{2+} was supplied, which is what I have observed (figure 5.3a & b).

5.3.4 Ability of Mg^{2+} to halt GTP and Ca^{2+} induced secretion

The inhibition of ongoing secretion from cells stimulated by GTP (but not GTP-γ-S) by late addition of Mg^{2+} (figure 5.2) is likely to be due to activation of GTPase activity (which would have been inhibited in the absence of Mg^{2+}). These experiments cannot directly reveal whether the cessation is due to depletion of GTP in the cell interior arising from the generalised activity of intracellular Mg^{2+} dependent nucleotidases or whether it is due to the conversion of GTP to GDP on the G_E protein itself. However, it seems improbable that the concentration of GTP (applied as high as 1mM) inside cells treated with SL-O and therefore readily accessible to solutes having the dimensions of LDH could drop so precipitously as to terminate secretion within seconds. Furthermore, were the cessation of secretion due to a generalised action of GTPases within the cells, one would anticipate that the rate of turn-off would be faster at the lower concentrations of GTP. This is not the case as the time for cessation of secretion shows no appreciable difference between 1mM, 100μM and 10μM GTP.

Another effect of GTP-hydrolysis on the kinetics of trimeric GTP-binding protein activation is a reduction in the maximal steady-state level of GTP-bound protein, regardless of the concentration of GTP supplied. By inactivating the GTPase, not only is the sensitivity to GTP increased, but also the maximum proportion of the GTP-binding protein in its active (GTP bound) form [Higashijima et al., 1987a]. I have found that the final extent of secretion induced by GTP in the presence of Mg^{2+} is reduced

(maximum 60%). This cannot be overcome by raising the concentration of GTP. This result is exactly that which would be predicted if it was the GTPase activity of G_E that was affected by Mg^{2+}, and conflicts with the expectation of the operation of non-specific GTPases.

For all these reasons I favour the idea that the secretion halts because of hydrolysis of GTP by the G_E protein itself. This being the case, the ability of late Mg^{2+} addition to halt ongoing secretion suggests that GTP hydrolysis is not conditional on an interaction with downstream targets. Such conditionality of GTP hydrolysis is a hallmark of the monomeric GTP-binding proteins. Although interaction of some α-subunits with their effectors can increase the rate of GTP hydrolysis, [Arshavsky & Bownds, 1992; Berstein et al., 1992], the basal rate of GTP hyrolysis acts as a timeswitch for trimeric GTP-binding proteins, controlling the length of time that the protein remains in the active GTP bound form. If the operation of G_E were similar to the monomeric GTP-binding proteins involved in vesicle trafficking then removal of Mg^{2+} should freeze secretion by inhibiting GTP hydrolysis, which is necessary for vesicle fusion. The granule fusions would cease in the absence of Mg^{2+}, and re-addition of Mg^{2+} would allow its continuation. In contrast to this, I find that removal of Mg^{2+} potentiates GTP induced secretion, and its readdition causes the cessation of secretion. The fact that non-hydrolysable analogues of GTP provide the most potent stimulus to secretion, whereas they inhibit vesicle trafficking, also supports this view. It therefore appears that GTP hydrolysis on G_E is like the trimeric "timeswitch" rather than the conditional monomeric type.

5.3.5 Summary of results

In summary, the absence of Mg^{2+} can be seen to have two main effects. Firstly it potentiates GTP triggered secretion, which for the reasons I have stated I believe is due to the inhibition of GTP hydrolysis on G_E. Inhibition of GTP hydrolysis potentiates trimeric GTP-binding protein function, but generally inhibits monomeric GTP-binding protein function. Therefore it appears that G_E behaves more like a trimeric than a monomeric GTP-binding protein.

Secondly the absence of Mg^{2+} causes delays in the onset of GTP-γ-S induced secretion, particularly if GTP-γ-S is used as the triggering stimulus after permeabilised cells have been equilibrated with Ca^{2+}. The absence of

Mg^{2+} produces opposite effects on the activation of trimeric and monomeric GTP-binding proteins. For trimeric GTP-binding proteins, activation is slowed down as the off-rate of GTP (and particularly GTP-γ-S which is normally irreversibly bound) is increased. For monomeric GTP-binding proteins activation is speeded up, as the off-rate of GDP is increased. As activation of G_E appears to be delayed in the absence of Mg^{2+}, the effect of removal of Mg^{2+} on G_E bears similarity to the expected effect of the removal of Mg^{2+} on trimeric GTP-binding proteins.

In addition, these Mg^{2+} dependent delays appear to have a guanine nucleotide concentration dependent and a concentration independent component. The concentration dependent component of the delay probably reflects some aspect of the nucleotide binding reaction. The length of the concentration independent component of the delay is dependent on the identity of the activating guanine nucleotide, with the rank order for length of delay GppNHp> GTP-γ-S > GTP. Being that it is independent of the guanine nucleotide concentration, this portion of the delay may reflect some post-binding activation event. Similar delays are seen in the generation of cAMP by adenylyl cyclase stimulated by guanine nucleotides in the absence of Mg^{2+}. The length of these delays is also dependent on the identity of the guanine nucleotide with the same rank order for length of delay. Again, the behaviour of G_E in the absence of Mg^{2+} resembles the behaviour of trimeric GTP-binding proteins under the same conditions.

5.3.6 Limitations to the comparison of secretion with G_s regulated adenylyl cyclase

In this discussion I have tried to present my data concerning the kinetics of the exocytotic mechanism in the light of knowledge concerning adenylyl cyclase. I have stressed the similarities but there remain some problems of both a conceptual and a practical nature. In particular, it should be realised that in the investigation of exocytosis one is confronted with a process which proceeds as a series of all or none fusion events [Neher & Marty, 1982; Lindau & Gomperts, 1991; Fernandez et al., 1984] and is therefore not fully amenable to the conventions of kinetic analysis which are applicable to homogeneous systems. However, I have concentrated on the preceding events, not the exocytotic process itself. Furthermore, exocytosis by its very nature is a process which must necessarily

terminate when the granules become exhausted, and in most of my experiments this has allowed me a time window for measurement of about 2 minutes compared with the almost limitless duration (often 60 minutes) for sampling in a typical cyclase experiment. Thus although there are obvious similarities, there are also some serious limitations to the comparison, and as more becomes known of the kinetics of other GTP-binding protein mediated processes such as phospholipase C activation, other features of convergence and difference may become apparent.

In comparing my results with the established paradigms of other GTP-binding proteins, there is the further problem that there is little information available concerning the kinetics of activation of the monomeric proteins of the *ras* family for the reason that with a single exception ($p21^{rac}$, [Abo et al., 1991; Knaus et al., 1991]) the immediate downstream (effector) reactions are not yet known. However, I believe that I have presented a strong case for thinking that G_E, which mediates regulated exocytosis in myeloid cells, possesses some of the functional characteristics exemplified by G_S, and not those of the *ras/rab* families of monomeric GTP-binding proteins. An additional argument is the finding that exocytosis from permeabilised mast cells can be stimulated by aluminium fluoride [Sorimachi et al., 1988]. This is understood to activate the trimeric GTP-binding proteins (G_S, G_i, G_o, G_q and transducin) in their GDP-bound form [Ferguson et al., 1986] while having no discernible effect on low molecular weight GTP-binding proteins such as ADP-ribosylation factor (*arf*) or members of the *ras, rap* and *rab* families [Kahn, 1991].

Among the family of $\alpha\beta\gamma$ trimers, G_S may not finally prove to be the closest functional homologue, but for now it is the only one for which there is sufficient experience, especially relating to the kinetics of effector coupling, with which to make a critical comparison.

Chapter 6

Labelling GTP-binding proteins Inside SL-O Permeabilised Cells

With such a detailed description of G_E at a cell physiological level, the next step was to use this information to attempt to identify proteins with the predicted properties of G_E. From the cell physiology data contained in the last 3 chapters I would predict that G_E might have 4 distinct properties that would aid in its detection:-

1. Ca^{2+} might stimulate guanine nucleotide exchange on G_E (Chapters 4 and 5).

2. G_E might bind guanine nucleotides more strongly in glutamate than chloride based buffers (Chapter 4).

3. In the absence of Mg^{2+}, G_E may require Ca^{2+} to bind guanine nucleotides (Chapters 4 and 5).

4. In the presence of ATP, the affinity of G_E for guanine nucleotides might increase (Chapter 3).

6.1 Photo-affinity labelling of GTP-binding proteins

Since all these predicted properties of G_E involve guanine nucleotides, I have attempted to develop a method for affinity labelling GTP-binding proteins inside permeabilised cells with radioactive guanine nucleotides. Initially I used a photoaffinity analogue, azido-analido-GTP (supplied by Dr M. Rasenick of the University of Chicago, Illinois). This stimulates secretion rather poorly (30% hexosaminidase release maximum), and ^{32}P labelled compound failed to produce convincing labelling of GTP-binding proteins. Due to these poor initial results and the difficulty in obtaining the compound in sufficient quantities (particularly the radioactive compound,

which caused problems with customs clearance), I have developed a method of covalently linking unmodified guanine nucleotides to their binding sites inside permeabilised cells.

6.2 Affinity labelling of GTP-binding proteins by in situ oxidisation of guanine nucleotides

This method was developed for the labelling of the GTP binding site of pure GTP-binding proteins, such as EfTu [Peter et al., 1988], and has recently been applied to labelling GTP-binding proteins in cell extracts [Low et al., 1992]. The reaction scheme of the method is briefly outlined in Figure 6.1, and involves incubating permeabilised cells with α^{32}P-GTP (NEN DuPont) and then oxidising the GTP in situ with Sodium Periodate. The deoxyGTP resulting from this forms a Schiff's base with the amino terminus of the conserved lysine residue in the GTP binding site. Addition of Sodium Cyanoborohydride followed by Sodium Borohydride reduces this Schiff's base to a secondary amine, thus covalently linking the GTP to the protein. Radioactively labelled proteins can then be detected as described in the methods chapter.

The first experiments utilised ^{35}S-GTP-γ-S, as this is the most potent stimulus to secretion. This labelled a number of intracellular proteins (as SL-O was required for labelling), but a number of bands were not dependent on periodate for labelling. I suspected that this labelling was due to phosphorylation as it was inhibited by addition of cold ATP and incubation of cells with ^{35}S-ATP-γ-S produced a similar pattern of periodate independent labelling. I concluded that rat mast cells must possess a nucleotide diphosphate kinase which is capable of removing the terminal ^{35}S-labelled phosphate from GTP-γ-S and attaching it to residual ADP. The ATP-γ-S generated can then be used to thiophosphorylate proteins, thus labelling them independently of periodate. For this reason I switched to using α^{32}P labelled GTP, and this renders all labelled bands dependent on provision of periodate.

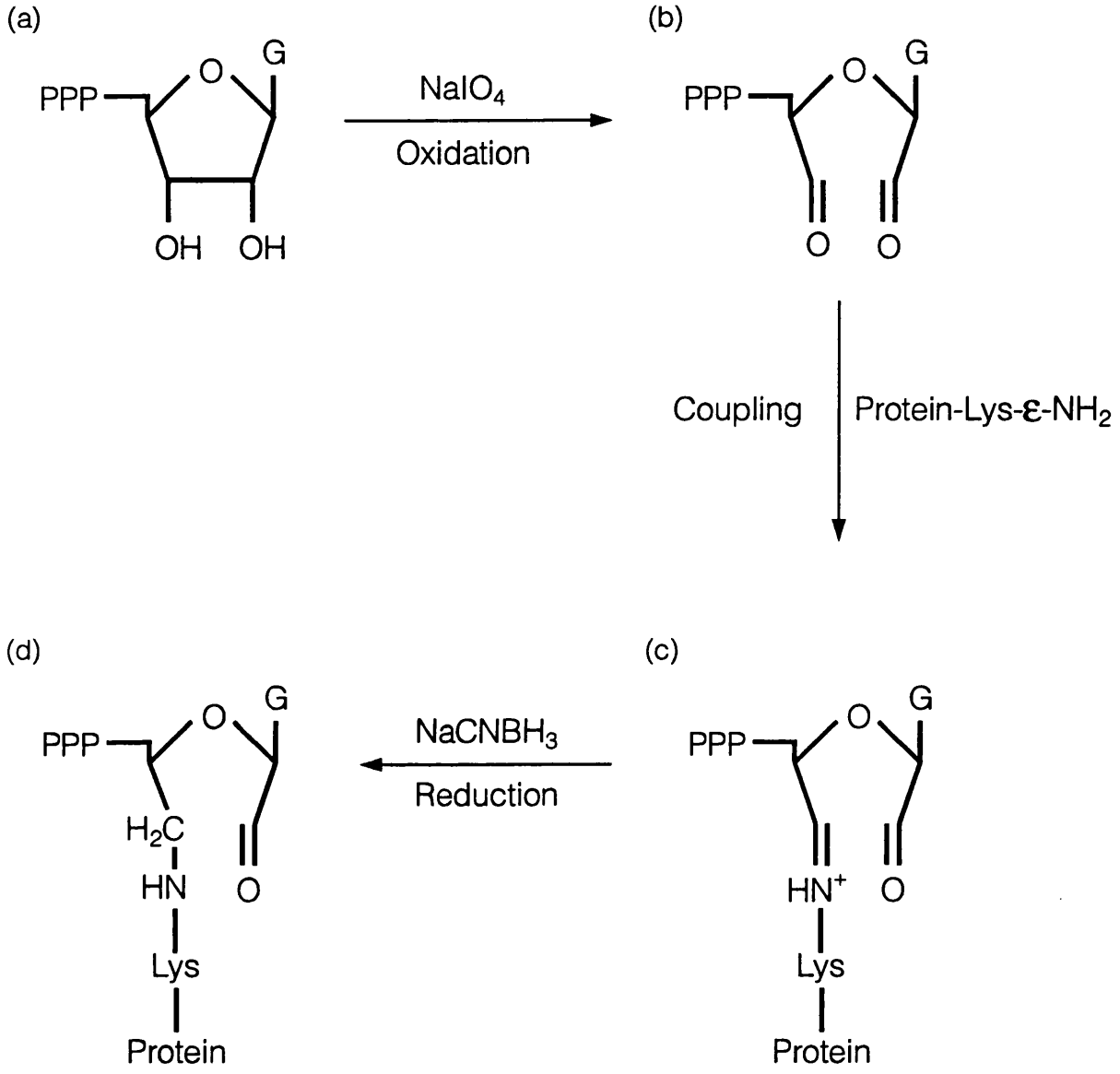


Figure 6.1: Reaction scheme of cross-linking [α-^{32}P]GTP to the invariant NKXD sequence of GTP-binding proteins.

(a) Protein-bound [α-^{32}P]GTP before and (b) after oxidation with Sodium Periodate; (c) initial azomethine reaction product after cross-linking to the ε-amino group of an active site lysine side chain; (d) stabilisation of the intermediate Schiff's base with Sodium Cyanoborohydride by conversion to the corresponding secondary amine.

Modified from Löw et al, 1992

6.3 Results

Figure 6.2 illustrates the appearance of a typical autoradiogram from an experiment using α^{32}P-GTP labelled permeabilised mast cells. The cells were exposed to label for 1 minute before being sedimented and then oxidising the GTP in situ. The method appears to label GTP-binding proteins selectively as labelling is unaffected by 100,000 fold excess (100μM) of cold ATP (lanes 3 and 4), but is inhibited by GTP (lanes 7-10), GDP (lanes 5 and 6), GTP-γ-S (lanes 11 and 12), XTP (lanes 13 and 14) and ITP (lanes 15 and 16). GTP-binding proteins of both low (20-30 KDa) and high (45-55 KDa) molecular weights are labelled, as is tubulin (55 Kda) which was positively identified by western blotting with a monoclonal anti-tubulin antibody (data not shown). However there is no consistent effect of altering the concentration of Ca^{2+} from pCa7 to pCa5.

In order to develop the technique further I have now started to use permeabilised rat hepatocytes. These cells possess glucagon receptors, linked via G_s to adenylyl cyclase. With this system it should be possible to detect changes in GTP binding in response to the provision of glucagon. By using this model system to improve the affinity labelling method, I hoped to define conditions under which I can reliably search for alterations in GTP binding in response to Ca^{2+} in mast cells. Although I have not perfected this method yet, the initial results are encouraging. Figure 6.3a shows an autoradiograph of an SDS-PAGE analysis of α^{32}P-GTP labelled permeabilised rat hepatocytes, and figure 6.4a and b show densitometer scans from the autoradiograph. In the presence of glucagon (10μgml^{-1}) an approximately 53 Kda band becomes more heavily labelled (figure 6.3 lanes 1 and 2, and the densitometer scan in figure 6.4a). The presence of ATP appears to enhance this response (figure 6.3 lanes 3 and 4 and figure 6.4b), whilst also reducing the intensity of some of the higher molecular weight bands. The higher molecular weight form of α_s has a molecular weight of 52KDa and should respond to stimulation of the glucagon receptor by exchanging bound GDP for GTP. For these reasons I believe that the band I have identified, which increases its GTP binding in response to glucagon, may well be α_s.

To be able to detect this stimulation it is necessary to leave the cells with the radioactive GTP and glucagon for at least 5 minutes, compared with

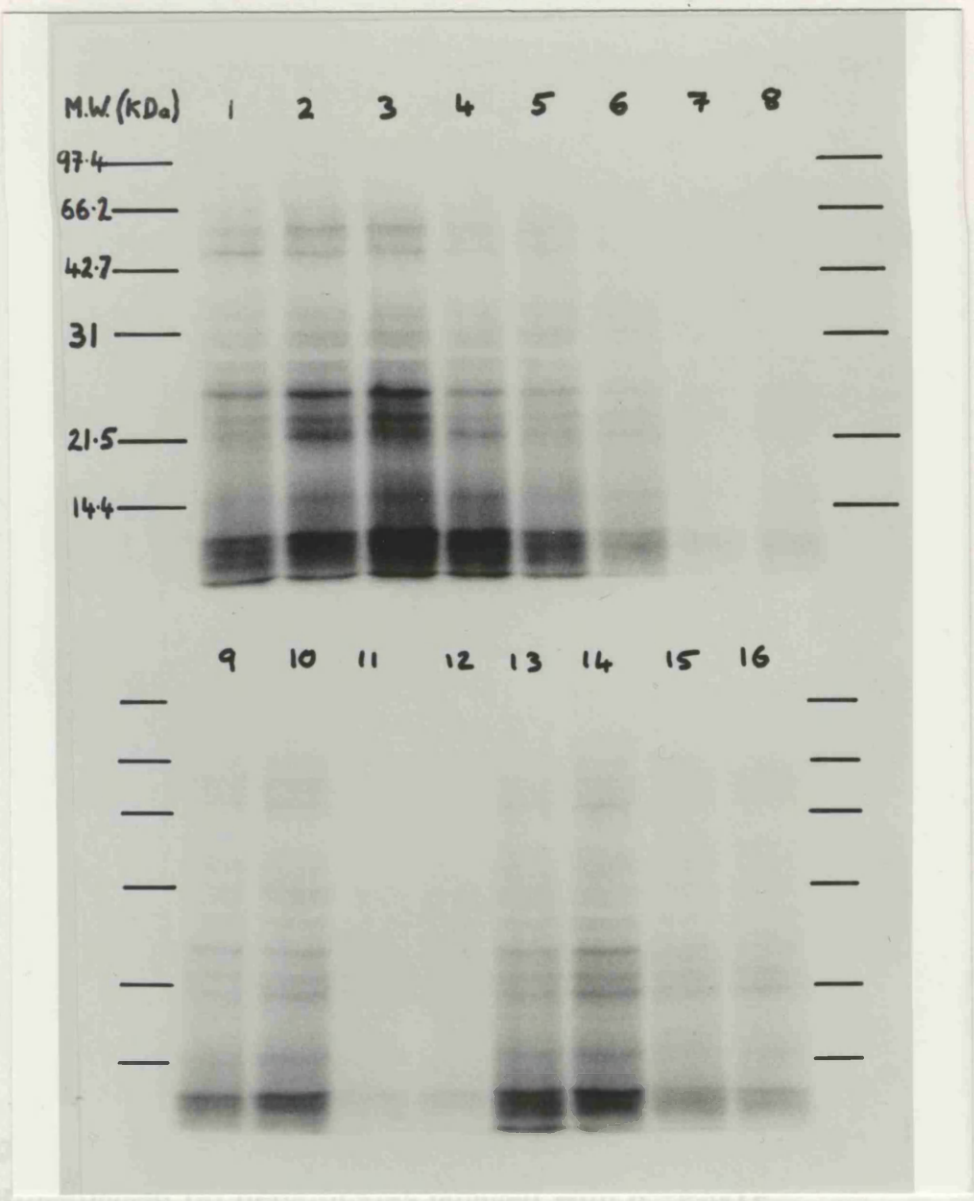


Figure 6.2. Autoradiograph of a 12.5% SDS-PAGE analysis of SL-O permeabilised rat mast cells labelled with α^{32}P-GTP.

Metabolically inhibited and DFP treated rat mast cells were permeabilised with SL-O, and incubated with α^{32}P-GTP for 1 min in the presence of 2mM Mg^{2+} and 5mM EGTA (pCa7 or pCa5 as indicated), under various conditions as indicated below. Lane 1: pCa7; Lane 2: pCa5;
Lane 3: pCa7, 100μM ATP; Lane 4: pCa5, 100μM ATP;
Lane 5: pCa7, 1μM GDP; Lane 6: pCa5, 1μM GDP;
Lane 7: pCa7, 10μM GTP; Lane 8: pCa5, 10μM GTP;
Lane 9: pCa7, 1μM GTP; Lane 10: pCa5, 1μM GTP;
Lane 11: pCa7, 10μM GTP-γ-S; Lane 12: pCa5, 10μM GTP-γ-S;
Lane 13: pCa7, 100μM XTP; Lane 14: pCa5, 100μM XTP;
Lane 15: pCa7, 100μM ITP; Lane 16: pCa5, 100μM ITP.

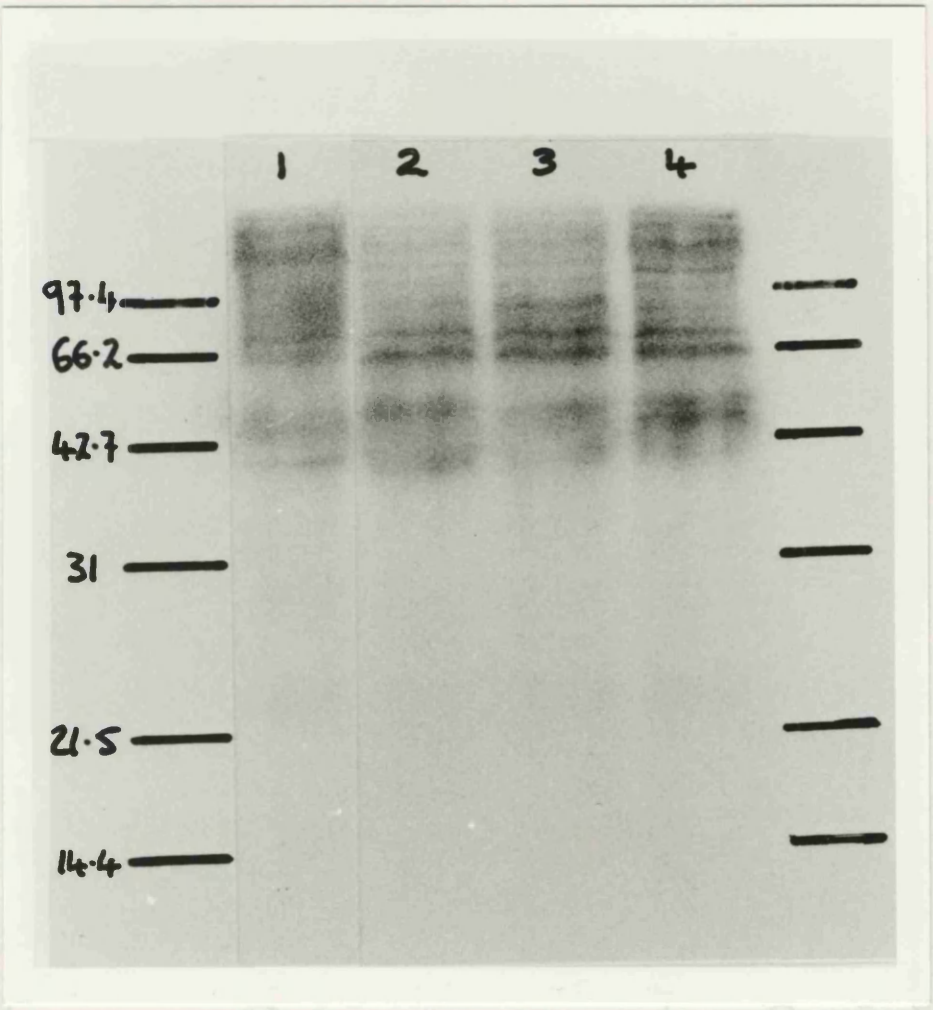


Figure 6.3. Autoradiograph of a 12.5% SDS-PAGE analysis of SL-O permeabilised rat hepatocytes labelled with α^{32}P-GTP.

Metabolically inhibited and DFP treated rat hepatocytes were permeabilised with SL-O, and incubated with α^{32}P-GTP for 5 min in the presence of 2mM Mg^{2+} and 5mM EGTA (pCa7), under various conditions as indicated below. Lane 1: no glucagon; Lane 2: 10μgml^{-1} Glucagon;
Lane 3: 100μM ATP, no Glucagon;
Lane 4; 100μM ATP, 10μgml^{-1} Glucagon

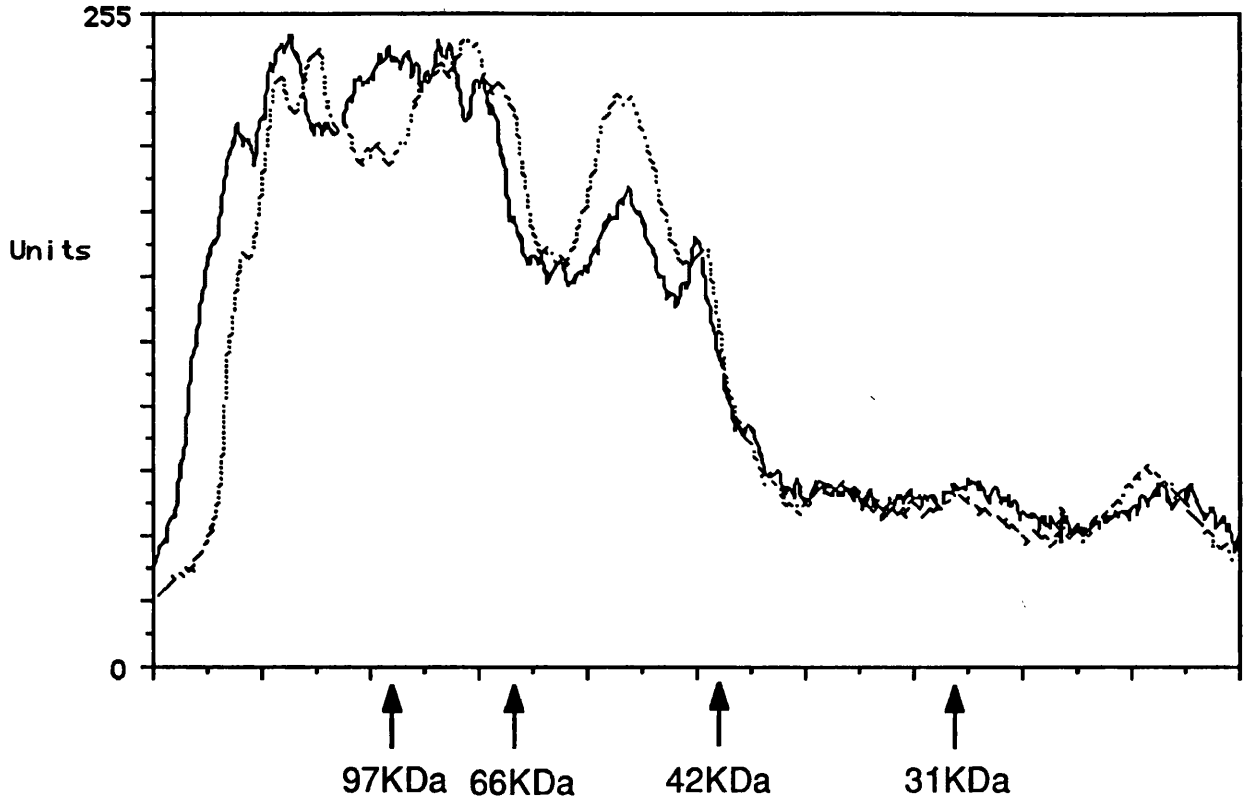


Figure 6.4a. Densitometry traces of lanes 1 and 2 from the autoradiograph illustrated in figure 6.3. Lanes 1 and 2 of the autoradiograph illustrated in figure 6.3 were scanned on a Hoeffer 370 densitometer, and the data stored on a Macintosh SE30 computer. The data were then analysed and compared using Hoeffer software. Solid line: Lane 1, no glucagon; Dotted line: Lane 2, 10 μ g/ml glucagon.

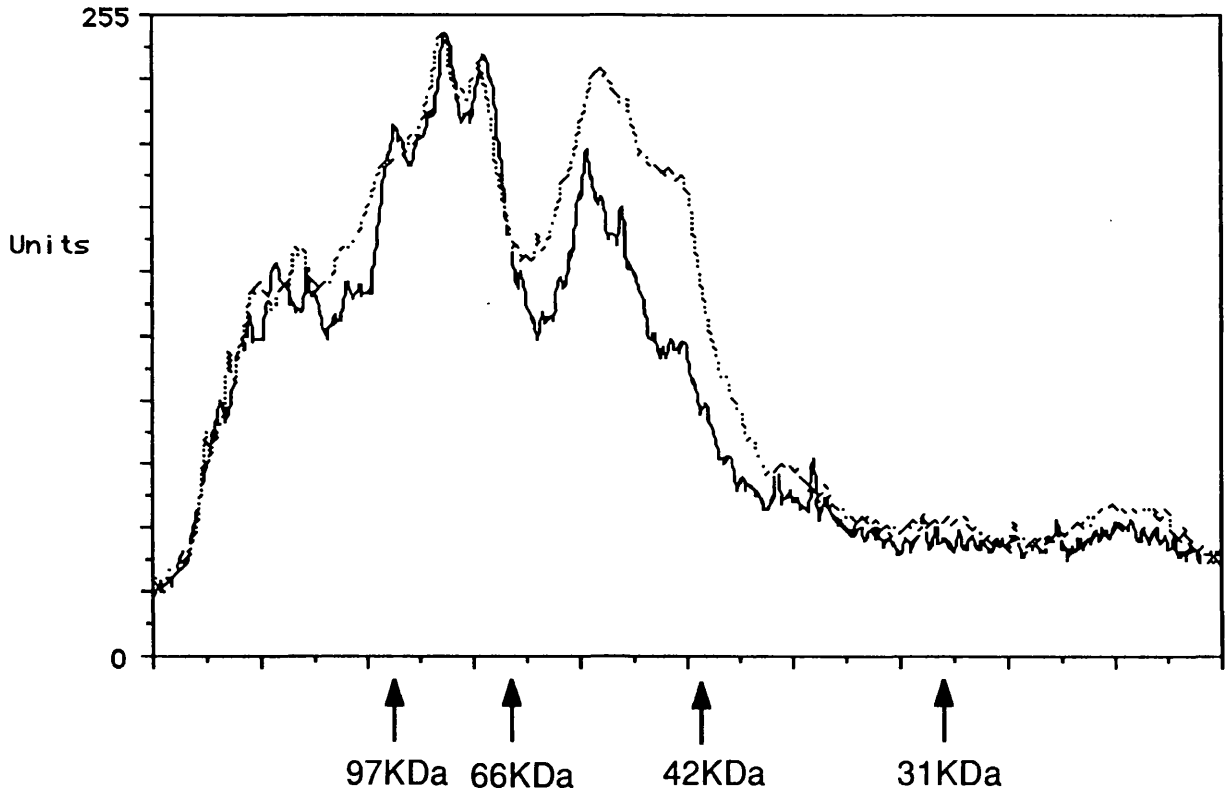


Figure 6.4b. Densitometry traces of lanes 3 and 4 from the autoradiograph illustrated in figure 6.3. Lanes 3 and 4 of the autoradiograph illustrated in figure 6.3 were scanned on a Hoeffer 370 densitometer, and the data stored on a Macintosh SE30 computer. The data were then analysed and compared using Hoeffer software. Solid line: Lane 3, 100 μM ATP, no glucagon; Dotted line: Lane 4, 100 μM ATP, 10μ g/ml glucagon.

the 1 minute incubation of initial mast cell experiments. It may therefore prove necessary to leave mast cells for a longer period of time with Ca^{2+} and α^{32}P-GTP in order to observe changes in labelling.

A further refinement that will hopefully improve the results is to attempt to remove any residual bound glucagon from the cells before permeabilising them. It is necessary to wash hepatocyte membranes to remove bound glucagon before attempting adenylyl cyclase assays, as otherwise there is a high background level of stimulation. Removal of bound glucagon can be achieved by repeatedly washing the cells, preferably at pH 7.8, before permeabilisng them (Prof J.D.Judah, personal communication). Such washing should reduce the level of GTP binding to G_S in unstimulated cells, and thus allow detection of a more substantial increase in GTP binding in response to glucagon.

Interestingly, experiments in mast cells, guinea pig eosinophils and hepatocytes show that glutamate enhances GTP binding to GTP-binding proteins in general. Although my experiments have predicted that glutamate might increase the affinity of G_E for guanine nucleotides, I did not expect it to be a general effect. This would appear to be a direct effect of glutamate on GTP-binding proteins, as only the initial incubation step is carried out in different buffers. Subsequent steps are all carried out under identical conditions.

6.4 Labelling ATP binding proteins

As this method can potentially be used to covalently link any ribonucleotide to a binding protein, ATP-binding proteins are another possible target for identification by this method. As ATP can cause the onset of exocytosis to be delayed, it would also be of interest to identify the protein that mediates this response. As the effect of ATP occurs in the absence of Mg^{2+} this may allow its identification, and certainly in the absence of Mg^{2+} there are several bands which can be labelled by this method with α^{32}P-ATP. Obviously some of these bands may well be kinases, which will bind ATP in the absence of Mg^{2+}, being unable to remove the terminal phosphate in the absence of Mg^{2+}. Further development of this technique may allow identification of both novel GTP and ATP-binding proteins.

Chapter 7

Conclusions

The streptolysin-O permeabilised mast cells provide an excellent preparation with which to study the regulation of exocytosis [Lindau & Gomperts, 1991]. In these cells, the requirement for Ca^{2+} and a guanine nucleotide in the terminal pathway of exocytosis indicates the involvement of a binding protein(s) for Ca^{2+} (C_E, as yet unidentified) and a GTP-binding protein(s) (G_E, also unidentified) [Howell et al., 1987; Cockcroft et al., 1987]. The work in this thesis has been aimed at characterising and identifying the GTP-binding protein G_E.

7.1 Phosphorylation of G_E

In chapter 3, I showed that ATP, by a reaction catalysed by PK-C, enhances the sensitivity of the secretory mechanism to Ca^{2+} and GTP-γ-S. The simplest (though by no means the only) explanation for the increase in sensitivity to GTP-γ-S is that G_E is phosphorylated by PKC, enhancing its guanine nucleotide binding affinity. This idea is strengthened by the fact that ATP also enhances GTP-γ-S *single effector* secretion from cells permeabilised in glutamate (ie in the absence of Ca^{2+}), as shown in chapter 4. Although ATP can generate GTP in glutamate based buffers, there are three pieces of evidence that suggest that this is not the mechanism of the enhancement of GTP-γ-S stimulated secretion by ATP:-

1. In the absence of Ca^{2+} and in the presence of Mg^{2+}, GTP is unable to induce single effector secretion.

2. Only ATP can enhance GTP-γ-S induced secretion. Other nucleotides that support Ca^{2+} induced secretion (presumably by the generation of GTP catalysed by nucleoside diphosphate kinase) cannot do this.

3. Previous reports have shown that the enhancement of GTP-γ-S single effector secretion is a PKC dependent reaction [Churcher & Gomperts, 1990] and therefore should require ATP as the phosphoryl donor.

From these considerations it seems possible that this ATP enhancement of GTP-γ-S induced secretion in glutamate based buffers is a reflection of the same PKC mediated reaction which increases the sensitivity to the dual-effectors in Cl^- based buffers. There is certainly no *a priori* reason to believe that the Ca^{2+}-binding protein, C_E, is subject to modulation. This being the case, it seems reasonable to expect G_E, or another protein regulating G_E, to be the target of a PKC mediated phosphorylation that increases its affinity for GTP and its non-hydrolysable analogues.

7.2 Glutamate enhances guanine nucleotide sensitivity

By working with cells permeabilised in iso-osmotic solutions formulated with glutamate rather than chloride I have found that an activating guanine nucleotide is the *sine qua non* for exocytosis. Ca^{2+} is dispensable (chapter 4). In mast cells it would appear that activation of G_E is essential for regulated secretion to occur. One of the effects of the glutamate buffer appears to be an increase in the sensitivity of G_E to guanine nucleotides and this may underlie the ability of GTP-γ-S to induce single effector secretion in glutamate but not in chloride. Conversely, it also gives an explanation as to why Ca^{2+} (with ATP) cannot stimulate exocytosis in Cl^- based buffers, since insufficient GTP is generated to stimulate secretion. For cells permeabilised in Cl^-, a minimum of $10^{-4.5}$M GTP is needed to stimulate secretion (figure 4.1) and (judging by the level of hexosaminidase release in glutamate induced by Ca^{2+} and ATP, figure 4.7) this is probably about the maximum level than can be generated.

7.3 G_E shares some characteristics of trimeric GTP-binding proteins

Based on my observations of the effects of Mg^{2+} on secretion, I have proposed that G_E shares some characteristics with the trimeric GTP-binding proteins. In particular, the binding and activation of G_E may be controlled by a Ca^{2+} binding protein (C_E) involved in exocytosis and which therefore can be regarded as the counterpart of a plasma membrane receptor or *pseudo-receptor* (chapter 4 and [Lillie & Gomperts, 1992a]).

There are a number of lines of evidence to support this idea, which I have taken from the various results chapters and summarised below:-

1. The ability of GTP to support exocytosis, and particularly the magnitude of vesicle release, is suppressed in the presence of Mg^{2+}. This suggests that G_E possesses an unconditional GTPase activity (ie that GTP hydrolysis is not required to allow continued activity), more characteristic of trimeric than monomeric GTP-binding proteins.

2. GTP-γ-S induced, single effector secretion in glutamate buffers occurs in a manner strongly supported by Mg^{2+}. Similarly, activation of G_S by GTP-γ-S (alone, in the absence of an activating ligand) is dependent on Mg^{2+}.

3. When Ca^{2+} is also supplied, the dependence on Mg^{2+} is lost and elevation of the Ca^{2+} concentration abbreviates both the delay before onset of secretion and the time taken for its completion. This is reminiscent of the effect of glucagon on stimulation of G_S by GTP-γ-S, which removes the dependence on Mg^{2+} and also abolishes delays before the onset of cAMP generation.

4. The absence of Mg^{2+} delays the onset of GTP-γ-S triggered secretion from cells permeabilised in chloride solutions. Deprivation of Mg^{2+} has similar effects on the generation of cAMP by G_S stimulated adenylyl cyclase, probably due to the requirement of Mg^{2+} for GTP-γ-S binding and activation of G_S subsequent to binding. This effect argues that G_E bears more similarity to the trimeric (in which the absence of Mg^{2+} promotes dissociation of GTP-γ-S, and thus inhibits activation) than the monomeric GTP-binding proteins (in which the absence of Mg^{2+} promotes GDP dissociation and thus promotes activation).

5. When GTP-γ-S is used to activate G_S (measured as cAMP generation) it provides a remarkably poor level of stimulation. It is only when glucagon is also added that activation become substantial and rapid. As I have shown, in the mast cell, GTP-γ-S induced single effector secretion is slow and of a low magnitude. Substantial and rapid activation with GTP-γ-S only occurs in the presence of Ca^{2+} (this is true for both Cl^- and glutamate buffers).

This suggests that Ca^{2+} (being the only other addition necessary for secretion) affects the activation of G_E. Thus the calcium binding protein C_E may act as an upstream modulator, regulating the activity of the GTP-binding protein G_E.

In addition, there is yet another reason why I have suggested the modulation of G_E function by Ca^{2+} in the mast cell. Activation of a protein requires some change in its qualities. In the case of a GTP-binding protein this must be a consequence of either an increase in GTP binding, a decrease in GTP hydrolysis or (rather improbably) rephosphorylation of bound GDP. As the addition of Ca^{2+} is the only requirement other than a guanine nucleotide which is needed to stimulate secretion, it would seem that Ca^{2+} must affect the activation of G_E in some way. Of the three known possibilities of activation, which route of activation does Ca^{2+} utilise? That Ca^{2+} should decrease the rate of hydrolysis of GTP by G_E seems unlikely, since secretion can be induced by non-hydrolysable analogues, and these still require the presence of Ca^{2+} (an absolute requirement in Cl^-, and a marked enhancement in glutamate). Removal of Mg^{2+}, which should inhibit GTP hydrolysis, does not alleviate the need for Ca^{2+}, but potentiates release stimulated by GTP and Ca^{2+}. Therefore a Ca^{2+} dependent inhibition of GTP hydrolysis seems unlikely. Except under the special circumstances of the experiment shown in figure 3.9, Ca^{2+} stimulated rephosphorylation of bound GDP is also an unlikely mechanism of activation since ATP is not required. This leaves an effect of Ca^{2+} on guanine nucleotide exchange as the activating mechanism, which I have chosen to express as the concept of a *pseudo-receptor*. This is illustrated in figure 7.1.

The identification of trimeric GTP-binding proteins on Golgi membranes apparently controlling vesicle trafficking also raises the question of how these are controlled. One possibility would be an intracellular receptor along the lines I have suggested. Despite all these arguments, I cannot rule out the possibility that activation of G_E is via a totally novel mechanism.

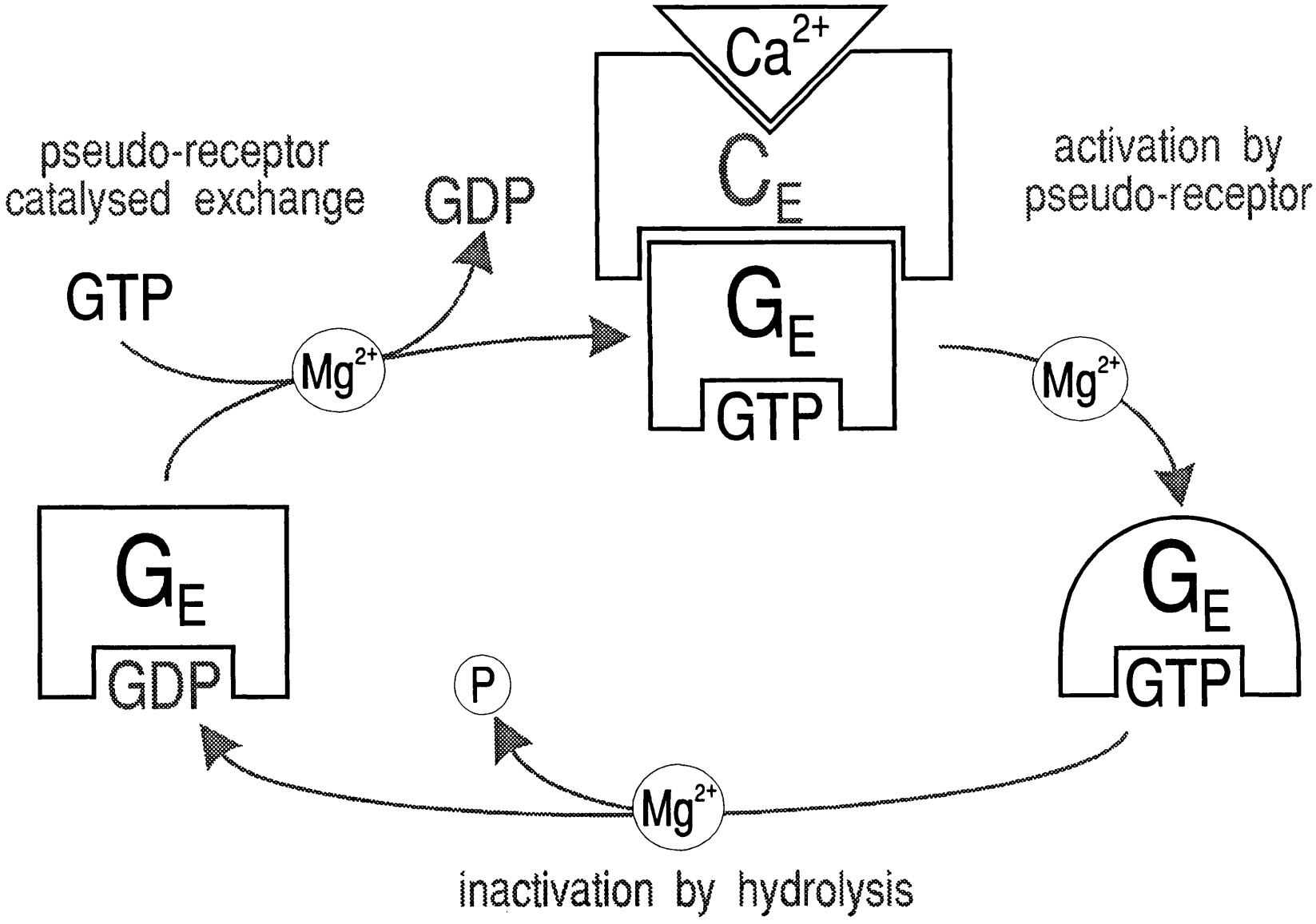


Figure 7.1: Schematic representaioin of the possible interaction between G_E and C_E.

7.4 Summary of the expected characteristics of G_E

From the arguments outlined above, G_E might be expected to have four identifiable properties:-

1. ATP should enhance its affinity for guanine nucleotides.

2. Its affinity for guanine nucleotides should be greater in glutamate than Cl^- based buffers.

3. At least in glutamate based buffers, G_E may only irreversibly bind GTP-γ-S in the presence of Mg^{2+}, if Ca^{2+} is absent.

4. The presence of Ca^{2+} at 10μM might increase the affinity for guanine nucleotides if a Ca^{2+}-binding protein is involved as postulated.

I would also predict from my data that G_E might well be a trimeric rather than a monomeric GTP-binding protein. This is based on the effects of Mg^{2+} on guanine nucleotide binding and GTP hydrolysis I have summised from my data (as outlined above).

7.5 Searching for G_E, and further...

Having defined some possible identifying characteristics of G_E, I have attempted to detect a GTP-binding protein possessing some of these qualities. Although this search has not yet proved fruitful, the method I am developing appears to be capable of detecting the activation of a GTP binding protein, possibly G_S, by glucagon in rat hepatocytes. When I have further refined the method in this well characterised system it may be possible to return to the task of identifying G_E with more confidence. In particular, it remains necessary to confirm the identity of the activated GTP-binding protein as G_S by western blotting using anti-G_s α-subunit peptide antibodies.

If the development of this method does prove successful then I anticipate many other possible applications. In cells which can be permeabilised, it may allow the identification of the specific GTP-binding proteins that are functionally linked to various surface receptors. In addition, it would also allow the identification of previously unknown GTP-binding protein linked receptors. Since the method appears to be able to label monomeric as well

as trimeric GTP-binding proteins, it might be possible to test whether receptor tyrosine kinases increase guanine nucleotide exchange (rather than decreasing GTP hydrolysis) on $p21^{ras}$ or related proteins.

Since the binding of any ribonucleotide can be detected, the method also provides for the detection of ATP-binding proteins. I have already mentioned the possibility that an ATP-binding protein may modulate exocytosis in mast cells, and I have started preliminary α-^{32}P-ATP labelling experiments. I have also passed on the method to Dr Frances Ashcroft (at Oxford University) who is attempting to identify the internal ATP^{4-} sensor in islet ß-cells. This sensor controls a K^+ channel, which closes when ATP is in abundance (indicating that glucose is plentiful), depolarises the cell and leads to insulin secretion. This method may allow identification of this protein.

7.6 Why have a GTP-binding protein in the control of secretion?

One question that arises when studying the role of GTP-binding proteins in exocytosis, particularly in cells where Ca^{2+} is the prime stimulus, is why should it be necessary to invoke the presence of a GTP-binding protein in the control pathway of exocytosis. Put more simply, why use two signal transduction pathways rather than one? Although I can offer no definite answer, there are a few possible reasons.

Even in cells where Ca^{2+} is clearly the prime stimulus to secretion, such as the adrenal chromaffin cell, the time course of the Ca^{2+} transient and the delay before this transient triggers exocytosis suggests that Ca^{2+} may activate a train of events leading to vesicle fusion [Chow et al., 1992]. As increments in intracellular Ca^{2+} can often also be highly localised [Cheek. et al., 1989], triggering activation of a GTP-binding protein (or other mechanisms) offers the possibility of amplifying and prolonging this signal.

This may be particularly important for cells which exist in vivo as isolated entities rather than a gland, such as the mast cell. In this case, substantial secretion of granule contents (containing mediators of inflammation) must occur in order for the alarm signal to be effectively raised. The release of only a few granules (as is suitable for the release of catecholamines from the adrenal medulla) will simply have no effect. As the transient Ca^{2+} spike observed in mast cells is over before exocytosis ever occurs, it must

trigger some secondary train of events, possibly the activation of a GTP-binding protein, which then allows total exocytotic release.

Another possible reason for involving GTP-binding proteins in regulated exocytosis would be to regulate vesicle docking and assembly of the fusion apparatus, in a manner analagous to vesicle trafficking. Although I believe that I have presented a strong case for a trimeric-like GTP-binding protein activity in mast cells, I cannot rule out a secondary role for monomeric GTP-binding proteins in vesicle fusion. As the secretory process in mast cells is a "one way trip" for vesicles, ie there is no requirement for recycling for exocytosis to occur, GTP-γ-S would not necessarily halt secretion. This opens the possibility that there could be two roles for GTP-binding proteins in regulated exocytosis as there appear to be in vesicle trafficking. This has been implicitly suggested in kinetic analysis of single granule fusions in patch clamp studies on beige mouse mast cells [Alvarez de Toledo & Fernandez, 1990; Alvarez de Toledo & Fernandez, 1988]. In other cell types the possibility remains that the actions of guanine nucleotides could reflect the role of monomeric GTP-binding proteins in vesicle docking and fusion.

7.7 What is the role of Ca^{2+} and guanine nucleotides in whole cells?

Throughout this thesis I have stressed that in permeabilised cells Ca^{2+} is ultimately dispensible for secretion (albeit of a rather low level) to occur, and that guanine nucleotides are essential. One of the major benefits of the permeabilised cell system is the ability to control the concentration and identity of guanine nucleotides within the cell, and particularly to introduce non-hydrolysable analogues. However in whole cells the concentration of GTP will not change markedly, and regulation of a process by varying the GTP concentration (as I have done in permeabilised cells) is impossible. Thus, raising the intracellular concentration of Ca^{2+} may well be the first step towards granule release in the mast cell.

The physiological trigger to mast cell degranulation, stimulation of the IgE receptor, does indeed cause a Ca^{2+} spike [Cunha-Melo et al., 1989]. As mentioned in the introduction, a rise in intracellular Ca^{2+} (triggered by either IgE stimulation or ionophore) appears to be dependent on the presence of GTP within the cell to stimulate secretion [Marquardt et al., 1987; Wilson et al., 1988; Wilson et al., 1989; Mulkins et al., 1992]. This

indicates a role for GTP, and thus a GTP-binding protein, at a later stage in stimulus secretion coupling than the rise in Ca^{2+}. The work in this thesis has been aimed at elucidating the regulation of this late acting GTP-binding protein(s), G_E, in excoytosis. My results indicate that under approximately intracellular conditions (pCa7, 1mM Mg^{2+}, 100μM GTP), GTP is unable to induce secretion (figure 4.9), and equally that Ca^{2+} cannot trigger secretion in the absence of GTP (figure 4.7). These results agree perfectly with the data from intact mast cells. Mast cells do not degranulate without a rise in Ca^{2+} (even though their intracellular GTP is high), and equally cannot degranulate in response to Ca^{2+} if GTP production has been inhibited.

Thus, for intact mast cells, a rise in intracellular Ca^{2+} is probably the prime stimulus to secretion, as it is in many other systems. This brief rise in intracellular Ca^{2+} must set in train other events, which can amplify the signal to cause the release of a large number of granules. One possibility is the pathway I have proposed in this thesis, that Ca^{2+} stimulates guanine nucleotide exchange on a GTP-binding protein, G_E, which when activated causes exocytotic release of the granule contents.

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Chapter 8

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