THE ROLE OF THE PHOSPHATIDYLINOSITOL TRANSFER PROTEIN AND THE SMALL GTP-ASE ARF IN REGULATED EXOCYTOSIS FROM RBL-2H3 MAST CELLS

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Abstract

Two proteins have been identified previously, based on their ability to restore secretion from granulocytes (neutrophils and HL60 cells): the ADP-ribosylation factor (ARF), and the phosphatidyl-inositol transfer protein (PI-TP). If granulocytes are permeabilised before stimulation there is a decline in the secretory response. This is associated with the leakage of cytosolic proteins, and implies that some of the lost proteins are involved in the regulation of secretion.

Previous work has relied upon the use of the artificial stimulus, GTPγS. RBL-2H3 mast cells may be stimulated experimentally via their tyrosine-kinase linked FcεR1 receptors, using the physiological stimulus. Therefore the primary goal of this project was to demonstrate that the effects of myr.rARF-1 and rPI-TP on secretion were of physiological relevance.

When conditions for depleting RBL-2H3 mast cell cytosol were established, ARF and PI-TP leakage were found to coincide temporally with the gradual decline in secretion. In reconstitution studies, cytosol-depleted cells were incubated with recombinant myr.ARF-1 and both α and β isoforms of rPI-TP; each of these proteins successfully restored lost responsiveness. Myr.rARF-1 was also found to restore lost phospholipase D activity following permeabilisation.

The mechanisms through which these proteins contribute to exocytosis were investigated: it was found that while ARF appears to exert its effect via PLD activation, PI-TP operates via a different pathway. However, analysis of polyphosphoinositide levels in reconstituted cells indicates a possible common mechanism, namely the increased synthesis of phosphatidylinositol-4,5-bisphosphate (PIP_2). To identify the site of PIP_2 synthesis, the subcellular location of the PI-TP's was examined. It was discovered that antigen-stimulation mediates a translocation of PI-TPα to the cells' cortex.

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Abbreviations and Symbols

°C	Degrees Celsius
α	alpha
APS	Ammonium Persulphate
ARF	ADP-Ribosylation Factor
ATP	Adenosine-5' triphosphate
β	beta
β-hex substrate	beta-methylumbelliferyl-N-acetyl-β-D-glucosaminide
BSA	bovine serum albumin
CaM	Calmodulin
DAG	Diacylglycerol
δ	delta
DMEM	Dulbecco's modified Eagle's Medium
DNP-HSA	Di-Nitrophenol conjugated to Human serum albumin
dpm	Disintegrations per minute
E.Coli	Escherichia coli
EGTA	Ethyleneglycol-bis-(β–aminoethylether) N,N,N,N tetra acetic acid
FcεR1	High affinity receptor for IgE
FCS	Foetal Calf Serum
γ	gamma
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitor
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GTP	Guanosine-5' triphosphate
GTPγS	Guanosine-5'-O-(3-thiophosphate)
HEPES	N-[2-Hidroxyethyl]piperazine-N' [2-ethanesulphonic acid]
IgE	Immunoglobulin E
IgEαDNP	Immunoglobulin E, directed against dinitrophenol
IP_3	Inositol 1,4,5-trisphosphate
IPTG	Isopropyl β-D-thiogalactoside
iu	International Units
μ	micro
NSF	N-ethyl maleimide sensitive fusion protein
PA	Phosphatidic Acid
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PC12 (cells)	Pheochromocytoma (cells)
pCa	$-\log_{10}[Ca^{2+}]$
PEt	Phosphatidylethanol
PG	Phosphatidylglycerol
PH domain	Pleckstrin homology domain
PI	Phosphatidylinositol
PI 3-kinase	Phosphatidylinositol-3-Kinase
PI 4-kinase	Phosphatidylinositol-4-Kinase
PIP	Phosphatidylinositol-4-Phosphate
PIP_2	Phosphatidylinositol-4,5-bisphosphate

PIP$_3$	Phosphatidylinositol-3,4,5-trisphosphate
PI4P 5-kinase	Phosphatidylinositol-4-Phosphate 5-Kinase
PIPES	Piperazine-N.N'-bis[2-ethylsulphonic acid]
PI-TP	Phosphatidylinositol transfer protein
PKA	Protein kinase A
PKC	Protein kinase C
PLA$_2$	Phospholipase A$_2$
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol-12-myristate-13-acetate
PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
RBL-2H3 (cells)	Rat basophilic leukaemia (cells)
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SH2/3 domain	src-homology domains 2/3
SLO	Streptolysin-O
SNAP	Soluble NSF attachment protein
SNARE	SNAP receptor
TCA	Trichloroacteic acid
Temed	N,N,N',N'-Tetra methyl ethylene diamine
TLC	Thin layer chromatography
Tween 20	Polyoxyethylene sorbitan monolaurate

CHAPTER ONE

INTRODUCTION

Chapter 1 Introduction

1.1 Mast Cells

1.1.1 The physiological role of Mast cells

Mast cells are secretory cells found at relatively low abundance in the body. They mediate an immune response known as the allergic or acute hypersensitivity reaction. They arise in the bone marrow, and migrate via the circulation as immature precursers. There are two distinct populations of mast cells; those on mucosal sites that differentiate under the influence of T-cell derived cytokines such as interleukin-3 (1), and those in connective tissue that differentiate under the influence of fibroblast-derived mediators (2). Though they are "terminally" differentiated *in situ*, each population retains the potential to transdifferentiate into the other (3). When in suspension they are rounded in appearance and approximately 6-13μm in diameter. Mast cells have a distinctive appearance when examined using transmission electron microscopy: caused by the presence of up to 1000 dense secretory granules.

The secretory granules store preformed inflammatory mediators. They are derived from lysosomes, and some of their contents reflect this origin. In addition to histamine, serotonin, neutral proteases such as tryptase and chymase, and chemotactic factors such as tumour necrosis factor-α (TNFα), the granules contain acidic hydrolases including β-hexosaminidase. The structure of the granule is maintained prior to release by proteoglycans such as heparin and chondroitin. Interaction with the glycosaminoglycans side-chains of these proteins maintain the protein mediators in stable and active configurations in the quiescent cell (4). Granule contents are released by exocytosis, stimulated via a high affinity receptor for immunoglobulin E borne on the cell surface.

In addition to the mediators released by exocytosis, mast cells stimulate the release of other factors as a consequence of gene transcription. Thus activation of these cells results in the *de novo* production of other mediators, for example TNFα (5) and other cytokines. Also, mediators derived from arachidonic acid are produced following activation of PLA$_2$; resulting in

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KEY:

Human Serum Albumin

Dinitrophenol

FcεR1 receptor

IgEαDNP

Lyn

Syk
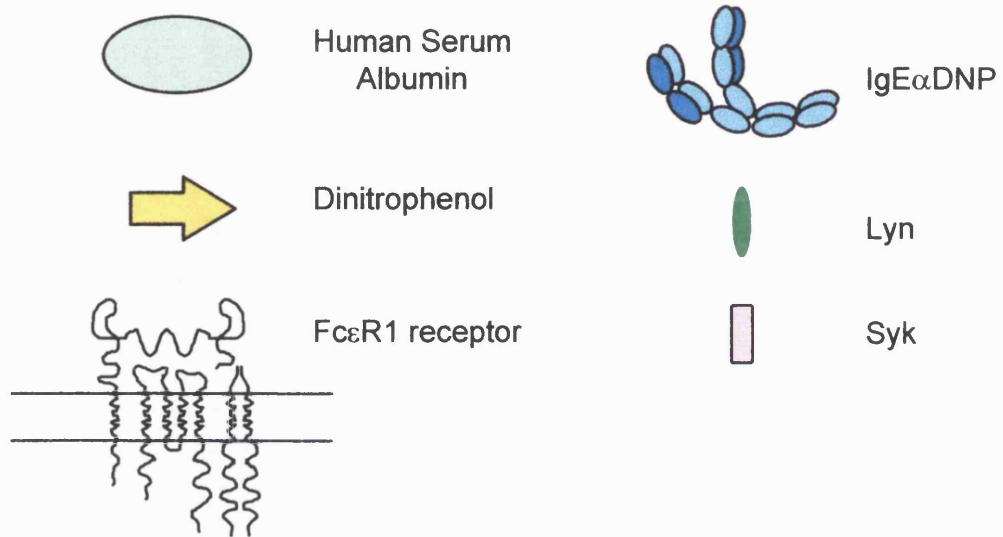
Fig. 1.1 FcεR1 receptor structure and aggregation

1. Diagrammatic representation of the monomeric FcεR1, showing the relative orientation of the subunits, and association with *lyn*

2. Binding of IgE to the α-subunit of FcεR1

3. Activation of via cross-linking of adjacent FcεR1's on a single multivalent antigen.

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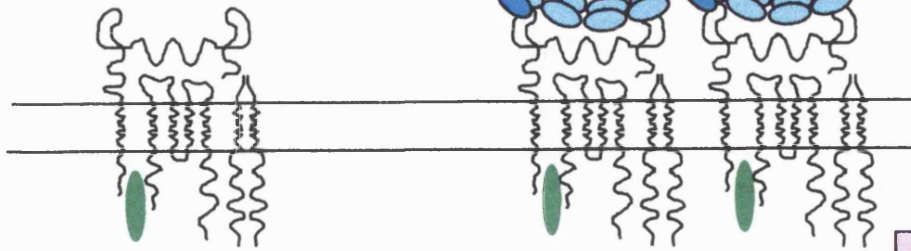
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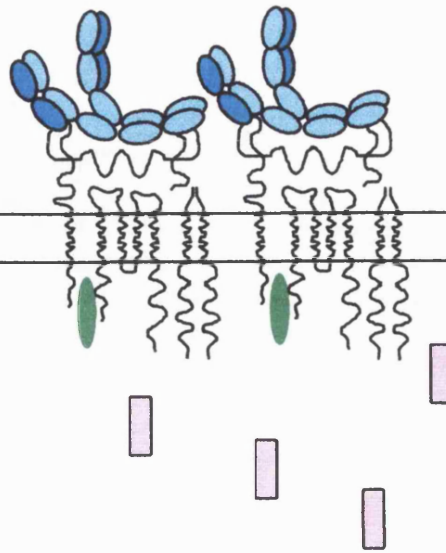
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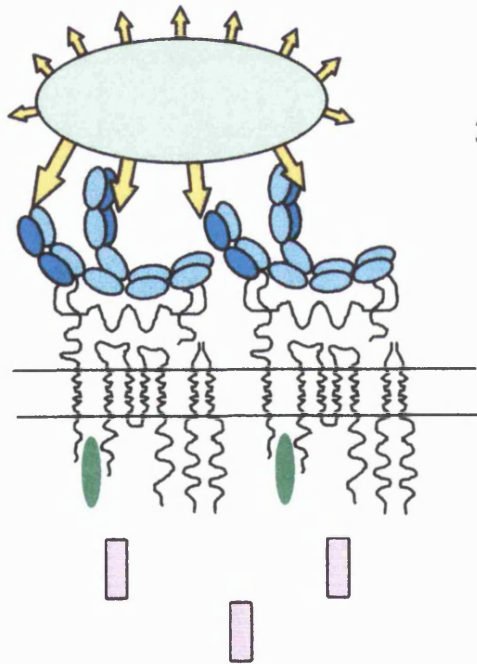
prostaglandin production by cyclo-oxygenase, and leukotriene production via lipoxygenase.

The combined actions of the pre-formed and newly synthesised mediators cause the range of symptoms comprising the allergic response. Some allergic episodes are particularly severe, resulting in the potentially fatal syndrome of systemic anaphylaxis. This condition arises from an exaggerated mast cell response to an allergen, where the normal mediators are released but are more widespread in their action. Thus all mast cell-mediated responses may appear to be at best troublesome, and at worst potentially lethal. However these cells play a vital role in the amplification of acute inflammatory responses, arising from non-immunological origins (6). The mast cells' role is thought to be mediated by TNFα released into the vicinity of an infection (7). This mediator increases vascular permeability and so allows leukocyte migration and infiltration into the affected area. This effect has been shown to be vital for rapid bacterial clearance (8,9) and so defence against infection.

1.1.2 Activation of Mast cells

Mast cells possess FcεR1 high affinity receptors for IgE in their plasma membranes. The FcεR1 receptor is a heterotetrameric glycoprotein, consisting of one α-chain, a β-chain and a dimer of disulphide linked γ-chains (see Fig.1.1) (10). Initial attempts to purify the receptor (11) yielded only the 50-60kDa α-chain, which was later found to be non-covalently associated with the 33kDa β-chain (12) and the γ-chains of 7-9kDa each (13). The α-chain contains one membrane-spanning region, and an extra-cellular domain which comprises the single IgE-binding site. The short cytoplasmic tail is apparently dispensable, as its deletion does not compromise receptor activity (14). The β-chain has four membrane-spanning domains; studies using monoclonal antibodies suggest that both the amino- and carboxy-termini are positioned in the cytosol. The γ-chains have one membrane-spanning domain each, with long cytoplasmic tails. They are linked by a disulphide bond close to the amino terminal, on the very short extracellular portion (15). Neither the β-chain or the γ-chain have intrinsic enzymatic activity, however mutation of their
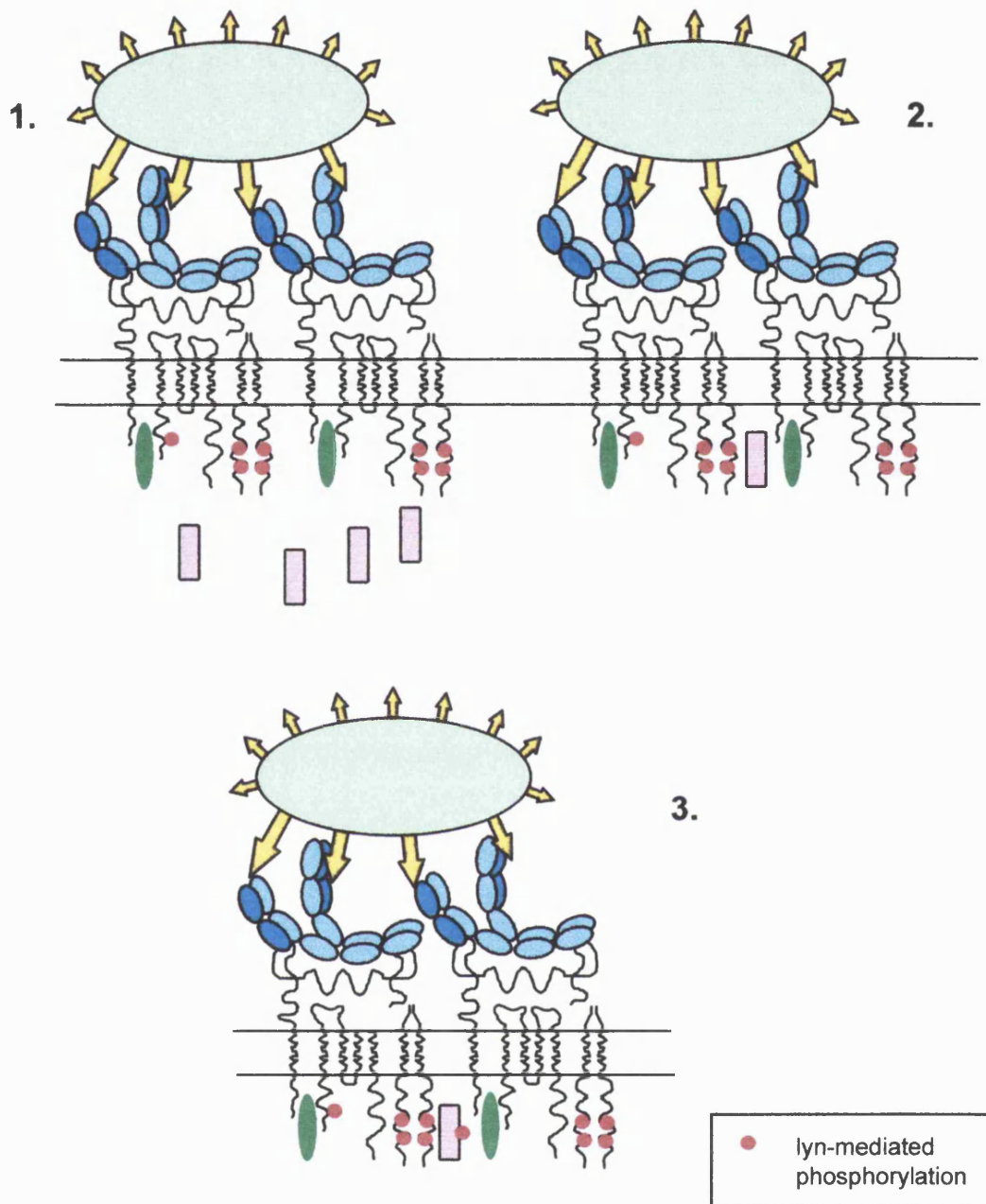
Fig. 1.2 Activation of FcεR1

Lyn phosphorylates the cytoplasmic domains of the receptor. Recruitment and phosphorylation of *syk* follows, and the receptor complex becomes immobilized in the membrane. Downstream of this phosphorylated receptor/tyrosine kinase complex, signalling enzymes are activated, and exocytosis is initiated.

cytoplasmic regions significantly compromises receptor activity (16,17).

Mast cells are usually activated experimentally by first sensitizing the FcεR1 using a monoclonal IgE directed against a small hapten. The IgE:FcεR1 interaction has been mapped to the FcεR1 α-chain in its extracellular region (18,19). This interaction causes no measurable changes in the cell (20). Activation is achieved by addition of a multivalent antigen. In experiments in this thesis, a monoclonal IgE directed against dinitrophenol was used to sensitize cells, and human serum albumin linked to approximately twenty dinitrophenol molecules (DNP-HSA) was used for activation (21).

Prior to sensitization with IgE, FcεR1 receptors are monovalent and randomly distributed over the surface of the cell. No grouping occurs - less than 5% of the receptors within 5Å of each other at any time (22). The majority of these unliganded receptors are mobile in the plasma membrane (23). When activation occurs, receptors aggregate and become immobile in the plasma membrane, and two adjacent IgE:FcεR1 complexes bind to the same antigen. This "cross-linking" initiates the intracellular changes associated with stimulation. Aggregation of receptors is a progressive phenomenon, cross-linked receptors initially form chains (24), and finally large clusters of immobilized receptor complexes (25). However, despite the eventual aggregation of a large proportion of the surface receptors, maximal secretion occurs when just 10% of receptors are involved (26).

1.1.3 RBL-2H3 cultured cell line

The RBL-2H3 cultured cell line is based on a rat mastocytoma, and were isolated initially because of their ability to release large amounts of histamine (27,28). These cells are well-characterised, and their ease of culture have made them a popular model cell-line for mast cell studies (reviewed in (29)). The versatility of these cells has been confirmed by many studies that have exploited their relative ease of transfection (30,31). As shown in this thesis and in other work these cells form a monolayer which makes them ideal for immunofluorescent studies, yet they may also be stimulated in suspension.

1.2 Lipid signalling enzymes activated on antigen-stimulation

Tyrosine phosphorylation of various cellular proteins is the first detectable effect of receptor cross-linking, demonstrated within just 5 seconds (32). The first phosphorylated substrate identified was the FcεR1 receptor itself, with tyrosines phosphorylated on both the β- and γ-chains (see Fig.1.2) (33,34). In membrane preparations, the phosphorylation of FcεR1 was detected without addition of any cytosolic proteins (35). This suggests that the kinase responsible is associated with the receptor even before stimulation (36). In immunoprecipitates of FcεR1 from unstimulated cells the only tyrosine kinase detected is p53/56*lyn* (37). Up to 20% of FcεR1 receptors are associated with *lyn* in quiescent cells (38), which is anchored to the plasma membrane by an amino-terminal myristoylation (39). On stimulation this proportion increases by up to 10-fold and *lyn* activity is increased 12-fold, thus *lyn*'s tyrosine kinase activity towards the FcεR1's β- and γ-chains (40) increases by more than 100-fold. The recruitment of *lyn* to activated receptors is thought to involve an interaction between the phosphorylated FcεR1 receptor, and *lyn*'s SH2 domain (38). Further, it is suggested that *lyn* interacts specifically with the β-chain of FcεR1 and that this interaction occurs whether the β-chain is phosphorylated or not (41).

A second tyrosine kinase has been found associated with FcεR1 receptors, but in this case only when isolated from stimulated cells. This activity has been identified as p72*syk* (42). *Syk* is a cytosolic tyrosine kinase, that contains two SH2 domains (43). Both of these SH2 domains are implicated in the association of *syk* with FcεR1 (41). *Syk* appears to interact with both the β- and the γ-subunits, but has a clear preference for the γ-chain; however *syk* will associate with neither subunit if they are not tyrosine-phosphorylated (44). *Syk* is activated following receptor ligation, but not in the absence of *lyn* (45), and phosphorylation of a fragment of *syk* (kinase domain excised) by *lyn* has been demonstrated (46), thus *lyn* is clearly important in *syk* activation. However *syk* phosphorylation is diminished when its own kinase activity is abolished, thus *syk* is activated by both hetero- and auto-phosphorylation (45).

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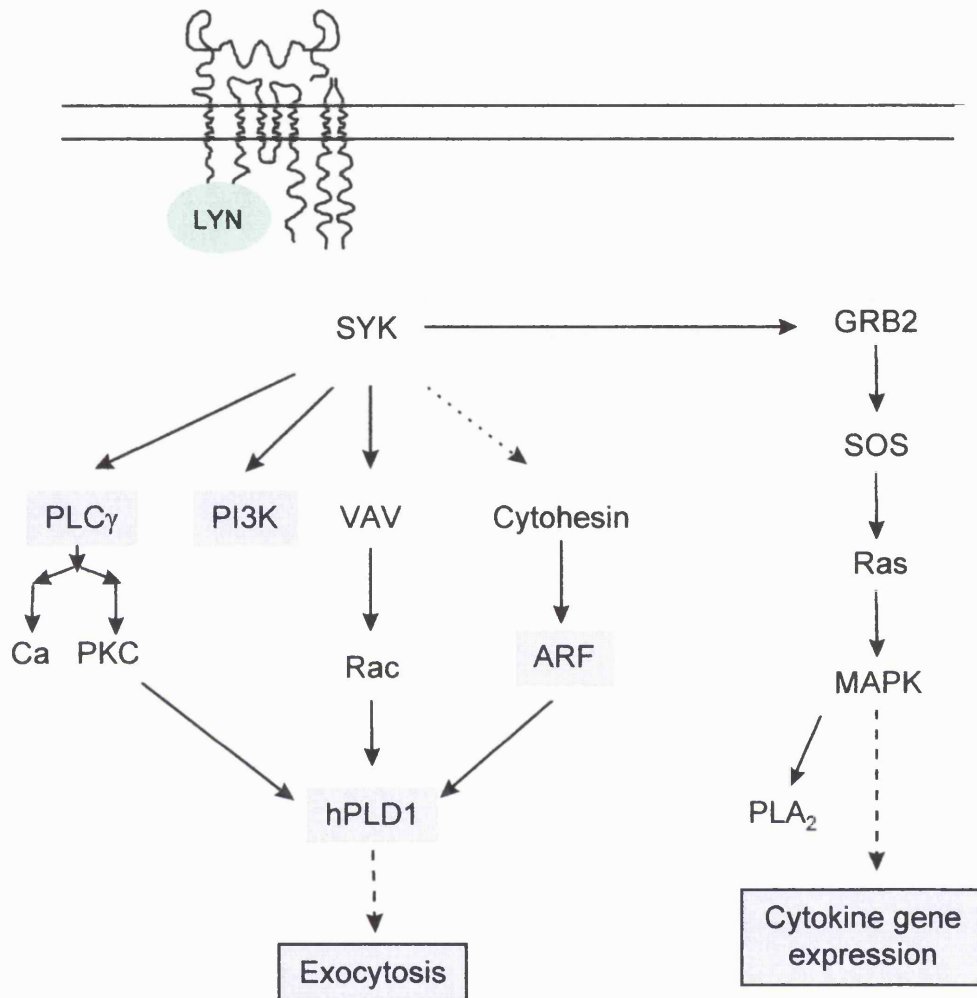
Fig 1.3 Signalling "map" in RBL-2H3 cells

syk activation leads to the activation of various down-stream lipid signalling enzymes - PI 3-kinase, PLA$_2$, PLCγ and PLD. Downstream of these events lies secretion, however these later signalling pathways have not been elucidated. (Arrows represent signalling interactions between proteins)

The importance of *syk* activation is demonstrated by studies using piceatannol (47) - an agent that inhibits *syk* but not *lyn*: in the presence of this agent antigen-induced FcεR1 tyrosine-phosphorylation was unaffected, however PLC activity and serotonin release were both inhibited. This work was supported by studies in *syk* deficient mast cells, that are unable to secrete allergic mediators in response to FcεR1 crosslinking (48). *Syk* activation results in the phosphorylation of a number of receptor-associated proteins, PLCγ1 and γ2 (49,50), the 85kDa subunit of phosphatidylinositol 3-kinase (51), Vav, a guanine nucleotide exchange factor for rac (52), the adaptor protein Grb2 - that forms complexes with another GEF, Sos in RBL-2H3 cells (53), and Bruton's tyrosine kinase (Btk) (54) (see Fig.1.3). Also recruited are tyrosine phosphatases, probably including CD45 (55), which may be responsible for dephosphorylating the receptor subunits, and the receptor-associated kinases (56).

1.2.1 Phospholipase C

PLC forms part of one of the earliest signalling mechanisms identified and studied. PLC catalyses the hydrolysis of PIP_2, forming diacylglycerol (DAG) and inositol trisphosphate (IP_3). IP_3 diffuses to the endoplasmic reticulum, where it activates specific channels responsible for the efflux of calcium, while DAG activates cPKC's and nPKC's.

There have been ten mammalian PLC isoforms identified to date (57). These may be divided into three subclasses: the four PLC-β's, two PLC-γ's and four PLC-δ's (58) (see Fig.1.4). All three isoforms contain a PH domain near the amino terminal (59), and all share two regions of high sequence homology - X and Y which constitute the catalytic domain (60). PLC-δ isoforms are smaller than the other types, and are also the group found in lower eukaryotes such as yeast (61); suggesting that PLC-δ may be an archetypal form. In PLC-δ and PLC-β, the X and Y regions are separated by just 50-70 amino acids. PLC-γ contains a long sequence in this region, containing two SH2 domains, one SH3 domain and an additional PH domain that is split by the SH domains. The differences in structure between the PLC subclasses underly the variety of regulatory mechanisms that control PLC activity.

PLC-β (β1-4)

PLC-δ (δ1-4)

PLC-γ (γ1and γ2)

	PH domain		C2 domain
	X and Y domains		EF hand
	SH2 domain		SH3 domain
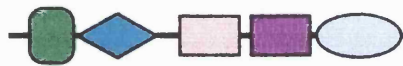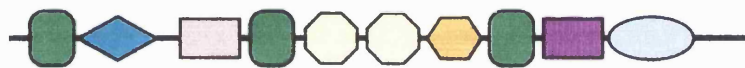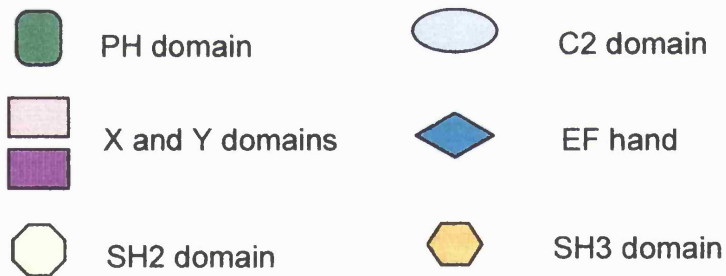
Fig 1.4   Linear representation of the PLC subfamilies

This diagram shows the various domains that are implicated in the regulation of their regulation. (Modified from (57))

PLC-β's are activated by the α-subunits of GTP-binding proteins of the G_q subfamily (62,63) with PLC-β1 slightly more sensitive than PLC-β3, and both significantly more sensitive than PLC-β2 (64). PLC-β isoforms are also activated by βγ-subunits of G-proteins (65) although with a different hierarchy of sensitivity (PLC-β3 > PLC-β2 > PLC-β1) (66,67). The clear specificity of PLC-β for activation by the α-subunits of the G_q subfamily is not observed in activation by βγ-subunits, with many combinations proving equally effective (68,69). Activation by $G_q α$ is thought to be mediated by an interaction with the carboxy-terminal (70), whereas βγ is thought to interact with the first half of the Y-domain (71).

PLC-δ is activated by calcium (72), however any other possible mechanisms by which PLC-δ may be regulated are not clear. Two mechanisms have been proposed recently. The first involves a novel class of G-proteins G_h, which have been shown to activate PLC-δ *in vitro* (73). The second is based on the observation that a polypeptide with homology to Rho-GAP activities can stimulate PLC-δ and not either PLC-β or PLC-γ (74). PLC-δ is not thought to be expressed in mast cells.

PLC-γ's are regulated by receptors that are linked to tyrosine kinases, for example the FcεR1 receptor in mast cells. The recruitment of *syk* to the γ-chain of FcεR1 results in the tyrosine phosphorylation and activation of PLC-γ1 and PLC-γ2 (29,50), leading to sustained PIP_2 hydrolysis (75). Inhibition of *syk* inhibits the phosphorylation of PLC-γ, however it has not been established if *syk* is directly or indirectly involved (47), and no interaction between PLC-γ and FcεR1 has been detected (76). Tyrosine phosphorylation causes the enzyme's translocation, and interaction with an unidentified component of the cytoskeleton via its SH3 domain (77). The SH3 domain of PLC-γ1 binds to a specific proline-rich motif, different to that recognised by other SH3 domains, (78) this specificity could indicate a specialised and as yet unknown function.

Activation of PKC follows PLCγ translocation to the membrane (79) - the resulting accumulation of DAG leads to PKC translocation and activation

(80,81). Mast cells express PKC's α, β, δ, ϵ, and ζ (82). PKCδ is thought to phosphorylate threonines in the γ-chain of FcϵR1 (83); this modification is implicated in the down-regulation of the receptor by endocytosis. PKC's α and ϵ are thought to be involved in the feedback inhibition of PLCγ1 (84), and PKC's β and δ are able to partially restore calcium-stimulated secretion, from cells previously permeabilised and cytosol-depleted (82). Despite these diverse roles, PKC activation does not appear to be sufficient or essential for secretion; inhibition of the PKC's by pan-specific agents causes only partial inhibition of secretion (85), and activation of PKC's by PMA is not sufficient to elicit a secretory response (81).

The calcium/calmodulin-dependent protein myosin light-chain kinase is activated in stimulated cells (86), and is implicated in the phosphorylation of myosin light-chain (87). There is a correlation between this modification and a PKC-mediated phosphorylation of myosin light- and heavy-chains (86), and both of these events correlate well with the rate of histamine release, and have been implicated in the regulation of this process.

1.2.2 Phospholipase A₂

Stimulation of mast cells leads to activation of PLA$_2$, which in turn leads to the generation of arachidonic acid (88). PLA$_2$ is activated via mitogen-activated protein kinase (89), which is itself activated following FcϵR1 crosslinking (90). Activation of MAPK is regulated via tyrosine phosphorylation of Grb2/Sos (53). Sos has guanine nucleotide exchange factor (GEF) activity for ras (91), therefore syk-mediated activation of Sos activates the Ras $\rightarrow$ Raf $\rightarrow$ MEK $\rightarrow$ MAPK pathway. This pathway is PKC-independent, and is distinct from the pathway controlling exocytosis in these cells (92)

1.2.3 PI 3-Kinase

PI 3-kinase activity has been implicated in many signalling pathways (reviewed in (93)) including secretion, however its role in mast cell exocytosis has not been established. PI 3-kinase is activated following receptor aggregation in B lymphocytes (94) and T cells (95). The regulation of PI 3-kinase is complex, apparently mediated via interaction with ras (96), and by tyrosine

phosphorylation of the regulatory p85 (97); the proline-rich domain of p85 is a potential site for *lyn*-phosphorylation (98). Inhibition of PI 3-kinase inhibits secretion from rat mast cells (99) and RBL-2H3 cells (100), and other effects such as membrane ruffling (51). This suggests the importance of this enzyme in exocytosis. However, inhibition of PI 3-kinase was achieved in each of these studies using wortmannin. This agent potently inhibits PI 3-kinase, however its specificity is now widely questioned. It has been demonstrated recently that wortmannin's effect on secretion may not be the result of its effect on PI 3-kinase (101,102).

1.2.4 Phospholipase D
1.2.4.1 Enzymology of PLD

Phospholipase D is a ubiquitously expressed enzyme, that hydrolyses PC releasing choline and PA (see Fig.1.5). PLD is regulated by a variety of hormones and growth factors. Receptor-mediated activation of PLD via receptors linked to both G-proteins (103) and tyrosine kinases (104) has been detected in almost every cell-type studied (reviewed in (105-108). PLD's product PA is thought to play a variety of signalling roles, some of which may be related its conversion to DAG, however no second messenger functions have been attributed to choline.

PLD was first identified in plants, and later in mammalian tissues (109). Efforts to purify the mammalian forms of the enzyme have so far proved mostly unsuccessful (110). Early purification work identified an oleate-sensitive isoform (111), which has been purified from lung tissue (110) although its sequence is not known. A second PLD activity - hPLD1a has been cloned from a HeLa cell cDNA library (112) that is activated by ARF, Rho, protein kinase C, and PIP_2 (112,113). Another, very similar isoform - hPLD1b has been cloned which is regulated in the same way. These two isoforms appear to be splice-variants, one of which is lacking a 38-residue sequence (113) (see Fig.1.6). Another cloned isoform hPLD2, appears to be constitutively active, and regulated by protein inhibitor(s) (114). Based on sequence analysis, none of the cloned enzymes appear to contain any recognized domains (SH2, SH3,

PH etc). (In the following sections, the PLD activity discussed is PLD1 - the ARF/Rho/PIP$_2$-dependent form, unless otherwise stated).

1.2.4.2 Regulation of PLD activity by monomeric G-proteins

Phospholipase D is activated in many cell-types including mast cells and RBL-2H3 cells activated by anti-IgE (115,116). Early work demonstrated that activation was maximal only in the presence of cytosol, or cytosolic proteins (117). Other investigations showed that PLD could be activated by GTPγS in permeabilised cells or membrane preparations, suggesting a dependence on a G-protein. These two observations together indicated that the GTP-binding protein involved was probably a member of the ras-related class of small monomeric G-proteins. Finally, two such factors were identified - ARF1 and ARF3 (118,119) (see Fig.1.7). These proteins are both members of the ARF (ADP-ribosylation factor) subfamily. ARF's ability to stimulate PLD was first demonstrated in HL60 cells, however the observation has been extended to a variety of other tissues, suggesting that it may be universal. Other members of the ARF family: ARF's 4, 5 and 6 have all been shown to have the same activity (105). The distinguishing feature of the ARF family of proteins is an extension at the N-terminal which includes a myristoylation site (120). This modification is thought to facilitate the membrane association of GTP-bound ARF (121). The myristoylation is not essential to ARF's ability to activate PLD, however its absence compromises ARF's potency (119).

Another small G-protein - Rho, has been identified as a modulator of PLD activity. This finding is based on the observation that when RhoGDI is used to strip Rho from membranes PLD activity is compromised, and the resulting inhibition is relieved by the addition of recombinant RhoA, Rac1 and Cdc42 (122). Direct inhibitors such as C3-toxin, which inhibits RhoA, RhoB and RhoC by ADP-ribosylation inhibits PLD (123). However this toxin was reported as having no effect in other studies (124,125). Another clostridial toxin, Toxin B, inhibits RhoA, Rac1 and Cdc42 by monoglucosylation and has been reported to inhibit PLD activity. Mixed results have been gathered from studies using purified proteins. A partially purified brain PLD was stimulated by RhoA and Cdc42 in the absence of ARF (126). In a separate study PLD partially purified

Phosphatidyl
Choline

O-
|
O—P—O —Choline
‖
O

Phosphatidic Acid

O-
|
O —P—O —OH
‖
O

+

Choline
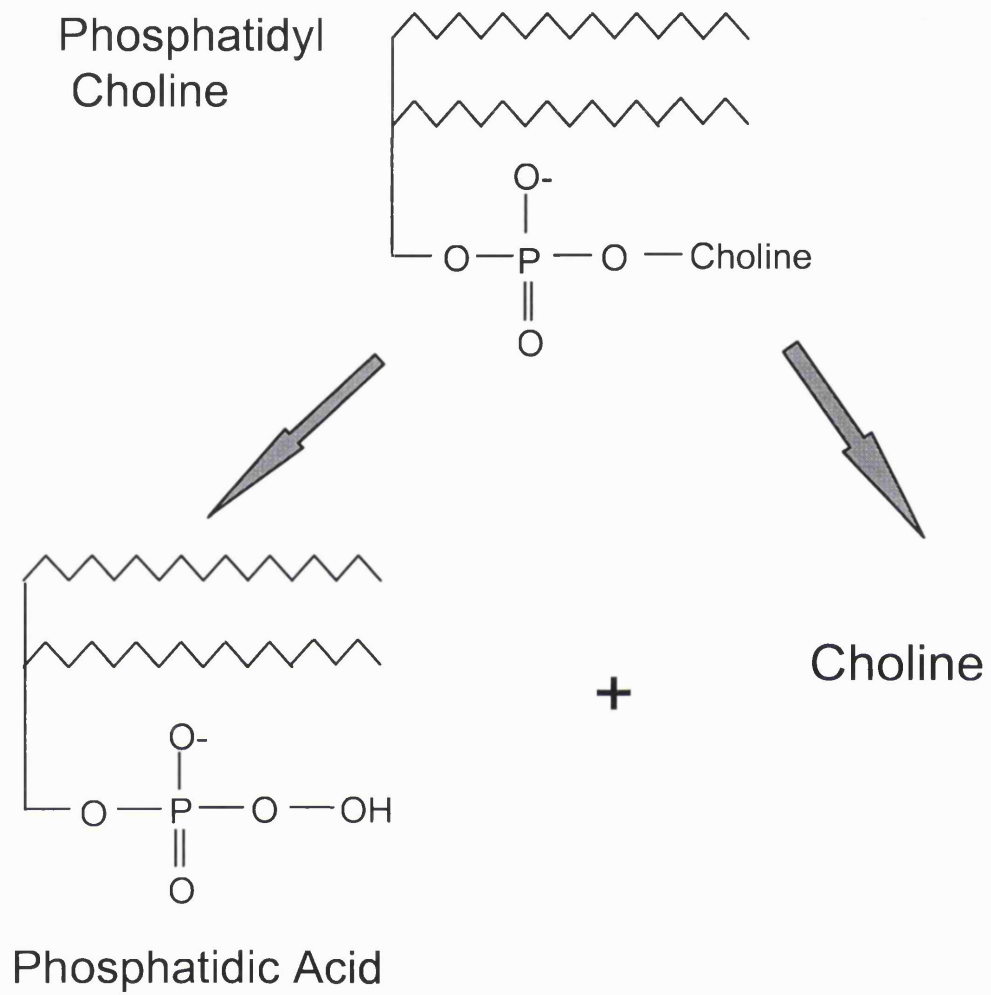
Fig 1.5 Representation of PLD activity

from human placenta reportedly lost the sensitivity to Rho observed in the crude extract, but retained its sensitivity to ARF (127). These results indicate that RhoA is likely to modulate PLD activity. In studies where ARF and Rho have been used together, there is evidence of a strong synergistic effect on PLD activation (113,123). This synergy is noted also in the presence of PKCα (113). These data suggest that these three activators of PLD interact with three distinct sites on the protein.

1.2.4.3 Regulation of PLD by PKC

There are many reports that PLD is regulated by PKC, based on treatment with phorbol esters, treatment with inhibitors and enzyme down-regulation and over-expression (reviewed in (108)). The expected mechanism of PKC-mediated PLD activation would involve PLD phosphorylation, however there is strong evidence to suggest that this does not occur (128,129). In studies using a recombinant PLD and individual PKC isoforms, PKCα and PKCβ were found to be effective activators of PLD, independent of ATP (113). The interaction between PLD and PKC is thought to take place between the PKC's regulatory domain (129), with activation achieved solely via an allosteric effect. In contradiction to these findings, studies in cell-free systems do show some effect of ATP (130), however these effects have been attributed to the effect of ATP on PIP_2 levels, on which PLD is dependent.

1.2.4.4 A requirement for PIP_2 in PLD activity

Initially the dependence of PLD on $PI-4,5-P_2$ was noted in *in vitro* assays (118), where the lipid was shown to be essential. The enzyme has been shown to be dependent on $PI-4,5-P_2$ synthesis in permeabilised cells also (131), however the importance of $PI-4,5-P_2$ in modulating PLD activity *in vivo* is not known. Several reports provide indirect evidence for this modulation: firstly that ARF-dependent PLD activity is strongly potentiated in the presence of millimolar MgATP (132), a concentration range that reflects that typically required by the lipid kinases. Neomycin, which sequesters PIP_2 with high affinity, therefore preventing interaction of the lipid with any potential target protein, strongly inhibits PLD activity (132). Finally, an antibody to PI 4-kinase which abolishes the enzyme's activity, and therefore prevents *de novo* production of PIP_2, also
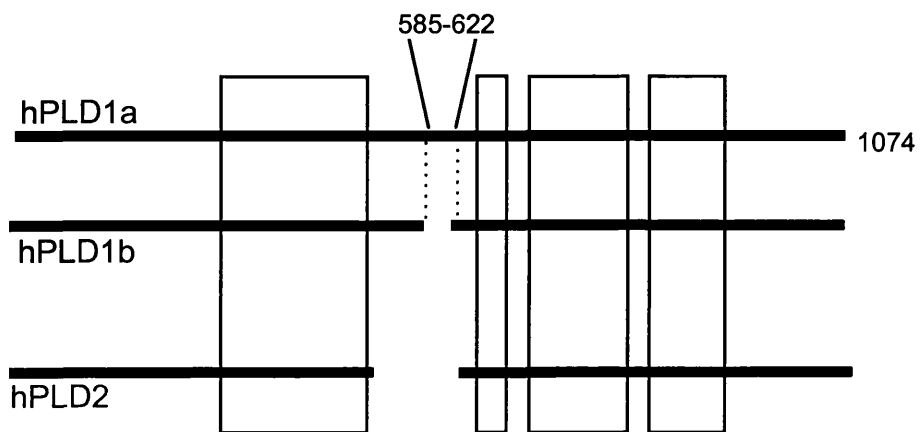
Fig 1.6 Diagrammatic representation of the cloned mammalian isoforms of PLD

Vertical boxes represent regions of extensive homology. The region in hPLD1a that corresponds the "missing" section in the hPLD1b splice variant is indicated. (Modified from (113))

inhibits PLD activity (131). It has been suggested that PLD's dependence on PIP_2 may be indirect, mediated via an effect of PIP_2 on ARF. PIP_2 stimulates an ARF-GEF, thus promoting the dissociation of GDP from ARF and hence the conversion of ARF to its active GTP-bound form (133). It is also possible that PIP_2 anchors PLD to the membrane, in contact with its substrate (134), though as PLD contains no recognised PIP_2-binding or PH-domains, it is not clear how this could occur.

1.2.4.5 ARF-regulated PLD in vesicular budding

ARF has been identified as an activator of PLD activity, however it also has a well-defined role in vesicular budding. ARF is a component of the COP1 or coatomer protein complex that coats vesicles passing between the ER and Golgi. The binding of ARF to the Golgi causes the recruitment of coatomer, and ultimately leads to vesiculation. The interaction of ARF with the membrane is facilitated by the N-terminal myristoylation, and the presence of a protein receptor has also been suggested (135). ARF is probably activated by a Golgi-associated ARF-GEF. Two ARF-GEF's Gea1 and Gea2 have been cloned from S.cerevisiae (136). These proteins were found to contain a sec7 domain, which activates nucleotide exchange on ARF (137). This domain is a region of homology to sec7, a protein that is involved in membrane traffic in yeast (138). Based on this sequence a human ARF-GEF - ARNO was cloned (137). Apart from its ARF-GEF activity, ARNO also contains a phospholipid-binding PH domain and is specifically activated by PIP_2. Other ARF-GEF's, such as cytohesin have been found to include PH-domains with different specificities, in this case for PIP_3 (139), perhaps indicating some complexity in the regulation of these proteins. ARNO is smaller than the equivalent proteins in yeast and lacks their sensitivity to the inhibitor Brefeldin A (140). Other ARF-GEF's have been identified that do show BFA-sensitivity (141), suggesting that there may be different classes of ARF-GEF's.

ARF is an essential component of vesicular budding from the Golgi. However, it is not clear whether ARF is involved directly, or whether it mediates its effect via an effector protein, for example PLD. This possibility is supported by the finding that if ARF is applied to membranes, then removed and replaced with

GTP   Ca²⁺   DG/PMA   GTP

ARFs   PKC   Rac
(1,3,4,5,6) (α & β)   Cdc42
                          RhoA

hPLD1

PC   Requires PIP₂   PA
      as a cofactor
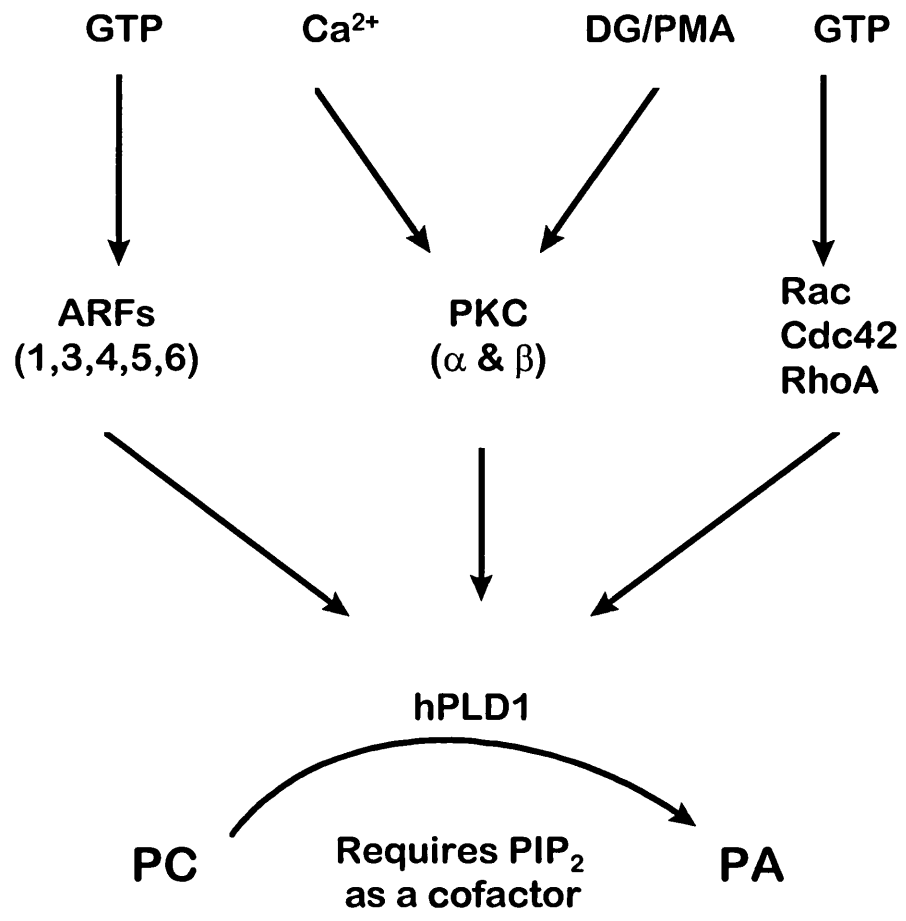
Fig. 1.7 Regulatory inputs leading to synergistic activation of PLD
(Modified from (105)).

coatomer, vesiculation proceeds (142). Thus ARF's function could be catalytic, causing PLD activation then becoming dispensable. However the stoichiometric complexes of ARF and coatomer that have been detected (143) suggest that ARF itself may also have a purely structural role. It is also possible that ARF acts via an effector protein other than PLD, for example arfaptin. ARF causes the recruitment of arfaptin onto the Golgi membranes (144), though the function of this protein is not known.

Supporting evidence for the importance of PLD in vesicle budding is based on the reported dependence on PKC. The PKC-dependent step in budding appears to be ATP-independent (145,146) and involves only the regulatory domain (147). This pattern of dependence is identical to that seen in PKC stimulation of PLD activity (see section 1.2.4.3), and the same isoform of PKC has been implicated in both processes - PKCα (148). It seems likely therefore that ARF-stimulated PLD activity is essential in vesicle budding. However, in yeast where this process is directly analogous, no ARF-regulated PLD's are expressed. It has been suggested therefore that ARF-regulated PLD represents a regulatory process that has been superimposed on a pre-existing, constitutive pathway.

If PLD is an essential component of vesicular budding, the products of PLD activity should potentiate the process. PLD hydrolyses PC, forming PA and choline. It has been suggested that the removal of PC could be as important to vesiculation as the formation of PA; in yeast membranes excess PC prevents vesicle formation (149). The importance of PA as a second messenger is becoming firmly established. PA facilitates coatomer binding (142), increases membrane curvature (150), and also activates PI4P 5-kinase (151). The effect of PA may therefore allow a positive feedback loop to become established at foci in the membrane: PA activates PI4P 5-kinase and so increases PIP_2 synthesis, PIP_2 potentiates PLD activity, leading to increased PA formation etc. Potentially these PIP_2-enriched foci could be important, particularly as coatomer binds to PIP_2 (142). Another potential role for PA could arise from its potential conversion to diacylglycerol (DAG), which has been shown to be essential in vesicle formation in yeast (152). Therefore

the importance of PLD in vesicular budding could reside in a variety of potential consequences of its activity.

1.2.4.6 The role of PLD in endocytosis

Two populations of clathrin-coated vesicles participate in the endocytic pathway - those that are derived from the plasma membrane, and those that bud from the trans-Golgi network. These vesicles are formed under the regulation of adaptor complexes, AP-1, AP-2 and AP-3. AP-2 participates in the formation of the plasma membrane-derived vesicles, AP-1 and AP-3 are involved in budding from the TGN. Their recruitment is dependent on ARF1 (153,154). There appears to be divergence between the mechanism of adaptor-recruitment to the plasma membrane, and recruitment to the TGN. AP-2 recruitment is mis-directed to the endosome by the addition of GTPγS, an effect that can be reversed by brefeldin-A mediated inhibition of ARF-GEF (155). This disruption can be achieved also by the addition of a constitutively active bacterial PLD (154). Therefore, though the exact details of the mechanism remain obscure it seems likely that ARF-mediated AP-2 recruitment is achieved via ARF's effect on PLD activity. AP-1 recruitment is not affected by the addition of bacterial PLD, nor by the addition of PLD-stimulating PIP_2 (see above). However AP-1 recruitment is dependent on ARF (156). Thus ARF clearly has some importance in vesicle formation independent of its effect on PLD.

One member of the ARF family - ARF-6, has been specifically implicated in endocytosis (157,158). Although ARF-6 can activate PLD, it has not been demonstrated to what extent PLD activation mediates the effect of ARF-6 in this process.

1.2.4.7 The role of PLD in exocytosis

Initial evidence for the importance of PLD activity in exocytosis was based on the widely-reported observation that low concentrations of ethanol block exocytosis, an effect that has been noted in RBL-2H3 cells (115,159). Ethanol subverts PLD activity, by substituting for water in the hydrolysis of PC, thus phosphatidylethanol (PEt) is formed in place of PA. Ethanol is not an ideal

reagent, its substitution in the transphosphatidylation reaction is partial so some PA is still formed, and it is not a true inhibitor: PC is still hydrolysed, which may itself be an important aspect of PLD activity. Exocytosis is stimulated when exogenous PLD is added to intact cells (160), however this approach is also problematic: it may result in inappropriate localisation of effects, in a system where spatial aspects of signalling may be important.

Thus the most compelling evidence for PLD's importance in exocytosis comes from work using HL60 cells. HL60 cells are of haematopoietic origin, related to neutrophils. Permeabilisation of HL60 cells leads to a loss of cytosolic proteins, and a parallel loss of responsiveness to stimulation. The kinetics of ARF leakage from these cells correlates very closely with the decline in both the secretory response and the extent of PLD activation (161). When cytosol-depleted cells are supplied with either purified recombinant or native ARF, the secretory response and PLD activation can be recovered under similar conditions (161). These data strongly suggest an important role for PLD in exocytosis.

Further support for this idea is gathered from various reports concerning the regulation of exocytosis and PLD. As noted in previous sections, PLD activity is modulated by the conventional PKC's and by Rho. Both of these regulators also affect exocytosis from permeabilised mast cells. Constitutively active Rac and Rho mutants have been constructed and introduced into mast cells, producing enhanced release (162,163). Rac1 has been shown to retard the decline in responsiveness measured following permeabilisation (164). The conventional PKC's also have established regulatory function in mast cell exocytosis. Thus all known regulators of PLD activity regulate exocytosis also.

1.3 Factors involved in regulating RBL-2H3 mast cell exocytosis
1.3.1 Calcium
Calcium is a regulator of exocytosis in many cells. In stimulated mast cells, intracellular calcium concentration is increased. Initially stimulation leads to PLC activation, resulting in IP_3 production (165,166). This leads to calcium release from IP_3-sensitive stores (167). Mast cell exocytosis stimulated by IgE

is dependent on calcium influx (168,169), however exocytosis stimulated by receptor-mimetic agents such as 48/80 is not (170). In IgE-stimulated exocytosis, calcium enters the cell via non-voltage activated calcium channels (171), which are possibly regulated by a GTP-binding protein (172).

Studies using permeabilised mast cells where buffered calcium solutions are applied, confirm that micromolar concentrations of intracellular calcium are required (173). However, similar studies have suggested that calcium plays a modulatory role, not sufficient to trigger secretion alone (174).

Insight into what the precise function of calcium in secretion could be, was gained following work in neuroendocrine cells. Calcium-dependent triggering of exocytosis, which in neuroendocrine cells and neurons occurs rapidly - within milliseconds is probably mediated via a specific calcium-sensor. Though several candidates for the exocytosis trigger have been suggested, no single ubiquitous protein has yet been identified. This may in fact reflect a difference in organisation between the various types of secretory cells. In neurones a small number of vesicles are docked to neurotransmitter release sites, close to the pre-synaptic calcium channels. In fact it has been suggested that synaptic vesicles may be directly tethered to calcium channels (175). Therefore following an action potential, when calcium enters through these channels, the local calcium concentration can reach the high micromolar range. (In retinal neurons, exocytosis is thought to be half maximal in the presence of 190μM calcium (176)). Therefore any sensor that must react to a change of concentration in this range must have a relatively low affinity for calcium. In neuroendocrine cells also docked vesicles may be close to the site of calcium influx. However calcium buffering is very efficient in cytosol, and localised "hot-spots" of high calcium concentration do not extend far (177). Thus a calcium sensor in these cells must be sensitive to a relatively low range of calcium concentrations (adrenal chromaffin cell secretion is half-maximal at 10-20μM calcium (178)). Thus a variety of calcium-sensing proteins may exist, alternatively different isoforms of the same protein to reflect these different triggering affinities. Such a family of calcium-sensing proteins has been identified, the synaptotagmins (179).

Synaptotagmin is expressed on vesicles from chromaffin cells, synapses and other endocrine cells - suggesting that synaptotagmin's role is universal. Many isoforms of synaptotagmin have been identified. All of these contain two cytoplasmic domains that are homologous to PKC's C_2 calcium- and phospholipid-binding domains, however not all of the synaptotagmins bind calcium and phospholipid (180). The synaptotagmins also vary with respect to their binding to syntaxin. Synaptotagmins I, II and V bind in the presence of 200-500μM calcium, synaptotagmins III and VII bind at 10μM calcium while synaptotagmins IV, VI and VIII do not bind syntaxin at all (180). The mechanism for the synaptotagmins' role is not known, however the proteins' importance in exocytosis is indicated by the inhibition of PC12 cell exocytosis by anti-synaptotagmin antibody (181). A possible effect of phospholipids on synaptotagmins has been suggested. In quiescent nerve terminals, synaptotagmin binds specifically with PI-3,4,5-P_3, but at the high calcium concentration seen following activation this specificity swaps to PI-4,5-P_2 (182). It has been suggested that this exchange from PIP_3 to PIP_2 actually represents the change in synaptotagmin binding to only the vesicle in resting cells, to binding to PIP_2-enriched patches on the acceptor membrane after stimulation. It is possible that specific fusion sites are defined by their phospholipid contents, and identified by appropriate phospholipid-binding domains on vesicle proteins. In support of this idea is the finding that addition of IP_6 - which competes for PIP_2 on synaptotagmin's phospholipid binding domain, prevents vesicle fusion (182), perhaps by effectively "blinding" synaptotagmin to the presence of the acceptor membrane. The presence of synaptotagmin in mast cells has not been demonstrated.

Calmodulin has been implicated in exocytosis from many cell-types. The protein binds to synaptotagmin (183), suggesting that it could act via a direct interaction with the exocytic machinery. In mast cells the importance of calmodulin is not clear. Much of the literature discussing calmodulin's importance in exocytosis is based on experiments using inhibitors such as W7, this is true of the work carried out in rat mast cells (184,185). These agents do not show the high degree of specificity that was initially claimed, thus this work

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must be viewed with caution, therefore it must be concluded that a role for calmodulin in mast cell secretion has not been proven. It has been suggested that calmodulin augments ARF-regulated PLD activity in neutrophils, an interesting finding, given the putative role of PLD in secretion (186).

Apart from its importance in triggering, calcium has been implicated as a modulator of priming (187), where the putative calcium-binding priming protein has an affinity for calcium in the low micromolar range (188). Thus it is possible that calcium mediates multiple steps in the exocytic pathway, interacting with a variety of proteins with different affinities.

1.3.2 Guanine Nucleotides

The importance of a G-protein in rat mast cell exocytosis was indicated by the findings that calcium, together with GTPγS - a non-hydrolyseable analogue of GTP, was required to induce maximal exocytosis (189,190), and in fact GTPγS can elicit a secretory response from rat mast cells even in the effective absence of calcium (174). The dependence of rat mast cell exocytosis on GTP can also be demonstrated via the application of inhibitors of IMP-dehydrogenase, the rate-limiting enzyme in *de novo* GTP biosynthesis (191). Rat mast cell exocytosis is completely inhibited by such agents, and this blockade is seen whether exocytosis is elicited by mastoparan, IgE:FcεR1, or calcium ionophores (192-194).

Heterotrimeric G-proteins consist of α-, β- and γ-subunits. Stimulation of cells lead to activation of G-proteins, as G-proteins are usually held in close association with receptors in quiescent cells. In their resting state, the three subunits of the G-protein are complexed together, with GDP bound to the α-subunit. Activation results in the release of GDP, the binding of GTP and in the dissociation of the α-subunit from the βγ-complex. This dissociation is followed by the activation of the two moieties' various effector proteins. The activation signal is terminated by the α-subunit's intrinsic GTP-ase activity hydrolysing GTP, ultimately leading to reassociation of the now GDP-bound α-subunit with the βγ-complex.

As already described, the application of pertussis toxin (PtX) indicated an important role for a heterotrimeric G-protein in exocytosis (195,196), PtX inactivates G_i's and G_o's specifically, by ADP-ribosylating the α-subunit. This agent inhibits mastoparan-induced exocytosis from rat mast cells (197), however exocytosis elicited via IgE:FcϵR1 is unaffected (198). Mastoparan is known to cause selective activation of heterotrimeric G-proteins (199). Thus its ability to elicit a secretory response is itself evidence that heterotrimeric G-proteins are involved in exocytosis. Like PtX, mastoparan shows specificity for G_i's and G_o's (in comparison with G_s and G_t (200)).

The application of this agent to adrenal chromaffin cells has led to the hypothesis that different stages in exocytosis may be regulated by different heterotrimeric G-proteins. Mastoparan inhibits MgATP-dependent calcium-induced exocytosis, but stimulates MgATP-independent exocytosis (201). This finding has been interpreted as a suggestion that one mastoparan-sensitive G-protein regulates calcium-induced triggering, while another regulates ATP-dependent priming. Support for this theory comes from work using anti-$G_o\alpha$ antibodies (202), which reverses mastoparan's inhibition of MgATP-dependent exocytosis, indicating that G_o regulates "priming" in these cells. Anti-$G\alpha_{i3}$ antibodies inhibit mastoparan-mediated stimulation of calcium-induced exocytosis, indicating that this protein might regulate "triggering".

In rat mast cells, no evidence exists for the involvement of G_o, however the importance of $G\alpha_{i3}$ is widely reported. This particular protein has been implicated because of its presence on rat mast cell plasma membranes, and because application of $G\alpha_{i3}$-peptides, or anti-$G\alpha_{i3}$ antibodies into rat mast cells blocks 48/80-induced exocytosis (203). This effect appears to be specific, with no effect reported for the equivalent reagents directed against $G\alpha_{i2}$. There appears to be two distinct pools of $G\alpha_{i3}$ in rat mast cells - one localised to the Golgi and one to the plasma membrane (203). Brefeldin A disrupts the Golgi when applied to cells. This agent has no effect on 48/80-stimulated rat mast cell exocytosis, indicating that only the plasma membrane associated pool is important in rat mast cell exocytosis. This finding is supported by studies in

RBL-2H3 cells, where the plasma membrane pool is absent, and the cells not activated by 48/80. Co-culture of RBL-2H3 cells with 3T3 fibroblasts results in an acquired responsiveness to 48/80, accompanied by a partial redistribution of Golgi-associated $G\alpha_{i3}$ to the plasma membrane (204). The importance of localisation in $G\alpha_{i3}$'s activity is perhaps to be expected, as its predicted role is in triggering.

An additional aspect to signalling from heterotrimeric G-proteins is the effects of the $\beta\gamma$-complex. $\beta\gamma$-subunits can bind to PH-domains (205). The fact that several key enzymes in cell signalling contain these domains (reviewed in (206)) makes a variety of potential signalling role for the $\beta\gamma$-complex possible. In rat mast cells the importance of $\beta\gamma$-subunits is indicated by their ability to stimulate exocytosis (207). However $\beta\gamma$'s are a partial stimulus: GTP-dependence is still exhibited, presumably because it is required to promote the dissociation of the α-subunit from the $\beta\gamma$-complex.

A number of the sub-types of monomeric G-proteins have also been implicated in exocytosis. The monomeric ras-related G-proteins are divided into the Ras, Rho, Rab, ARF and Ran subfamilies. These proteins are analogous to the α-subunits of heterotrimeric G-proteins. They are activated following the release of GDP and the uptake of GTP, and lapse into a resting state following hydrolysis of GTP. However these proteins do not act alone, but in co-operation with a guanine-nucleotide exchange factor (GEF), and the low intrinsic GTP-ase activity is stimulated in the presence of a GTP-ase activating protein (GAP) (see Fig.1.8).

The ARF subfamily is divided into ARF's and ARF-like (Arl) proteins (208). The Arl's may be distinguished by their lack of the ARF activity that suggested their name - the ability to act as cofactors for cholera toxin-dependent ADP-ribosylation of the heterotrimeric G-protein G_s (209). Based on size, gene structure and primary sequence, the ARF's have been classified into three classes. Class one consists of ARF's 1, 2 and 3, class two contains ARF4 and ARF5, and ARF6 - the most structurally distinct is alone in class three (210). Though ARF's 1 and 3 were originally identified as activators of PLD (119),

members of all the classes show the same activity (211,212). The structure of ARF-GDP has been solved, and found to be similar to ras (213,214).

The first reports that suggested ARF's importance in exocytosis were based on studies carried out in RINm5F insulin-secreting cells, where stimulation and exocytosis correlated with ARF translocation to the Golgi and the plasma membrane (215). Later reports concerned the use of a synthetic peptide, corresponding to residues 2-17 from ARF's N-terminal (216). This peptide was reported as an inhibitor of PLD and exocytosis in HL60 cells, however the specificity of this peptide has been questioned (217). The most direct evidence for ARF's importance in exocytosis has come from reconstitution studies of PLD

and exocytosis (161). These findings have led to the recent hypothesis that ARF's effect on exocytosis may be a product of its effect on PLD.

There is one report of ras-stimulated secretion from mast cells (218). This study states that if oncogenic ras is introduced into mast cells in the presence of external calcium, secretion will be triggered without any further stimulus. However the mechanism of this effect is unclear, particularly as secretion is measured only 2.5-4 hours after the introduction of the protein. Thus if ras is involved in mast cell exocytosis, its effects are likely to be only indirect.

The rab family of G-proteins has been implicated in a variety of vesicular trafficking events (reviewed in (219)). Each rab is localised specifically to distinct areas of membrane, indicating perhaps that the proteins are responsible for ensuring the specificity of membrane fusion events. Most of the interest in the rab proteins' potential role in exocytosis has concentrated on rab3a. Early studies using rat mast cells indicated that rab3a might stimulate secretion. This work employed a peptide corresponding to the effector domain of rab3a (220), however the specificity of this agent has now been questioned (221). Over-expression of rab3a in RBL-2H3 cells inhibits exocytosis, stimulated by GTPγS and via the FcεR1 receptor (222). Thus it has been
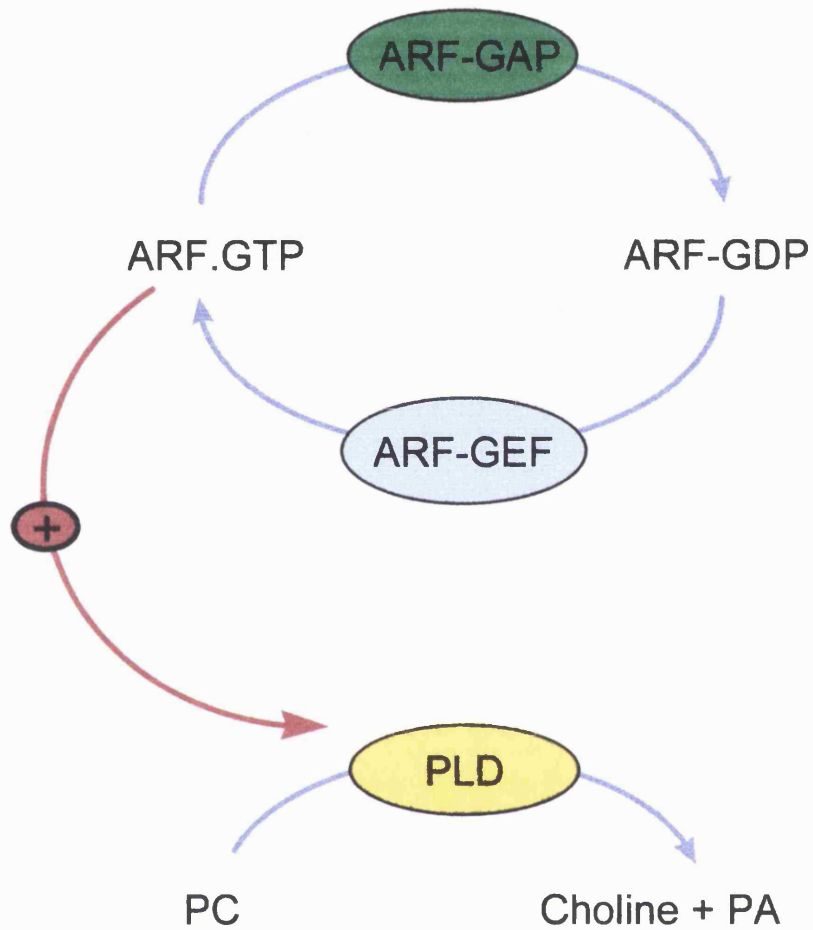
Fig 1.8 Cycle of ARF activation and inactivation

GDP is exchanged for GTP on ARF via interaction with ARF-GEF, GTP bound to ARF is hydrolysed via ARF-GAP. ARF-GEF activity is enhanced by PIP_2, ARF-GEF activity by PIP_2 and PA together.

suggested that rab3a is a negative regulator of exocytosis (223). However it has not been established that rab3a is expressed in rat mast cells *in vivo.*

The Rho family of G-proteins consists of Rho (A, B, and C), Rac (1 and 2), Cdc42, RhoE, RhoG and TC10. The most clearly established function of the Rho proteins is associated with their effects on the cytoskeleton. The proteins are each associated with a particular type of actin-structure assembly: Rac in membrane ruffles (224), Rho in stress fibres (225) and Cdc42 in filopodia (226). The importance of these proteins in exocytosis may be inferred from various reports. C3 clostridial toxin inhibits RhoA, RhoB and RhoC. Application of this agent to mast cells causes the inhibition of exocytosis (162). In addition, introduction of constitutively active RhoA - V14RhoA into permeabilised mast cells increased exocytosis, as does the addition of active Rac. The mechanisms of these protein's effects on exocytosis is not clear. It has been suggested that Rac and Rho may mediate their effects via modification of the cytoskeleton. However in rat mast cells, the proteins still enhance exocytosis when cytoskeletal changes are blocked (163).

The potential for Cdc42 to regulate exocytosis is unclear. Clostridial Toxin B, an inhibitor of RhoA and Cdc42 inhibits exocytosis from RBL-2H3 cells (227). In these experiments C3 was without effect. As C3 inhibits RhoA but not Cdc42 this finding indicates that Cdc42 is the target of Toxin B, and is active in exocytosis. However the lack of effect of C3 in this study contradicts other reports (228), therefore this data must be viewed with some caution.

In more recent work carried out in mast cells, a complex was identified that retarded the loss of sensitivity to stimulation seen following permeabilisation (164). This complex was identified as Rac and RhoGDI. RhoGDI alone accelerates the onset of the refractory state, probably because it strips Rho proteins from the membrane. Wild-type Rac alone is without effect, but Rac with RhoGDI is effective - perhaps because the GDI ensures presentation of Rac to appropriate cellular targets. Constitutively active Rac alone is also effective, indicating perhaps that the recombinant wild-type rac is not correctly modified (162).

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The requirement for GTP observed in mast cells is also seen in other cells. However there is a great variance in the literature as to the relative importance of GTP or calcium in exocytosis - probably reflecting fundamental differences between the types of cells studied. In general, cells of haematopoietic lineage, for example mast cells, are more dependent on G-proteins, whereas neuronal systems show a greater dependence on calcium.

1.3.3 ATP

Treatment of intact rat mast cells with metabolic inhibitors to deplete intracellular ATP inhibits exocytosis induced by IgE:FcεR1. However, permeabilised rat mast cells, stimulated by calcium and GTPγS show no dependence on MgATP levels (189,190). When calcium alone is used as a stimulus however, ATP dependence is seen. These findings taken together indicate that the role of ATP may be to support the synthesis of GTP. Another possibility is that any dependence of rat mast cells on MgATP could be due to the dependence on PKC-mediated phosphorylations (229). This suggestion is supported by the findings that secretion stimulated by calcium alone may be inhibited by inhibitors of PKC (174). The importance of MgATP in supporting PKC-mediated phosphorylations is also suggested by the similar effects of added MgATP and PMA (an activator of PKC), both causing the cells to become more sensitive to the presence of GTPγS or calcium, rather than acting as direct stimuli themselves (190,230).

An interesting possibility is suggested by the effect of MgATP on cytosol-depleted rat mast cells. Rat mast cells become unresponsive to stimulus after permeabilisation, an effect that is explained by the gradual exit of the cytosolic proteins. This decline in responsiveness is accelerated in the absence of MgATP (231), and this acceleration may be reversed upon late addition of MgATP. If MgATP were responsible for retarding the loss of a protein mediator of exocytosis, the ability of late-added MgATP to partially restore responsiveness would not be predicted. Instead, this effect suggests that MgATP's role is to maintain some non-cytosolic cellular component - either

protein or lipid, in a phosphorylated -perhaps a "primed" state that in the absence of MgATP becomes reversed.

MgATP is primarily of importance in exocytosis during the preparative step which precedes triggering, known as "priming" (232). This term describes perhaps a variety of processes dependent on ATP and cytosolic proteins. Three such proteins have been described in PC12 cells (232), and two of these proteins have been isolated and identified. The first of these was the phosphatidylinositol transfer protein (PI-TP) (233).

PI-TP was originally named because of its *in vitro* activity in transferring phosphatidylinositol through an aqueous phase from one membrane compartment to another (see Fig.1.9). The protein is a member of a family that includes PC-TP - a phosphatidylcholine transfer protein, and the non-specific lipid transfer protein nsL-TP (234,235). The protein has an apparent size of 35kDa in SDS-PAGE (234) with one phospholipid binding site. Different isoforms have been detected, of which two - PI-TPα and PI-TPβ are found in mammalian cells (236,237). These proteins show no sequence homology to any other protein except each other, with 70% identity and 94% similarity between the two isoforms. Despite the lack of sequence homology, yeast isoforms such as SEC14p display the same characteristic lipid transfer activity (238,239).

PI-TP's single phospholipid binding site is specific for either PI or PC, however in the absence of either lipid, for example when recombinant protein is isolated from E.Coli, the protein can bind to other phospholipids such as phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) (240). The protein displays different affinities for different phospholipids: PI>PC>PG>PE, having an affinity for PI sixteen times that for PC (241). Recently, the crystal structure of SEC14p has been solved (242). The protein has a large hydrophobic pocket, assumed to form the binding site for the phospholipid's acyl-chains. In addition a highly hydrophobic region protrudes away from this pocket: it is suggested that this protrusion interacts with, and possibly inserts partially into the membrane, and in effect "scoops" the phospholipid into the

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binding pocket. The lack of sequence homology between mammalian PI-TP and SEC14p precludes any assumption that their tertiary structures may match, however the convergence in their function does make some similarity likely.

PI-TP is thought to have a number of important signalling roles, identified partially through the application of the permeabilised cell assay. The original work using this protocol, was carried out in GTPγS-stimulated HL60 cells (243). Prolonged permeabilisation, and cytosol depletion resulted in a decline in PLC-mediated PIP_2-hydrolysis, a decline that was reversed in the presence of cytosol.

Fractionation of cytosol lead to the purification and identification of the protein that "rescued" the response. Thus the importance of PI-TP in PLC-β activity was established. This observation was later extended to PLC-γ1 (244,245). In these studies, because PLC-γ1 itself was found to exit cells, a double reconstitution system was used, where both PLC-γ1 and PI-TP were required to fully restore IP_3 production. A member of the third subfamily of PLC's - PLC-δ1 is also regulated by PI-TP (72). PLC-δ1, like PLC-γ1 leaks from cells and must be replaced; if PI-TP is replaced alongside PLC-δ1, calcium-stimulated IP_3 production is restored. This ability of PI-TP to regulate members of all the PLC subfamilies, despite other differences in their regulation indicates that PI-TP may function at a very fundamental level of PLC activity. One possibility is that PI-TP facilitates the provision of PI to the PI 4-kinase and subsequently to PI4P 5-kinase. The production of PIP_2 by these two enzymes is thought to occur at the plasma membrane, whereas PI is synthesised at the endoplasmic reticulum (246). Thus transport of PI to the plasma membrane must be a vital step in phosphoinositide signalling, a step which is entirely compatible with PI-TP's *in vitro* activity. That *de novo* PIP_2 synthesis occurs in stimulated cells may be inferred by the findings that the amounts of IP_3 produced exceeds the amount of PIP_2 present in quiescent cells (247), despite the fact that PIP_2 levels decrease only transiently during stimulation.
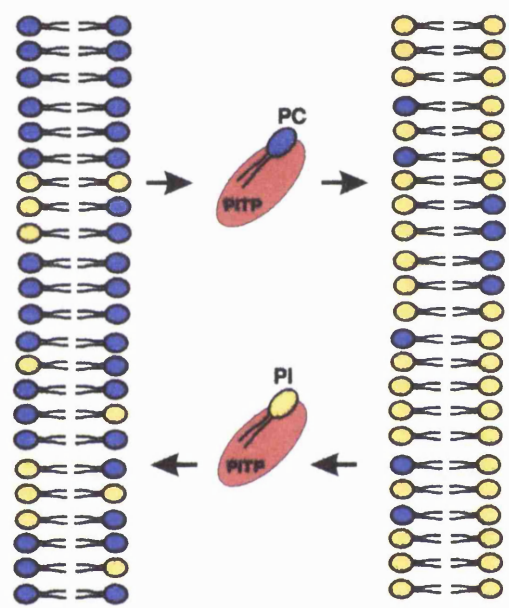
Figure 1.9 *In vitro* transfer activity of PI-TP

PITP transfers PI and PC from donor to acceptor membranes. For each PI/PC molecule delivered, another is removed. Thus *in vitro* at least PI-TP's phospholipid binding site of PITP is always occupied.

Thus it is possible that PI-TP's ability to bind and transport PI is the sole explanation for PI-TP's role in PLC signalling. This idea is supported by experiments using SEC14p - in which the protein, with no sequence homology to PI-TP, but an equivalent ability to transfer PI, was shown to be equally effective in PLC signalling as both PI-TPα and PI-TPβ (245). However this idea is not supported by the inability of nsL-TP to support PLC activity (248), as nsL-TP is also able to bind and transport PI. Thus PI-transfer activity may be necessary for PLC signalling, but is not sufficient.

It has been suggested that PI-TP regulation of PLC activity may be more subtle than simply supplying PI to regions of membrane, where PIP_2-hydrolysis by PLC has lowered the local concentrations. Further examination of PLC signalling attempted to identify a "rate-limiting step". In the presence of GTPγS, but not PI-TP, the rate of PLC-mediated PIP_2 hydrolysis is increased markedly when exogenous PLC-β is introduced (247). Thus the amount of enzyme appears to be limiting, and not the amount of substrate. In this system, PI-TP increase the rate of hydrolysis at all concentrations of PLC-β. This suggests that the presence of PI-TP sustained a faster rate of synthesis and hydrolysis of PIP_2, even when the rate of these reactions is not dictated directly by its transfer activity. This idea is expressed in the "cofactor" hypothesis of PI-TP function. This hypothesis proposes that the lipid carried by PI-TP is the preferred substrate for the lipid kinases, which are organised into a signalling complex.

There is evidence for the formation of these signalling complexes in EGF signalling, with PI-TP found associated with the EGF receptor, PLC-γ and PI 4-kinase (244). The advantage of these signalling complexes, in terms of the increased speed and efficiency they confer on multi-enzyme cascades is obvious. In PI-TP-sustained PLC signalling for example, PI bound to PI-TP could be sequentially phosphorylated by the lipid kinases, then PI-TP associated PIP_2 passed to PLC for hydrolysis (247). Thus reaction intermediates, such as PI4-P is passed to PI4P 5-kinase, without equilibration with, and dilution in the bulk environment. Support for this idea comes from analysis of deletion mutants of PI-TPα. Deletion of 5 amino-acids from the C-

terminus completely abolishes PI-TP's ability to restore PLC signalling, however this deletion does not affect transfer activity for PC and decreases transfer activity for PI by only 30% (240).

The permeabilised cell protocol revealed a second important activity of PI-TP, the ability of this protein to restore secretion in cytosol-depleted cells. Initially this work was carried out in GTPγS-stimulated HL60 cells, where addition of both PI-TPα and PI-TPβ restored a secretory response to stimulus that had been lost following prolonged permeabilisation (161). This response was found to be dependent on millimolar concentrations of MgATP, suggesting the importance of a phosphorylation event.

The second priming activity identified from studies in PC12 cells was the type 1 PI4P 5-Kinase (249). PI-TP and PI4P 5-kinase support priming when used alone, and are strongly synergistic when used together, suggesting that they participate in a single process. The identity of these proteins raise intriguing questions about the importance in priming of the inositol phospholipids, in particular PIP_2, as both of these activities participate in the formation of this lipid. Thus an important aspect of priming could be to increase the levels of PIP_2 in the cell. The failure of neomycin to inhibit exocytosis from rat mast cells however, means that these cells are unlikely to require this lipid for exocytosis.

An essential aspect of the PPI's importance in cell signalling and membrane traffic may be their potential for modulating the properties of distinct areas of membrane. This potential for PPI's to form distinct micro-domains has recently been demonstrated. Caveolae are small invaginations from the plasma membrane. Recently the protein and lipid constituents of these entities has been examined: heterotrimeric G-proteins and receptors were detected in a lipid environment that was highly PIP_2-enriched (250,251), suggesting that the lipid could perhaps form an assembly point, for the formation of a signalling complex. Localisation of PIP_2 at membrane ruffles and focal contacts in 3T3 cells has also been demonstrated, as well as a co-localisation of α-actinin to these regions (252).

Site-specific synthesis of PPI's requires correct localisation of the enzymes that catalyse their formation. Certainly PI 4-kinase has been shown to localise to many different membrane compartments (reviewed in (253)). The presence of a PH-domain on this protein makes its targeting to specific area possible (254). PI4-P 5-kinase also has a potential for specifically localized PIP_2 production. The type1 isoform, which is implicated in the production of PIP_2 in secretory vesicle priming (249) is activated by interaction with Rho-family G-proteins. It has been suggested the enzyme could be targeted to, and immobilized on specific areas of membrane via this interaction (255,256). This may have important implications for membrane trafficking, in view of the importance of Rho G-proteins in mediating changes in the actin cytoskeleton.

In thrombin-stimulated platelets, activation of Rac results in the formation of lamellae. The increased actin polymerisation is initiated following the uncapping of actin. It is thought that this could be achieved via Rac activating a PI4-P 5-kinase, leading to increased synthesis of $PI-4,5-P_2$, which inhibits the capping protein (257). In an analogous mechanism, it has been suggested that activation of PI 3-kinase could lead to the formation of a different membrane modification. This difference could be mediated by the different binding affinities that various capping proteins show for different PPI's. For example gelsolin is inhibited more potently by $PI-3,4-P_2$ and $PI-3,4,5-P_3$ than by $PI-4,5-P_2$, and production of these lipids is associated with formation of filopodia, not lamellae (257).

1.4 SNAPs and SNAREs

These proteins have been implicated mainly in constitutive secretion, or in exocytosis from neuroendocrine cells, their importance in secretion from haematopoietic cells, including rat mast cells has not been established. The original identification of these proteins was achieved because of the effects of a group of clostridial neurotoxins. These toxins - all possessing zinc endopeptidase activity, inhibit neurotransmission. The substrates of these proteases were identified, and shown to be essential components of the exocytic machinery (258-261) - the vesicle associated membrane protein

(VAMP or synaptobrevin), synaptosome associated 25kDa protein (SNAP-25) and syntaxin - the latter two found associated with the plasma membrane (262,263). These proteins associate with two soluble proteins, the N-ethylmaleimide (NEM)-sensitive fusion protein (NSF), and the NSF-attachment proteins (SNAPs). NSF has ATP-ase activity (264), which is enhance by SNAP (265). The SNARES associated with the target membrane - or t-SNARE's interact with VAMP, the vesicle- or v-SNARE in a 7S complex. This complex then recruits α-SNAP and NSF, forming a 20S complex (263). ATP hydrolysis by NSF results in disassembly of the 20S complex, in a process thought to be essential for exocytosis.

The differences between v-SNARE's and t-SNARE's, i.e. that donor and acceptor membranes could be distinguished, led to the SNARE hypothesis (262). This hypothesis states that the specificity of these interactions ensures that the fusion events are tightly regulated. However this hypothesis is now doubted, not least because syntaxin-1 and SNAP-25 - supposedly t-SNARE's, are also found on vesicle membranes (266). The finding that rab proteins can modulate SNARE complex formation (267), viewed in light of rab's postulated role in dictating donor-acceptor membrane specificity (219) suggests a possible modification of the SNARE hypothesis: to include rabs as the proteins responsible for accurate targeting of fusion.

Despite the early demonstrations of NSF's importance in fusion, more recent work has suggested that NSF may be important only in priming. There appears to be a distinct NSF/α-SNAP dependent stage in exocytosis, after which removal of NSF does not inhibit fusion. This idea is supported by evidence that suggests that NSF may be essential in the pre-fusion, ATP-dependent priming step of exocytosis (268).

Three isoforms of SNAP - α,β and γ have been identified (269). There are some contradictory reports of the activation of different SNAPs: unique role for α-SNAP and β-SNAP have been identified with synaptotagmin binding specifically to β-SNAP but not α-SNAP (270), however in adrenal chromaffin cells the two proteins had indistinguishable effects (271). γ-SNAP shows only

25% identity to α- and β-SNAP and binds to a distinct region of the SNARE complex (272). The SNAP's do appear to be essential in exocytosis (273), and like NSF they are thought to be important specifically in the MgATP-dependent priming steps (274). However SNAP-25's importance in exocytosis from PC12 cells, is thought to lie particularly in calcium-dependent triggering (275).

The essential nature of these proteins in neuronal cells, and adrenal chromaffin cells has been demonstrated. The finding of yeast homologues of synaptobrevin (276) syntaxin (277) and SNAP-25 (278) suggests that they may participate in an evolutionarily ancient process. However the importance of the proteins in secretion from mast cells has not been demonstrated.

1.5 Aims of the project

1. To ascertain whether the observations made in HL60 cells, as to ARF's ability to reconstitute GTPγS-stimulated PLD activity and secretion, and PI-TP's ability to reconstitute GTPγS-stimulated secretion could be extended to RBL-2H3 cells. To show that the effects in HL60 cells were not anomalous, but represent a more universal observation.

2. To apply these proteins to antigen-stimulated cells. GTPγS is an effective stimulus but not a physiological one. If ARF and PI-TP are to be regarded as essential components of the secretory machinery in complex cellular responses such as the acute hypersensitivity reaction from mast cells, it must be determined whether they are effective under physiological stimulus.

3. To determine what the role of these proteins is in secretion. For example, does ARF mediate its effects via potentiation of PLD activity, or via a different route, and do these proteins operate in different pathways, or in a common mechanism.

CHAPTER TWO

MATERIALS AND METHODS

Chapter 2   Materials and Methods

2.1 Materials

DMEM	Sigma
Foetal Calf Serum	Advanced Protein Products
[^{3}H]-choline chloride	Amersham
[^{14}C]-sodium acetate	Amersham
[^{3}H]-myo-Inositol	Amersham
[^{32}P-γ]-ATP	Amersham
Streptolysin-O	Murex Diagnostics Ltd
GTPγS	Boehringer
IgE anti-DNP	Sigma
DNP-HSA	Sigma
ATP	Boehringer
BioRex 70 (mesh 200-400)	BioRad
Ultima Gold XR scintillant	Canberra-Packard
Dowex 1-X8 resin (formate form)	Sigma
Ultima Gold scintillant	Canberra-Packard
Kiesegel TLC plates	Merck
IPTG	Appligene
Luria Broth	Gibco
8-well glass slides	ICN
Mouse anti-αPI-TP	S.Cockcroft
Mouse anti-βPI-TP	S.Cockcroft
Rat anti-ARF	S.Cockcroft
Biotinylated goat anti-mouse	Sigma
CY3-streptavidin	Molecular probes
Acrylamide	BioRad
PVDF membrane	Millipore
Mowiol	Harco

All plastic-ware used for tissue culture was obtained from Falcon

Laboratory reagents were obtained from Sigma, solvents from BDH.

2.2 Cells

2.2.1 Culturing RBL-2H3 cells

RBL-2H3 cells were grown in Dulbecco's Modified Minimal Media (DMEM). This basic media must be supplemented with foetal calf serum (FCS), glutamine and the antibiotics penicillin and streptomycin. FCS was heat-inactivated by incubating in a pre-warmed water-bath at 56°C for one hour. FCS was added to DMEM at a final concentration of 12.5%, penicillin was used at a final concentration of 50iu/ml, streptomycin 50μg/ml and glutamine 4mM. RBL-2H3 cells were grown as a monolayer in 175cm^2 vented flasks, at 37°C with 5% CO_2 and 100% humidity. Each confluent flask contained approximately 4x10^7 cells; RBL-2H3 cells were considered confluent when 80% of the flask surface was obscured by cells and their processes. The cells remained active until passage 40-45, after this they were discarded. Experiments were performed using one box of confluent cells unless otherwise indicated.

2.2.2 Labelling RBL-2H3 cells

Before labelling the cells were passaged, and diluted in 50mls fresh medium to reach confluence over the appropriate labelling interval. [^{3}H]-choline chloride was added to the medium at a final concentration of 0.5μCi/ml, and cells incubated for 48 hours. [^{14}C]-sodium acetate was added to at a final concentration of 0.5μCi/ml, and the cells were incubated for 4days before use. ^{3}H-myo-Inositol was used at a final concentration of 1.0μCi/ml, and the cells were incubated for 48 hours before use.

2.3 Assays using intact and permeabilised cells

2.3.1 Sensitization of RBL-2H3's with IgEαDNP

Cell monolayers were washed twice in DMEM and finally scraped into 5mls of fresh medium. IgEαDNP was added at a final concentration of 2μg/ml. The cell suspension was incubated for one hour at 37°C, with occasional mixing to avoid sedimentation of the cells. After incubation, the suspension was centrifuged at 450g for 5mins at room temperature. The supernatant was discarded, and the cell pellet resuspended in buffer.

2.3.2 Stimulation of intact cells

After washing the cells were resuspended in an appropriate volume of buffer. The buffer used was standard HEPES, modified for use with intact cells (see section 2.3.11). The cell suspension was added to previously-prepared assay tubes on ice, already containing antigen as the stimulus (see section 2.3.8). The cell suspension in all assays constituted 50% of the total assay volume. The assay tubes were transferred to a 37°C water-bath, and incubated for 25mins. After incubation, the tubes were transferred to an ice-filled water-bath and when cooled to 4°C, centrifuged to pellet the cells.

2.3.3 Stimulation of acutely-permeabilised cells

Cells were assayed in the presence of their cytosolic contents (see Fig 2.1). Cell monolayers were washed twice and finally scraped into 20mls of buffer. This suspension was centrifuged at 450g for 5mins at room temperature. The supernatant was discarded, and the cell pellet resuspended in fresh buffer. The cell suspension was added to previously prepared assay tubes on ice, already containing permeabilisation reagents and the stimulus. Permeabilisation and stimulation were carried out in either standard PIPES or HEPES buffer (see section 2.3.11), supplemented with 2mM $MgCl_2$, 1mM MgATP, 0.4iu/ml SLO, calcium solution buffered by 3mM EGTA, +/- stimulus (see sections 2.3.7 and 2.3.8). The tubes were transferred to a 37°C water-bath, and incubated for 25mins. After incubation, the tubes were transferred to an ice-filled water-bath. When cooled to 4°C, the assay tubes were centrifuged to pellet the cells.

2.3.4 Measuring declining responsiveness after permeabilisation

4mls of washed cells were added to 1ml permeabilisation mixture, containing 2mM $MgCl_2$, 1mM MgATP, 0.4iu/ml SLO, and 100nM calcium buffered by 0.3mM EGTA, on ice. Immediately, aliquots of cells were removed and added to pre-prepared assay tubes, then transferred to a 37°C water-bath to provide a starting point for the "run-down". At t=0 the remaining cells were transferred to the 37°C water-bath. At timed intervals, further aliquots were removed and added to assay tubes. The assay tubes contained either standard PIPES or

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HEPES buffer (see Section 2.3.11), supplemented with 2mM $MgCl_2$, 1mM MgATP, 0.4iu/ml SLO, 10μM calcium buffered by 3mM EGTA, +/- stimulus (see Sections 2.3.7 and 2.3.8). All assay tubes were incubated for 25mins, and after incubation transferred to an ice-filled water-bath. When cooled to 4°C, the assay tubes were centrifuged to pellet the cells.

2.3.5 Reconstitution of cytosol-depleted cells

Cells were depleted of cytosol, and assayed in the presence of recombinant proteins, see Fig.2.1. 4mls of washed cells were added to 1ml permeabilisation mixture (as above), and aliquots removed to measure the acutely permeabilised response (see Section 2.3.4). At t=0, the remaining cells were transferred to the 37°C water-bath. After 5mins, if reconstitution of the receptor-stimulated secretion was to be attempted, or 10mins if GTPγS was used, the remaining cells were centrifuged at 4°C. The supernatant was discarded and the cell pellet resuspended in buffer, then dispensed to assay tubes. The assay tubes were prepared exactly as described above (see section 2.3.4). All assay tubes were incubated for 25mins, and after incubation transferred to an ice-filled water-bath. When cooled to 4°C, the assay tubes were centrifuged to pellet the cells.

2.3.6 Depletion of endogenous ATP

While intact, cells were washed into standard PIPES or HEPES buffer (see section 2.3.11), prepared without glucose. The cell suspension was transferred to a 37°C water-bath for 5mins equilibration. Antimycin A was added at a final concentration of 5μM, and deoxy-glucose at 1mg/ml, and the suspension incubated for a further 5mins. The cells were then transferred directly to assay tubes for intact cell measurements, or permeabilised as described above. All reagents were prepared in glucose-free buffer so as not to allow levels of endogenous ATP to recover prior to assay, however in reconstitution experiments, glucose was added as usual.
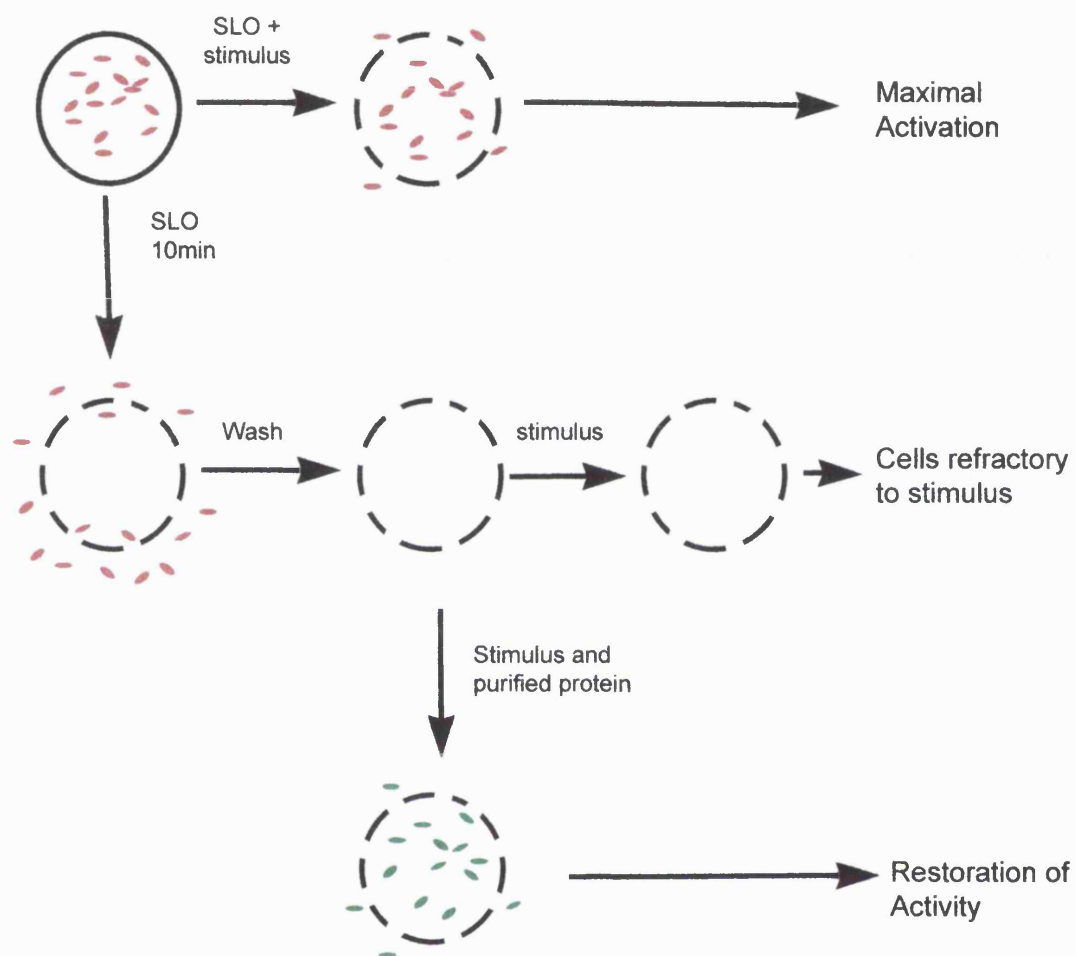
Fig.2.1 Diagrammatic representation of the "acutely-permeabilised" and "cytosol-depleted" cell protocols

2.3.7 Preparation of GTPγS

GTPγS was purchased as a 100mM solution and prepared as a 5mM stock solution in saline and stored at -20°C. GTPγS was used at a concentration of 10μM in all experiments, except where indicated otherwise.

2.3.8 Preparation of DNP-HSA

DNP-HSA was purchased as a solid and prepared as a stock solution in double-deionised water, at 1mg/ml. The stock was stored at 4°C for a maximum of three months, then discarded. DNP-HSA was used at a final concentration of 40ng/ml in all experiments, except where indicated otherwise.

2.3.9 Preparation of calcium buffers

Calcium buffers were prepared according to a method previously described (279). The buffers are EGTA-based, and used to strictly control the levels of free calcium in each experiment. Two solutions were used: 100mM EGTA, 20mM PIPES at pH6.8 and 100mM Ca^{2+}-EGTA, 20mM PIPES at pH6.8. These two solutions were combined in varying proportions to achieve a concentrated stock at the required concentration of calcium. The buffer stocks were stored at -20°C, and used at a final concentration of 3mM EGTA, and in the presence of 2mM free Mg^{2+}.

2.3.10 Preparation of MgATP

ATP was purchased as a solid and prepared as a stock at 100mM. The solid was dissolved in double-deionised water, in the presence of 100mM $MgCl_2$ and 200mM Tris-HCl pH 7.0. The pH of the solution was monitored throughout its preparation and kept close to neutral, as an excessively acidic solution can cause hydrolysis of ATP. The stock was stored at -20°C until required.

2.3.11 Preparation of PIPES and HEPES buffers

The standard PIPES and HEPES buffers used in all experiments were prepared in double-deionised water. PIPES buffer contains 20mM PIPES, 137mM NaCl, 3mM KCl, 1mg/ml glucose and 0.1mg/ml bovine serum albumin, and was prepared at pH 6.80. This buffer was used whenever GTPγS was used as the stimulus. HEPES, or Tyrodes (complete) buffer

contained 20mM HEPES, 137mM NaCl, 3mM KCl, 1mg/ml glucose and 1mg/ml BSA, and was prepared at pH 7.20; this buffer was used when the cells were activated via the receptor. When cells were stimulated when still intact, the buffer was supplemented with 1mM MgATP and 1mM $CaCl_2$.

2.3.12 Measuring secretion from attached cells

A confluent 175cm^2 flask of RBL-2H3 cells was prepared: the cell monolayer was washed twice and finally scraped into 36mls of DMEM. 24mls of this suspension was re-cultured, the remaining 12mls plated out in 24 well plates. The plates were incubated over-night, at 37°C in 5% CO_2 and 100% humidity before use, to ensure good cell adhesion. If stimulation via the receptor was required, the wells were supplemented with IgEαDNP in DMEM at a final concentration of 2μg/ml, one hour before use. Cell monolayers were washed gently in standard HEPES or PIPES buffer and placed on ice. The assay reagents were prepared at the same concentrations as for experiments carried out in suspended cells (see sections 2.3.3 and 2.3.2), the reagents were however prepared in a larger total volume (400μl) to prevent desiccation of the monolayer during incubation. The plates were transferred to a 37°C incubator for 25mins. After incubation, the plates were transferred to an ice-filled water-bath. When cooled to 4°C, a sample of the reaction mixture was removed, and centrifuged to pellet any detached cells.

2.4 Assaying secretion from RBL-2H3 cells

2.4.1 Ascertaining % β-hexosaminidase release

Following an assay, samples were centrifuged at 4°C to sediment the cells. 50μl of the supernatant was removed and transferred to a black microtitre plate. An equal volume of β-hexosaminidase substrate was added, the plate covered and placed at 37°C for 1hr 30mins. The reaction was quenched with 150μl 1M Tris (pH unadjusted), and fluorescence measured at 405nm. The amount of fluorescence was related to the total cellular content of β-hexosaminidase, and so percentage release. A sample was prepared, which consisted of 25% cell suspension and 1% Triton X-100. 50μl of this is added to the plate with β-hexosaminidase substrate. As the original assay samples

contained 50% cell suspension (see section 2.3.2), this sample contained an amount of β-hexosaminidase equivalent to 50% release. Triplicate samples of buffer were added to the plate and incubated with substrate. The value from these samples gave equivalent fluorescence to "0%" release.

2.4.2 Preparation of β-hexosaminidase substrate

The substrate used is 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide. This was prepared in 0.2M citrate buffer, at pH 4.5 at a final concentration of 1mM. The released β-hexosaminidase hydrolyses this substance, producing free 4-MU. At the high pH produced by the quenching buffer the fluorescence of this product may be measured at 405nm.

2.5 Assaying PLD activity by measuring [^{3}H]-choline release

2.5.1 Extraction of [^{3}H]-choline metabolites

PLD was measured from [^{3}H]-choline chloride labelled cells (see section 2.2.2), assayed as described in section 2.3. If PLD was to be measured from the same samples as secretion, the 50μl sample for the secretion assay were removed first, as described above. If PLD alone was to be assayed, the whole sample was quenched with no necessity for prior centrifugation. 100μl samples were quenched with 500μl chloroform/methanol (1:1) and vortexed thoroughly. This addition produced a single phase. The phases were split by addition of 125μl of water and again the samples vortexed and centrifuged.

2.5.2 Separation of free [^{3}H]-choline

Following centrifugation for 5mins at 4°C, 400μl of the aqueous top phase were applied to BioRex columns to separate free [^{3}H]-choline from its other aqueous metabolites. The 10ml columns contained 1ml of slurry, 50% BioRex 70 cation exchanger in water. Phosphorylcholine, glycero-phosphocholine and CDP-choline were eluted with 3mls of water and discarded. [^{3}H]-choline was eluted with 3ml 50mM glycine/500mM NaCl (pH 3), directly into scintillation vials. 3ml of Ultima Gold XR scintillation cocktail was added, and the samples vortexed thoroughly. The increase in labelled choline was expressed as a function of the total radioactivity (DPM) incorporated in the choline lipids. From control samples the total lipid chloroform extract was transferred to a

scintillation vial and the chloroform evaporated overnight on the bench. 500μl of methanol was added to the dried lipids, followed by 4ml of scintillation cocktail. The BioRex resin was regenerated by washing the resin with 3mls 0.5M NaOH, pH 9, followed by 10mls of water.

2.6 Assaying [^{14}C]-phosphatidyl ethanol (PEt) formation

2.6.1 Extraction of PEt

PEt formation was measured from [^{14}C]-sodium acetate labelled cells (see section 2.2.2), assayed as described in section 2.3, except that 1% ethanol was included in all assay tubes. 100μl samples were quenched with 600μl chloroform/methanol (1:1) and vortexed thoroughly. 5ml of PEt standard was added to each sample to aid visualisation of the lipids (see below). This extraction produced a single phase. The phases were split by addition of 170μl of water and again the samples vortexed and centrifuged. The entire organic phase was removed from each sample and transferred to a clean eppendorf tube.

2.6.2 Separation of lipids by TLC

Plates were heated at 120°C for 60 minutes prior to use, in order to remove all traces of water. The plates were marked into as many equal lanes as required (not exceeding 12 per plate), a 1cm margin was left unused at the each side of the plate. An origin line was drawn 2cm from the bottom of the plate, where samples were applied. Lipid samples were dried under vacuum, then resuspended in 50μl of chloroform. The samples were loaded onto plates in 5μl elliptical spots, with the whole spot lying within the central 50% of each lane. When all of the 50μl sample had been applied, the tube was rinsed with a further 20μl of chloroform and this was applied also. Each aliquot was dried by blowing gently to evaporate the chloroform before addition of the next. The plates were run at room temperature in a fully equilibrated solvent tank, until the solvent front moved to within approximately 1cm of the top of the plate. The plates were dried in a fume-hood for several hours (overnight).

2.6.3 Quantification of PEt formation

For accurate separation of PEt from other lipids, the following solvent was used: chloroform/methanol/glacial acetic acid/water (75:45:3:0.3). After separation and drying, the plates were placed into a sealed tank containing solid iodine. The iodine vapour was used to visualise the lipid spots, colouring them yellow. The spots were marked with pencil, then the iodine stain allowed to fade. The silica was dampened with distilled water and the PEt spots scraped into a scintillation vial. 500μls of methanol was added to release the labelled lipid from the silica. After mixing, 3mls of Ultima Gold scintillation cocktail was added, the samples vortexed thoroughly then counted.

2.6.4 Preparation of PEt standard

4mg of phosphatidylcholine was dissolved in 400μl 0.2M acetate buffer (pH 5.6). This was mixed with 1000 units of peanut PLD dissolved in 400μl 0.2M acetate buffer (pH 5.6). 100μl of 1M $CaCl_2$, 400μl of ether and ethanol to a final concentration of 2% were added, and the suspension mixed thoroughly. The preparation was left at room temperature for 12 hours (overnight). Ether was evaporated away, and the exact volume of the suspension measured. Per 1ml of sample, 3.75mls of chloroform/methanol (1:2) was added, followed by 1.25ml of chloroform and 1.25ml of water, then the sample vortexed thoroughly. The entire organic phase was transferred to a clean glass tube and dried down under vacuum. The lipids, including PC and PA but predominantly PEt were then resuspended in 500ml of chloroform and stored at -20°C.

2.7 Assaying PLC activity by measuring [³H]-IP₃ production

2.7.1 Extraction of [³H]-Inositol phosphates

PLC activity was measured from [³H]-Inositol labelled cells (see section 2.2.2), assayed as described in section 2.3, except that 1mM LiCl was included in all assay tubes. The lithium was included to ensure measurement of all the IP₃ produced, by preventing its conversion to inositol. 100μl samples were quenched with 375μl chloroform/methanol (1:2) and vortexed thoroughly to obtain a single phase. A further addition of 125μl of chloroform and 125μl of

water was then added to obtain a two phase system. After vigorous mixing, the samples were centrifuged for 5min.

2.7.2 Separation of [³H]-Inositol phosphates

Inositol phosphates were separated from free inositol and glycerophosphoinositol using Dowex 1-X8 anion exchange resin (formate form), packed into Pasteur pipettes (0.5 ml bed volume). The aqueous phase from the above extraction was applied to the column. The column was washed with 4ml of water to elute [³H]-inositol. Glycerophosphoinositol was removed with 3ml of 5mM sodium tetraborate/5 mM sodium formate. Finally IP$_3$ was eluted with 3ml of 1M ammonium formate/0.1M formic acid directly into scintillation vials. The radioactivity was measured after addition of 3ml Ultima Gold scintillation cocktail and thorough mixing. The columns were regenerated by washing with 4mls of 2M ammonium formate/0.1 formic acid followed by extensive washing with 15mls of water (15ml).

2.8 Preparation of recombinant myristoylated ARF

2.8.1 myr.ARF-1 expressing bacteria

E.Coli BL21 (DE3) were used. This strain contained the expression plasmid (generally pET22b) and pBB131. This plasmid expressed the *S. cerevisiae* myristoyl-CoA:protein N-myristoyltransferase (NMT). The bacteria were resistant to ampicillin and kanamycin. Stock bacteria were kept at -80°C in 15% glycerol. (Plasmids obtained from Qiagen - generated by D.Jones)

2.8.2 Growing bacteria

Two 200ml stocks and four 900ml stocks of Luria broth were prepared and autoclaved. When the flasks cooled, 100μg/ml ampicillin, 50μg/ml kanamycin and a 5μl aliquot of semi-thawed E.Coli glycerol stock was added to each of the 200ml stocks and the flasks sealed. The bacterial cultures were placed in a shaker overnight at 37°C. The following day, myristic acid was freshly prepared in ethanol, and added to each of the 900ml stocks at a final concentration of 100μM. These stocks were further supplemented with 100μg/ml ampicillin and 50μg/ml kanamycin. The growing cultures were removed from the shaker, and 100mls poured into each of the 900ml stocks.

These stocks were placed in the shaker for 2hours at 28°C. The expression vectors were induced by IPTG, added to each flask at a final concentration of 0.25mM. The induced bacteria were grown for a further 4hours. The bacteria were harvested by centrifugation. The pellets were retained and frozen overnight to aid cell lysis.

2.8.3 Extraction and purification of myr.ARF-1

Each frozen pellet from the 1 litre stocks was resuspended in 5mls of buffer A, supplemented with 5mg of lysozyme and left at room temperature for 20-30mins. 8ml of buffer B was added per 1litre equivalent. The solution was mixed and left at 4°C for 10-20mins. After lysis, the suspension was centrifuged at 4°C for 60mins and the supernatant collected and loaded onto a 200ml DE-52 column. 200mls of buffer C was added, and the column eluate collected throughout. This sample was concentrated to 5mls at 4°C under nitrogen. This sample was loaded onto a S-75 gel-filtration column fully equilibrated in filtered buffer D. Following a void volume of 158mls, forty 2ml fractions were collected. The four/five fractions with highest protein concentrations were pooled and the exact volume measured. In order to stabilise the protein, 1mM $MgCl_2$ and 1mM GDP were added. This dilute preparation was concentrated further, to 2-5mg/ml before freezing at -20°C for storage. (Buffer A: 50mM Tris, 40mM EDTA, 25% (w/v) sucrose, 0.02% NaN_3, pH 8.0. Buffer B: 50mM Tris, 100mM $MgCl_2$, 0.2%(w/v) Triton X-100, 0.02% NaN_3, pH 8.0. Buffer C: 20mM Tris, 50mM NaCl, 1mM DTT, pH 8.0. Buffer D: 20mM PIPES, 3mM KCl, 137mM NaCl, pH 6.8.).

2.9 Western Blotting

2.9.1 Detection of cytosol leakage from permeabilised cells

A 4mls suspension of washed cells was prepared, containing 5×10^7 cells. This was added to 1ml of permeabilisation buffer, containing 2mM $MgCl_2$, 1mM MgATP, 0.4iu/ml SLO and 100nM Ca^{2+} buffered by 0.3mM EGTA. All solutions were prepared in standard PIPES buffer (see section 2.3.11), but with no added BSA. Immediately a 1ml aliquot, containing 1×10^7 cells was removed and centrifuged at 4°C. This sample was used to probe the proteins in intact cells. At stated time points (5mins, 10mins and 30mins) further 1ml

samples were removed, centrifuged and rapidly processed as described below.

2.9.2 Translocation of cytosolic proteins after stimulation

Two 1ml samples were prepared, each sample containing 1.5×10^7 cells in HEPES buffer (see section 2.3.11). (These were pre-sensitised to antigen, by pre-treatment with IgEαDNP (see section 2.3.1)). At t=0, 250μl of stimulus was added to one tube producing a final concentration of DNP-HSA of 40ng/ml, and an equal volume of buffer added to the other. The tubes were then added to a 37°C water-bath for 10mins. After 10mins, a permeabilisation buffer was added, containing 0.4iu/ml SLO and 100nM calcium, buffered by 3mM EGTA. (The aliquot of permeabilisation buffer added to the tube of stimulated cells was supplemented with DNP-HSA to maintain the initial concentration). The cells were returned to a 37°C water bath for a further 10mins, then transferred to 4°C and centrifuged to sediment the cells. The samples were rapidly processed as described below.

2.9.3 Preparation of cytosol samples for electrophoresis

After centrifugation, the supernatant - containing leaked cytosolic contents were removed from the cell pellet. The supernatants were treated with TCA, to precipitate the protein. This allows the protein to be concentrated in a smaller, less dilute sample. The exact volume of the supernatants were measured, and sufficient 100% TCA added to produce a final concentration of 10% (v/v). This mixture was vortexed thoroughly then incubated at 4°C for 30mins. After incubation, the sample was centrifuged for 15mins at 4°C and the supernatants discarded. The pellet was resuspended in 50μl of 1M NaOH, then the volume measured accurately. This volume was doubled in 1M Tris pH6.8 to neutralise the sample. An appropriate volume of the 4X sample buffer was added, and the samples boiled at 98°C for 2mins to denature the proteins. (4X Sample buffer is 62.5mM Tris pH6.8, 10% glycerol (v/v), 2% SDS (w/v), 0.00125% Bromophenol blue (w/v) and 5% 2β-mercaptoethanol).

2.9.4 Preparation of membrane samples for electrophoresis

After centrifugation, the membrane pellet was resuspended in 100µl RIPA buffer. The suspension was vortexed thoroughly, and placed at 4°C for 30mins, with occasional mixing. After centrifugation for 15mins at 4°C, the supernatant was removed and retained and the pellet was discarded. The volume was concentrated by treatment with TCA, exactly as described in section 2.9.3. The resulting protein pellet was resuspended in NaOH and neutralised also as above, then treated with 4X sample buffer and boiled prior to application to a polyacrylamide gel. (RIPA buffer is 150mM NaCl, 1% Triton X-100 (v/v), 0.5% deoxycholate (w/v), 0.1% SDS (w/v) and 50mM Tris pH 7.5).

2.9.5 Gel electrophoresis

The total volume of each sample was corrected so that the same proportion of each may be used in the same volume. A volume of 15-20µl was loaded for each sample, equivalent to 10^6 cells. SDS-PAGE gels were prepared using the protocol described by Laemmli (280): a resolving gel of 14% acrylamide was made, and a 4% acrylamide gel was used for the stacking gel. Each gel was loaded with a set of molecular weight markers (see section 2.12.2). The gels were run at 150V for 60mins in running buffer. (Stacking gel is 4% acrylamide, 0.1% SDS, 0.04% APS, 0.1% TEMED, 250mM Tris pH 6.8; resolving gel is 14% acrylamide, 0.1% SDS, 0.08% APS, 0.08% TEMED, 375mM Tris pH 8.8; running buffer is 193mM glycine, 0.1% SDS, 25mM Tris pH 8.3)

2.9.6 Transfer of proteins to PVDF

After running, the gel was equilibrated in transfer buffer for 20mins. A blotting "sandwich" was assembled, with the gel in full contact with PVDF membrane and the transfer run at 50V for 50mins, or at 30V overnight (as indicated) in transfer buffer. (Transfer buffer is running buffer is 193mM glycine, 25mM Tris pH 8.3)

2.9.7 Antigen detection

Bands on the membrane were visualised by extended washing with India ink (0.1% in PBS/Tween). The ink was thoroughly washed from the membrane,

then the membrane submerged in blocking buffer for 60mins at room temperature. After blocking, the membrane was washed in PBS/Tween. The primary antibody was applied in PBS/Tween (for primary antibody concentrations and incubation times see section 2.12). After incubation with primary antibody, the membrane was thoroughly washed with PBS/Tween and a secondary antibody applied in blocking buffer for 30mins at room temperature. After extensive washing, with at least five changes of PBS/Tween, the proteins were probed with ECL. (PBS/Tween was standard phosphate buffered saline with 0.02% Tween; blocking buffer is PBS/Tween with 5% milk protein (w/v) and 2% goat serum (v/v)).

2.9.8 ECL detection

A mixture containing an equal volume of ECL solution one and solution two was prepared. The membranes were soaked in this mixture for 2mins, then exposed to photo-sensitive film. (ECL soln.1 is 0.45mg/ml luminol, 65μg/ml p-Coumaric acid in 0.1M Tris pH 8.5; soln.2 is 0.02% H_2O_2 in 0.1M Tris pH 8.5.)

2.10 Polyphosphoinositide analysis

2.10.1 Labelling of phosphatidylinositides with [^{32}P]-ATP

4mls of washed cells were placed on ice, and 1ml of permeabilisation buffer added containing 2mM $MgCl_2$, 1mM MgATP, 0.4iu/ml SLO and 100nM Ca^{2+} buffered by 0.3mM EGTA. At t=0, the cells were transferred to a 37°C water-bath. After the time indicated, the permeabilised cells were transferred to 4°C and centrifuged to sediment the cells. The supernatant was discarded, and the cells resuspended in buffer containing 2mM $MgCl_2$, 1mM MgATP and 10μM Ca^{2+} buffered by 0.3mM EGTA. Immediately before addition to assay tubes the cells were labelled with 20μCi/ml [^{32}P-γ]ATP. Cells were then added to assay tubes, already containing stimulus, and any protein to be used for reconstitution. The cells were incubated at 37°C for 20mins, the cooled on ice to 4°C.

2.10.2 Extraction and separation of phosphatidylinositide lipids

After incubation, the cells were placed on ice, and cooled to 4°C. A 100μl assay volume must be quenched with 375μl acidified chloroform/methanol/HCl

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(100:200:15), and the mixture vortexed thoroughly to obtain a single phase. 10μl of Folsch reagent was added to aid recovery of phosphatidylinositides. 125μl of chloroform and 125μl of 0.1M HCl was added to separate the sample into two phases. The samples were vortexed thoroughly and then centrifuged. The top phase was removed and replaced with the "synthetic" top phase of chloroform/methanol/0.1M HCl (1:1:0.9). This mixture was vortexed well, then centrifuged. The top phase was replaced twice more, to remove all the ^{32}P not incorporated into lipid. Finally all of the bottom phase was transferred to a clean eppendorf tube.

2.10.3 Quantification of phosphatidylinositide lipids

The samples were dried under vacuum and applied to TLC plates exactly as described in section 2.6.2. The plates were treated with oxalate, then dried overnight and heated at 120°C for 60mins before use. The lipids were separated in an equilibrated tank with chloroform/methanol/acetone /water/glacial acetic acid (40:13:15:7:12). The labelled lipids were quantified by densitometry, following analysis in a phosphorimager. The TLC plate was exposed to phosphorimager plates for 2hrs before reading. (Oxalate wash is 60% water (v/v), 40% methanol (v/v) and 10mg/ml potassium oxalate).

2.11 Immunofluorescence

2.11.1 Preparation of slides for Immunofluorescence

Glass slides with 8 wells, each of 5mm diameter were used. The slides were cleaned thoroughly in ethanol, then excess ethanol removed by flaming. The slides were placed in 60cm^2 Petri dishes. 2.5x10^6 cells, suspended in 10mls of DMEM were poured over the slides. The dishes of cells were placed at 37°C, 5% CO_2 and 100% humidity for 48hrs before use. Approximately 30,000 cells were in each well, of which 60-70% were still present at the end of the experiment. When cell numbers were significantly below these levels the experiment was discarded. As in experiments using suspended cells, a pre-incubation with IgEαDNP was necessary before stimulation. 1½ hrs before use, the plated cells were washed and 10mls of fresh DMEM added to the dishes, supplemented with IgEαDNP, at a final concentration of 2μg/ml.

2.11.2 Stimulation of cells

Before stimulation, the plated cells were rinsed with intact cell buffer (see below). The stimulus, DNP-HSA was added at 40ng/ml was applied to the appropriate wells. At the times indicated (between 0 and 20mins) the stimulus was removed and the cells rinsed well. Following stimulation the cells were fixed by 20mins incubation with 3% paraformaldehyde. After this the cells were permeabilised for 20mins with 80μg/ml lyso-phosphatidylcholine, in order to allow antibodies access into the cells' interior. To prevent non-specific interactions, the permeabilised cells were incubated for 30mins with 2.5% goat serum/2.5% foetal calf serum. This solution, as well as all further solutions was made in permeabilised cell buffer (see below). After blocking, the cells were incubated with a mouse-derived primary antibody for 45mins (for concentrations of primary antibodies used see section 2.12). Then a biotinylated anti-mouse antibody was applied at 20μg/ml to the cells for 45mins, and finally a fluorescent dye CY3, linked to streptavidin was applied at 20μg/ml for 45mins. After thorough washing, 5% mowiol was applied and a coverslip carefully placed over the cells. The mowiol was left to set at room temperature for 12hrs before images were gathered. (Intact cell buffer is 20mM HEPES, 3mM KCl, 137mM NaCl, 1mg/ml BSA, 1mg/ml glucose and 0.1mM EGTA, pH 7.2. Permeabilised cell buffer is 20mM PIPES, 3mM KCl, 137mM NaCl, 1mg/ml BSA, and 0.1mM EGTA, pH 6.8).

2.11.3 Immunofluorescence using confocal microscopy

Fluorescent images were obtained using a confocal laser scanning microscope equipped with an argon-ion laser, attached to a Leitz Fluovert-FU microscope (Leica). For visualisation of CY3-streptavidin, excitation was at 550nm and emission was measured at 565nm. Digital images were displayed using the "glowovun" colour look-up table from Leica.

2.12 Miscellaneous methods

2.12.1 Assaying protein with Bradford reagent

The concentration of protein in samples was measured by comparison with standards, prepared in a concentration range up to 400μg/ml. Samples expected to contain a higher concentration than this were diluted. 5μl of

sample was added to 200μl of Bradford reagent. The mixture was incubated at room temperature for 10mins, then the absorbance measured at 620nm. (Bradford reagent is 66.6μg/ml G_{250} Coomasie Brilliant Blue, 3.17% ethanol, 13.3% phosphoric acid).

2.12.2 Conditions for antibody use in Western blots

rat anti-ARF	4μg/ml	room temperature	1hr
mouse anti-PI-TPα	2μg/ml	room temperature	1hr
mouse anti-PI-TPβ	4μg/ml	room temperature	1hr
goat anti-mouse	0.2μg/ml	room temperature	30mins
goat anti-rat	0.5μg/ml	room temperature	30mins

All primary antibodies are purified monoclonal antibodies, raised in the laboratory of Prof. Cockcroft.

2.12.3 Recombinant PI-TP and PLC-δ1

Recombinant PI-TPα and PI-TPβ was prepared and purified as previously described in all cases by P.Swigart. Recombinant PLC-δ1 was prepared by V.Allen, and obtained from M.Katan.

2.12.4 Conditions for antibody use in Immunofluorescence

mouse anti-PI-TPα	0.1mg/ml	room temperature	45mins
anti-mouse/CY3	20μg/ml	room temperature	45mins

2.12.5 Molecular weight markers

(obtained from Sigma mw SDS 70L kit)

bovine albumin	66,000 Da
egg albumin	45,000 Da
glyceraldehyde 3-phosphate dehydrogenase	36,000 Da
carbonic anhydrase	29,000 Da
trypsinogen	24,000 Da
trypsin inhibitor	21,000 Da
α-lactalbumin	14,000 Da

2.13 Expression of data

The data presented in this thesis represent typical experiments. These experiments were repeated 2-5 times. "Error bars" on graphical data represent the spread of duplicate points.

CHAPTER THREE

CHARACTERISATION OF GTPγS-STIMULATED SECRETION

AND PLD ACTIVITY FROM RBL-2H3 CELLS

Chapter 3   Characterisation of GTPγS-stimulated secretion and PLD activity from RBL-2H3 cells

3.1 Introduction

Activation of permeabilised rat mast cells is possible, using the non-hydrolyseable GTP analogue GTPγS (190).   Assays where the cells are "acutely permeabilised" involve the simultaneous addition of stimulus with the permeabilisation agent, SLO. Thus the cells respond while still replete with all their cytosolic contents.   SLO allows access to the cells interior, thus concentrations of not only stimulus, but calcium and ATP may be manipulated; and any dependence on these factors demonstrated (see (173)).   The experiments presented in this chapter indicate that both secretion and PLD are dependent on micromolar concentrations of calcium and millimolar concentrations of ATP.   Experiments of this type have also been used to determine the effective concentrations of GTPγS, and optimal incubation times.

If SLO permeabilisation is carried out over an extended interval, cytosolic proteins leak from the cell.   Among these are proteins which may have essential roles in the secretory response, and in supporting the activity of PLD. Experiments presented here demonstrate the dependence of both the GTPγS-stimulated responses on cytosolic proteins. This dependence is implied by the gradual decline in secretion and PLD assayed over the permeabilisation interval.

ARF and PI-TP have been implicated previously as essential components of GTPγS-dependent secretion from neutrophils and HL60 cells (161).   The importance of either factor in a different cell-type had not been established, and this was a primary goal in the work presented here. Before reconstitution was attempted however, leakage of the native proteins was established. As shown in this chapter, ARF and both isoforms of PI-TP are present in intact RBL-2H3 cells, and all three proteins leak when the cells are permeabilised. The leakage of ARF, PI-TPα and PI-TPβ occur over different time courses. However, significant amounts of each protein exit RBL-2H3 cells during the same interval over which responsiveness declines.

In addition, experiments described in this chapter examine the ability of myr.rARF-1 to reconstitute secretion and PLD activity in cytosol-depleted RBL-2H3 cells, and the ability of both rPI-TPα and rPI-TPβ to reconstitute secretion. Part of this examination is concerned with deriving optimal conditions for reconstitution, with respect to concentration and the length of the pre-permeabilisation interval. In addition the effect of ATP concentration and calcium concentration on reconstitution is examined. GTPγS is used as the stimulus throughout, however the ability of GTP to stimulate cytosol-depleted cells, supplied with myr.rARF-1 was also examined.

3.2 GTPγS-stimulated secretion and PLD activity

As shown in Fig.3.2.1, secretion and PLD activity increased in the presence of GTPγS concentrations above 300nM. The variation of both responses to increases in GTPγS concentrations was similar. (A GTPγS concentration of 10μM was maximal for both parameters, and this concentration used routinely.) While a clear correlation between secretion and PLD activity was revealed by varying GTPγS concentrations, varying calcium concentrations produced quite different profiles. As shown in Fig.3.2.2, the secretory response was more sensitive to calcium levels, increasing at concentrations above 100nM. In the physiological concentration range - between 100nM and 1μM secretion doubled. At concentrations above 1μM this response was clearly saturated, whereas PLD activity continued to increase up to 100μM calcium. Thus at a concentration of 1μM, when the secretory response is near to maximal, PLD is active at only approximately 50% of its maximal rate.

This lack of exact correlation between secretion and PLD was seen also in response to MgATP. In the typical experiment shown in Fig.3.2.3, PLD activity was maximal at 1mM MgATP. In the presence of 3mM MgATP the secretory response was maximal, but at this concentration PLD activity fell sharply. This fall in PLD activity continued at 10mM MgATP, when GTPγS-stimulated activity was equal to control levels. Thus secretion was approximately three times less sensitive than PLD activity, however both responses were dependent on millimolar concentrations of MgATP. As shown in Fig.3.2.4, RBL-2H3 cells are

(a) Effect of GTPγS concentration on secretion

(b) Effect of GTPγS concentration on PLD activity
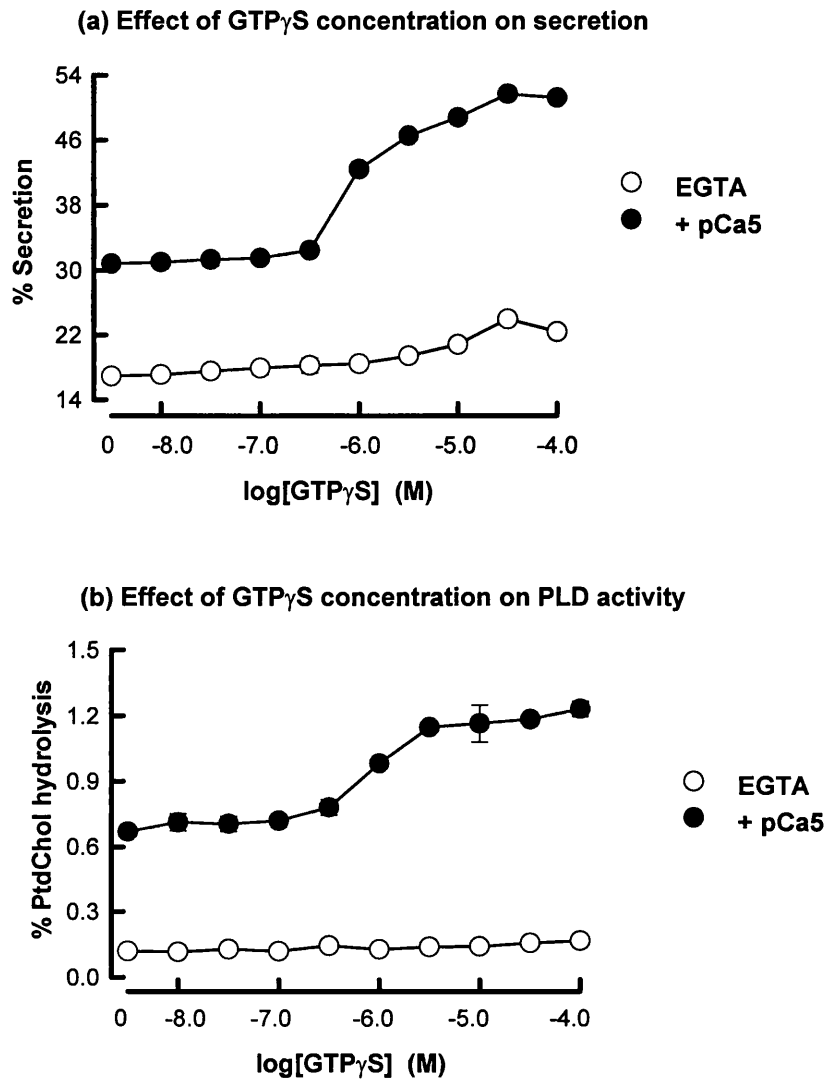
Figure 3.2.1. Effect of GTPγS concentration on PLD activity and secretion, measured in acutely permeabilised RBL-2H3 cells

Cells were assayed in PIPES buffer (pH 6.8), in a variety of GTPγS concentrations; in the absence (open symbols) and presence (closed symbols) of 10µM calcium, buffered by 3mM EGTA. The standard acutely permeabilised cell protocol was used (see section 2.3.3). % PC hydrolysis and secretion were measured from the same samples, as described in section 2.5.

(a) Effect of calcium concentration on secretion

(b) Effect of calcium concentration on PLD activity
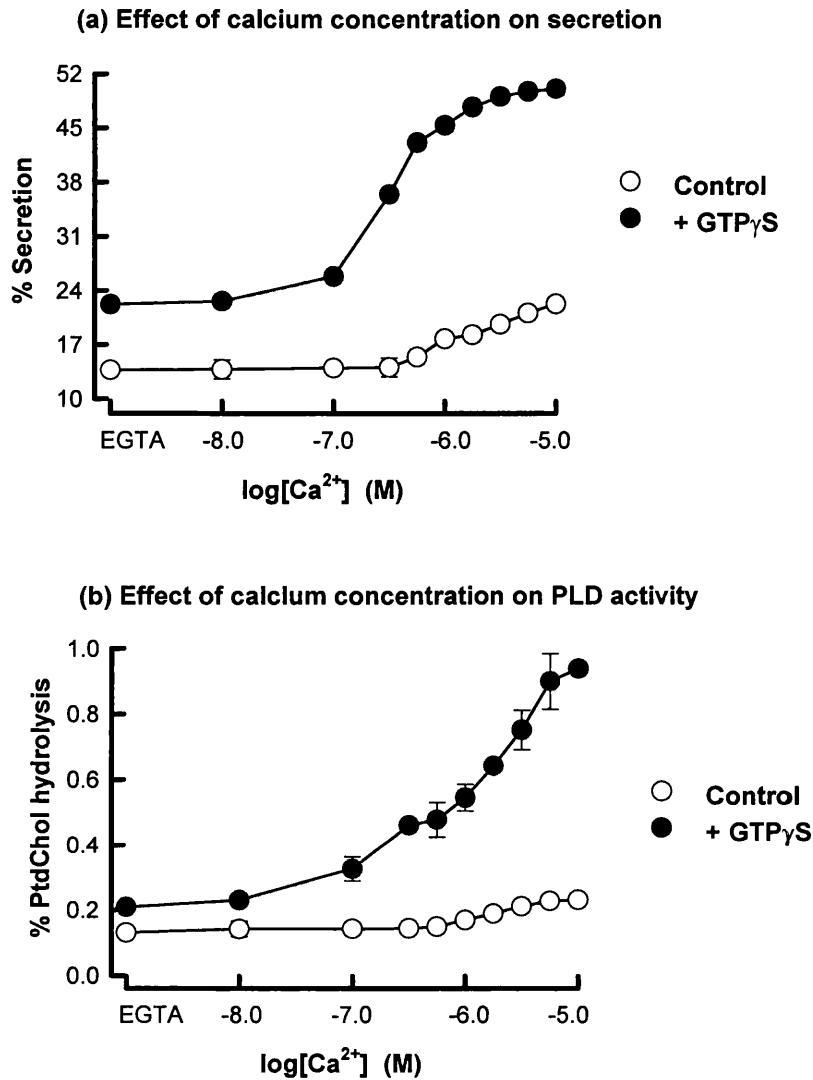
Figure 3.2.2. **Effect of Calcium concentration on secretion and PLD activity, measured in acutely permeabilised RBL-2H3 cells**

Cells were assayed in PIPES buffer (pH 6.8), in a variety of calcium concentrations (buffered by 3mM EGTA); in the absence (open symbols) and presence (closed symbols) of 10µM GTPγS. The standard acutely permeabilised protocol was used (see section 2.3.3). % PC hydrolysis and secretion were measured as described in sections 2.4 and 2.5.

(a) Effect of MgATP concentration on secretion

(b) Effect of MgATP concentration on PLD activity

Figure 3.2.3. **Effect of MgATP concentration on secretion and PLD activity, measured in acutely permeabilised RBL-2H3 cells**

Cells were metabolically inhibited prior to use, and cells and all reagents prepared in glucose-free buffer. Cells were assayed in PIPES buffer (pH 6.8) with rising concentrations of MgATP); in the absence (open symbols) and presence (closed symbols) of 10μM GTPγS. All assays included 1μM calcium, buffered by 3mM EGTA. The standard acutely permeabilised protocol was used (see section 2.3.3). % PC hydrolysis and secretion were measured as described in section 2.5.

sensitive also to millimolar concentrations of GTP, with both secretion and PLD activity triggered. However at all concentrations, the effect of GTP was modest compared to that of GTPγS.

As shown in the typical experiment in Fig.3.2.5, secretion may be measured from cells in suspension, and while attached. 5% release was measured from unstimulated, suspended cells. This was considered to be due to lysis of a small proportion of cells during preparation. The response from suspended cells was complete after 20mins, at which time the response from attached cells was only 75% of its maximum levels.

3.3 Dependence of secretion and PLD activity on cytosolic proteins

The rates of GTPγS-stimulated PLD and secretion rose initially after permeabilisation. This was presumably due to incomplete permeabilisation, resulting in a proportion of the cells excluding the stimulus. From two minutes onwards, the responses fell steadily. In the typical experiment shown in Fig. 3.3.1, 10% of secretion was lost during the first five minutes, after which the response fell more slowly. PLD activity also declined more quickly initially, with the rate of decline falling after 20mins. Both PLD and secretion were clearly responsive to GTPγS even after 45mins of permeabilisation.

As the fall in responsiveness to stimulus was assumed to be due to the leakage of cytosolic proteins following permeabilisation, the rates of leakage of ARF, PI-TPα and PI-TPβ were examined. As shown in Fig.3.3.2, both PI-TPα and PI-TPβ were abundant in intact cells, and both detected in the extracellular milieu after 5mins of permeabilisation. PI-TPα leaked rapidly, with over 50% detected outside the cells after 10mins. PI-TPβ leaked from RBL-2H3 cells more slowly: approximately 50% of the total PI-TPβ leaked from the cells after 10mins, however significantly more PI-TPβ remained in the cells after 30mins than PI-TPα. The rate of ARF leakage was similar to that of PI-TPα: over 50% of the ARF present in intact cells leaked out 10mins after permeabilisation. After 30mins ARF was detectable inside the cells, however in only very small amounts.

(a) Effect of GTP concentration on secretion

(b) Effect of GTP/GTPγS on PLD activity

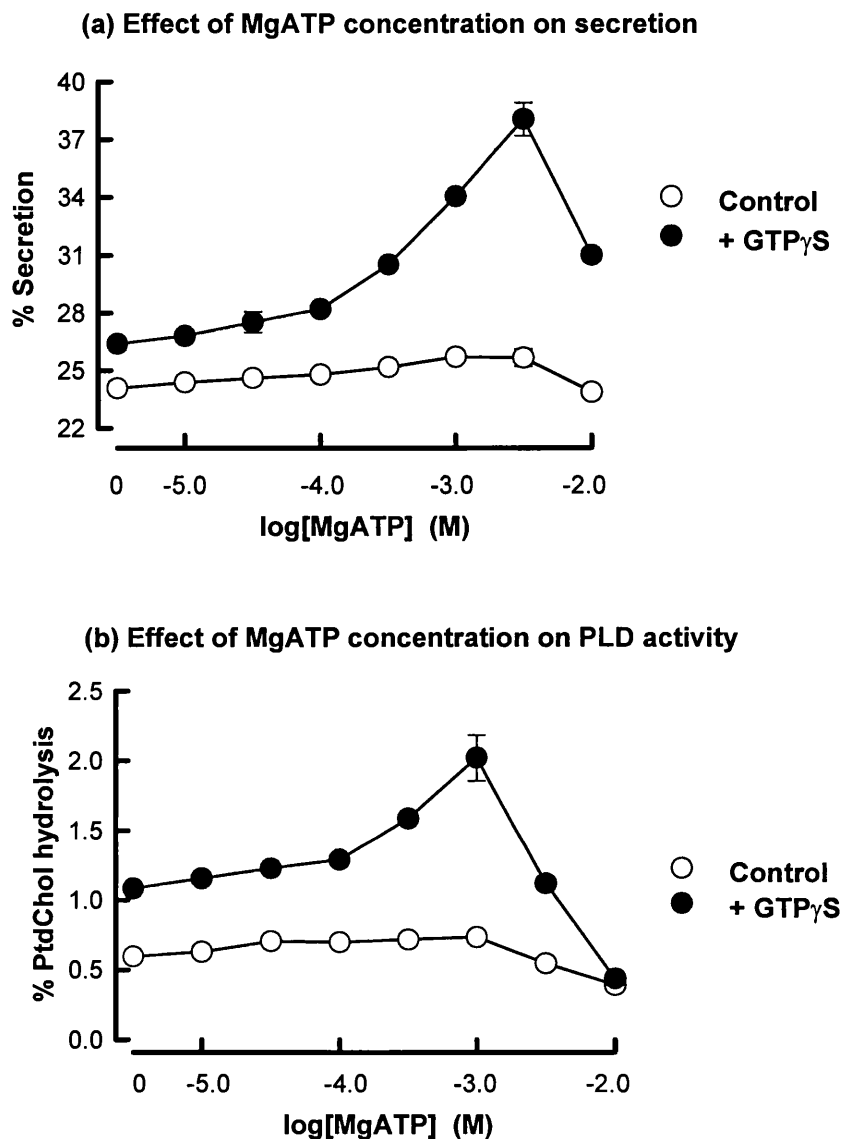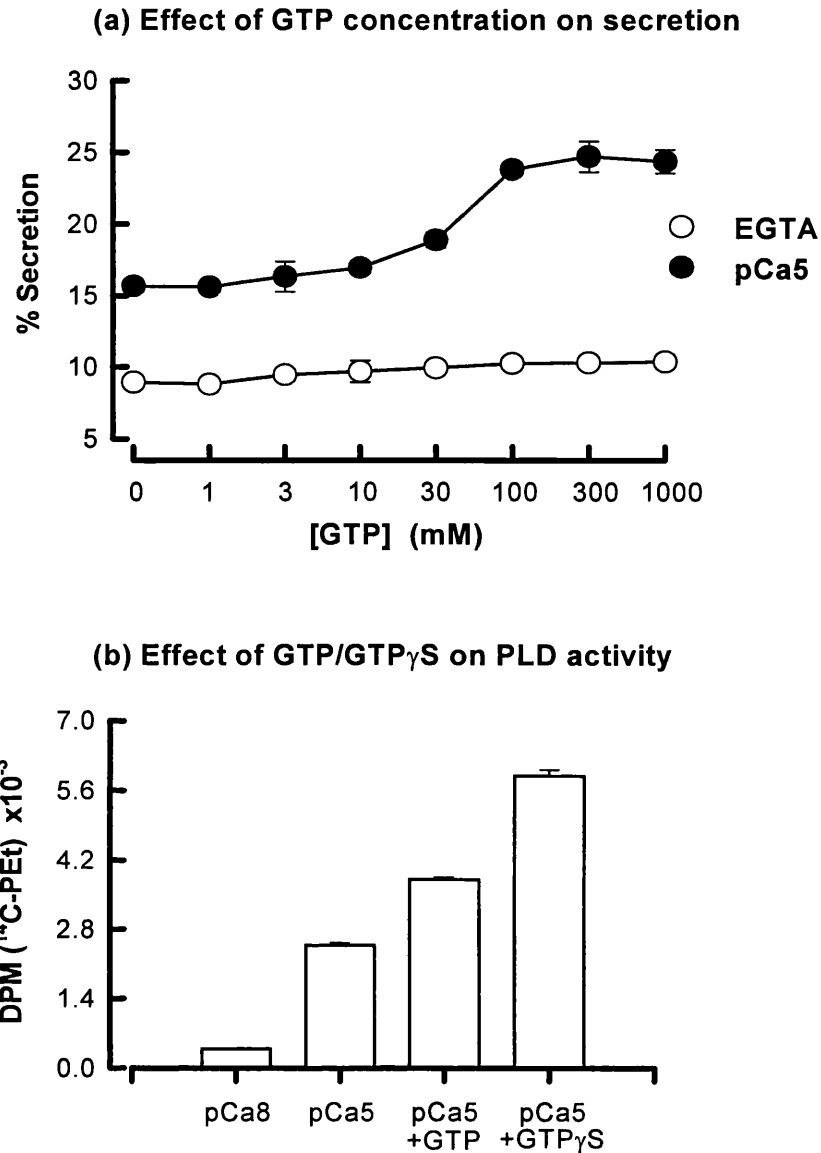**Figure 3.2.4.** **Effect of GTP on PLD activity and secretion, measured in acutely permeabilised RBL-2H3 cells**

Graph(a): Cells assayed in PIPES buffer (pH 6.8), with rising GTP concentrations; in the absence (open symbols) and presence (closed symbols) of 10μM calcium, buffered by 3mM EGTA. The standard acutely permeabilised cell protocol was used (see section 2.3.3), secretion measured as described in section 2.5. Graph (b) PLD activity assayed by measuring PEt formation (see section 2.6). Cells assayed in the presence of either 10μM or 10nM Ca2+ buffered by 3mM EGTA. 1% EtOH included in each sample. Cells were stimulated by 1mM GTP or 10μM GTPγS.

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(a) Effect of time on secretion from suspended cells

O Control
● + GTPγS

(b) Effect of time on secretion from attached cells

O Control
● + GTPγS
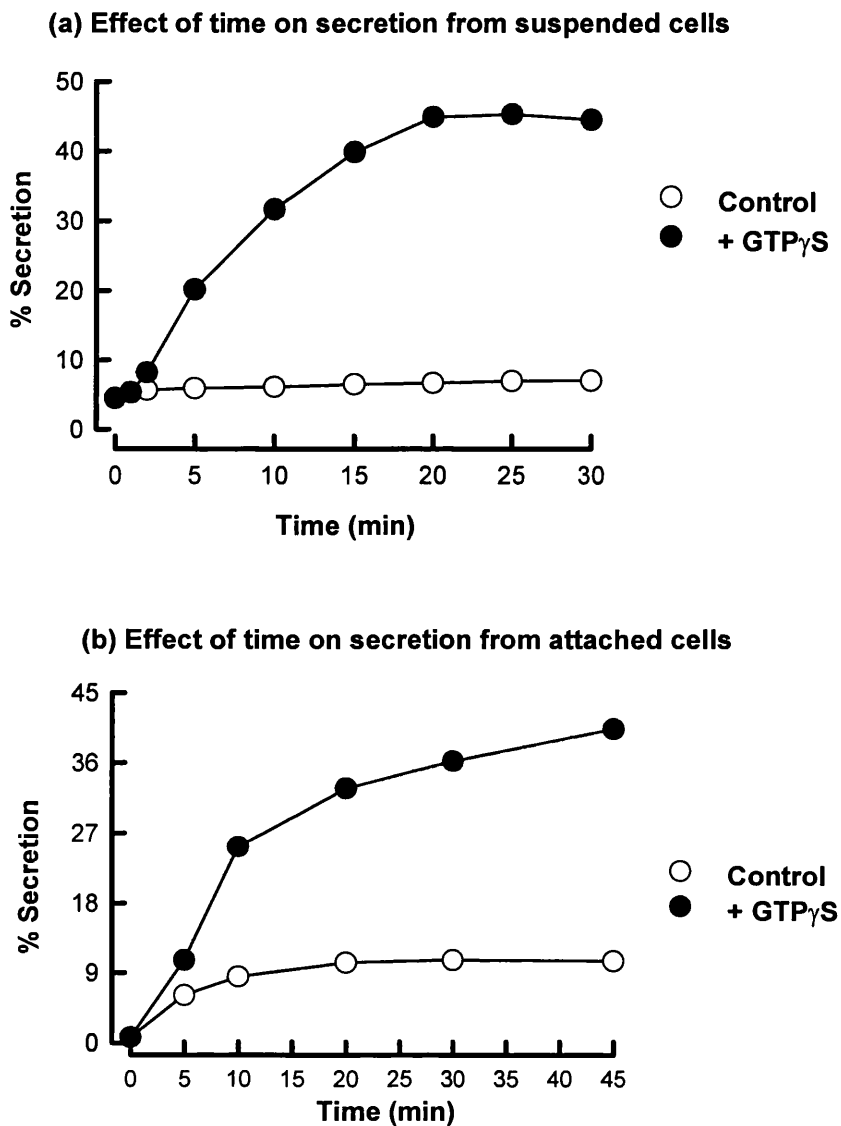
Figure 3.2.5. Time course of secretion, measured in acutely permeabilised RBL-2H3 cells, both attached and in suspension

Cells were assayed in PIPES buffer (pH 6.8), in the absence (open symbols) and presence (closed symbols) of 10μM GTPγS. All assays included 10μM calcium, buffered by 3mM EGTA and 1mM MgATP. Aliquots were removed at the stated time points. The standard acutely permeabilised protocol was used for graph (a) (see section 2.3.3), for graph (b) a modified protocol was used (see section 2.3.12). Secretion was measured as described in section 2.4.

(a) Effect of permeabilisation time on secretion

(b) Effect of permeabilisation time on PLD activity
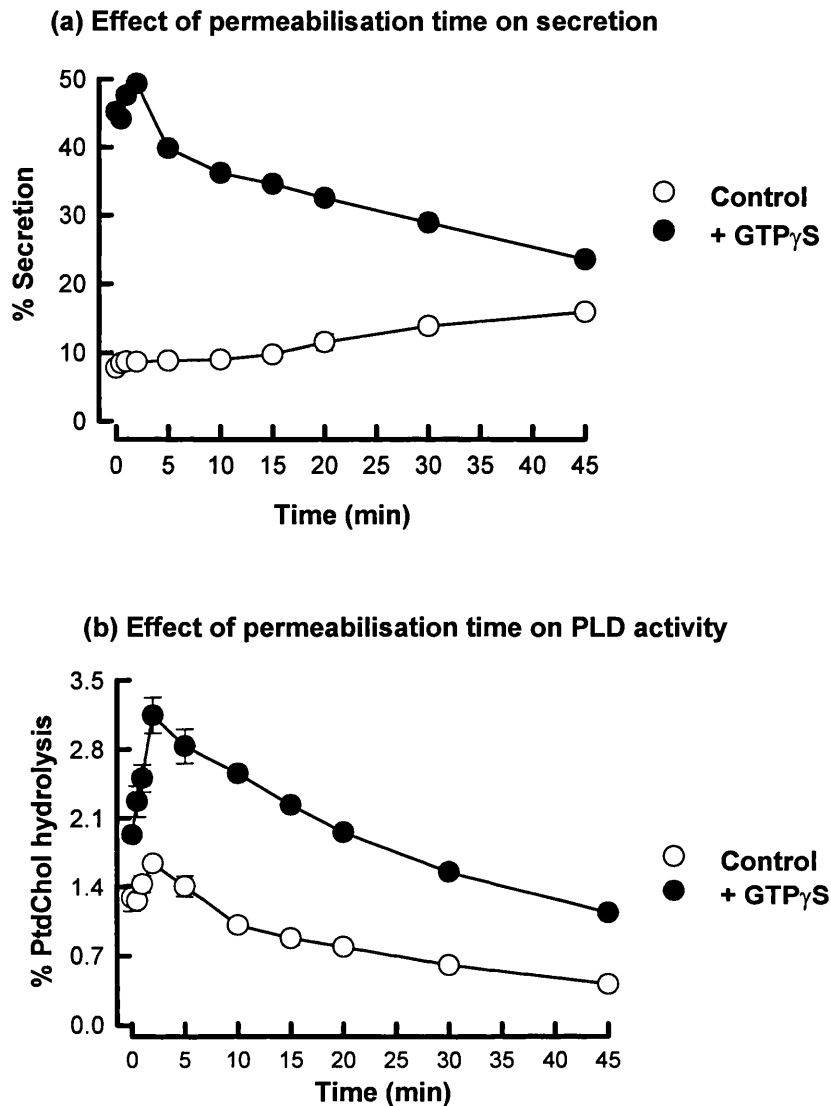
Figure 3.3.1. Effect of permeabilisation time on GTPγS-stimuiated secretion and PLD activity

Cells were assayed in PIPES buffer (pH 6.8), in the presence (closed symbols) and absence (open symbols) of 10μM GTPγS. All assays included 1mM MgATP, 2mM MgCl$_2$ and 10μM Ca^{2+} buffered by 3mM EGTA. Samples were assayed after various permeabilisation times (see section 2.3.4). % PC hydrolysis and secretion were measured from the same samples, as described in section 2.5.
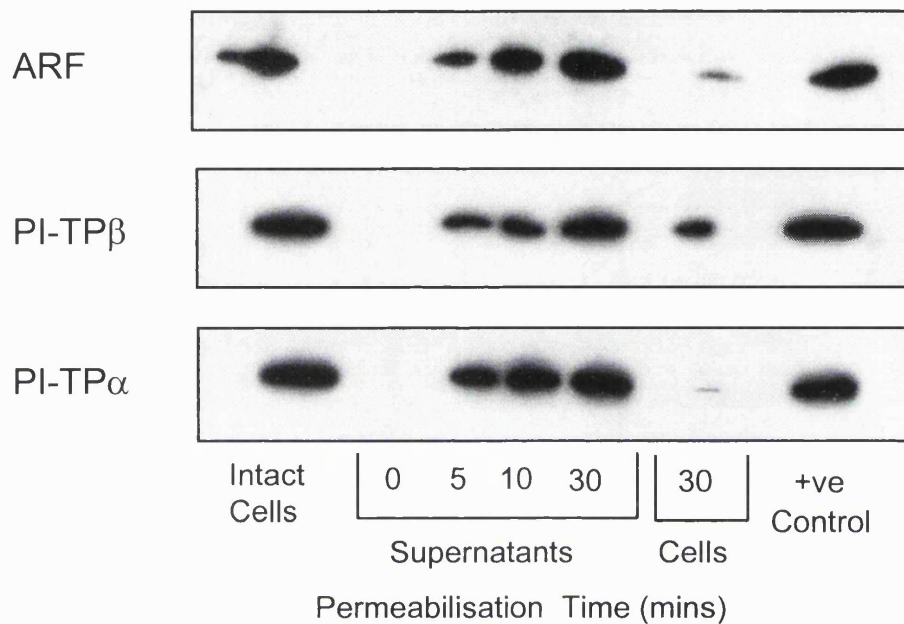
Figure 3.3.2. **Leakage of rPI-TPα, rPI-TPβ and ARF from permeabilised cells**

Cells were permeabilised, and aliquots removed after stated time-points (see section 2.9.1). Cell membranes were separated from their leaked cytosolic contents. The leaked material and membranes were prepared as samples, and probed by Western blotting with monoclonal antibodies directed against PI-TPα, ARF and PI-TPβ. (see section 2.9.3-2.9.8)

3.4 Reconstitution of secretion with myr.rARF-1

The fall in responsiveness to GTPγS over increasing permeabilisation time is clearly shown in Fig.3.4.1. This fall was more rapid than that seen in Fig.3.3.1. This acceleration was due to the inclusion of a centrifugation step in the protocol. The purpose of this step was to allow leaked cytosolic contents to be washed away from the cells before reconstitution. It is likely that this washing increased the rate of protein leakage and thus accelerated the onset of the refractory state.

Addition of myr.rARF-1 to acutely permeabilised cells caused a small increase (1%). This was noted in all reconstitution experiments involving myr.rARF-1 and was assumed to be an artefact. After five minutes of permeabilisation, the response to GTPγS had fallen, and the whole of this loss was recovered by addition of myr.rARF-1. After 10mins, reconstitution with myr.rARF-1 was only partial. Thus the ability of myr.rARF-1 to reconstitute secretion was dependent on the time at which the protein was introduced. If "run-down" was allowed to progress too far, secretory competence could not be recovered.

The extent to which myr.rARF-1 could reconstitute secretion was dependent on the amount of protein used (see Fig.3.4.2). Reconstitution was seen in the presence of 100μg/ml myr.rARF-1, and this was not significantly increased in the presence of 1mg/ml myr.rARF-1. (A concentration of 100μg/ml myr.rARF-1 was used in experiments unless otherwise indicated). Reconstitution of secretion by myr.rARF-1 was sensitive to calcium concentration also (see Fig.3.4.3). In the absence of myr.rARF-1 secretion increased in the presence of calcium concentrations above 1μM, and was maximal in the presence of 10μM calcium. Myr.rARF-1-reconstituted secretion was also maximal in the presence of 10μM calcium, however increases were measured at concentrations below 1μM. Thus myr.rARF-1 increased the sensitivity of this system to calcium. In the presence of myr.rARF-1, cytosol-depleted cells were sensitive to the same range of calcium concentrations as acutely permeabilised cells.
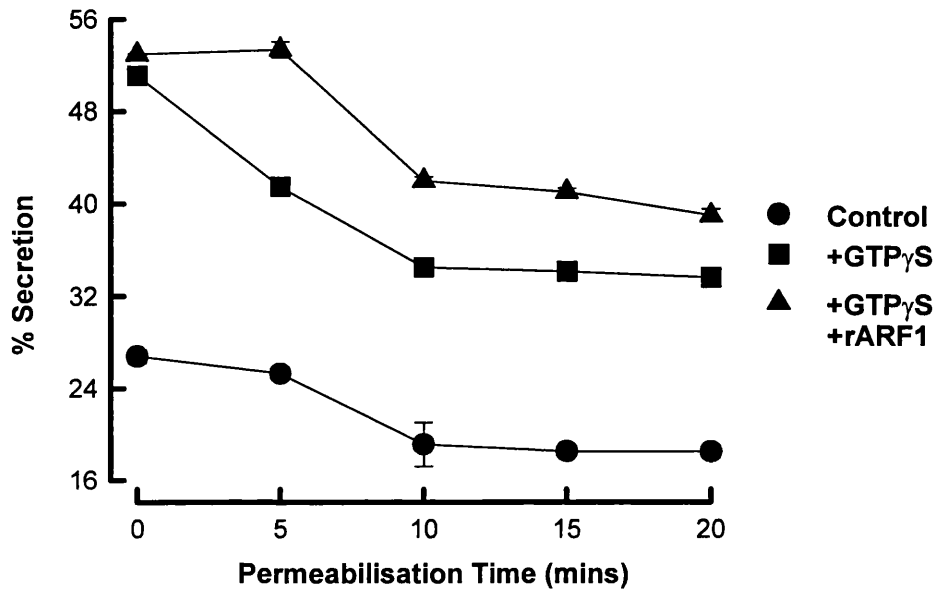
Figure 3.4.1. Effect of permeabilisation time on myr.rARF-1 reconstituted, GTPγS-stimulated secretion from RBL-2H3 cells

Cells were permeabilised in PIPES (pH 6.8) in the presence of 0.4iu/ml SLO, 1mM MgATP, 2mM $MgCl_2$ and 100nM Ca^{2+}, buffered by 0.3mM EGTA. At stated time-points, aliquots were removed and assayed in the presence of 1mM MgATP, 2mM $MgCl_2$ and 10μM Ca^{2+} buffered by 3mM EGTA (circles), with 10μM GTPγS (squares), or 10μM GTPγS and 100μg/ml myr.rARF-1 (triangles). Secretion was measured as described in section 2.4.
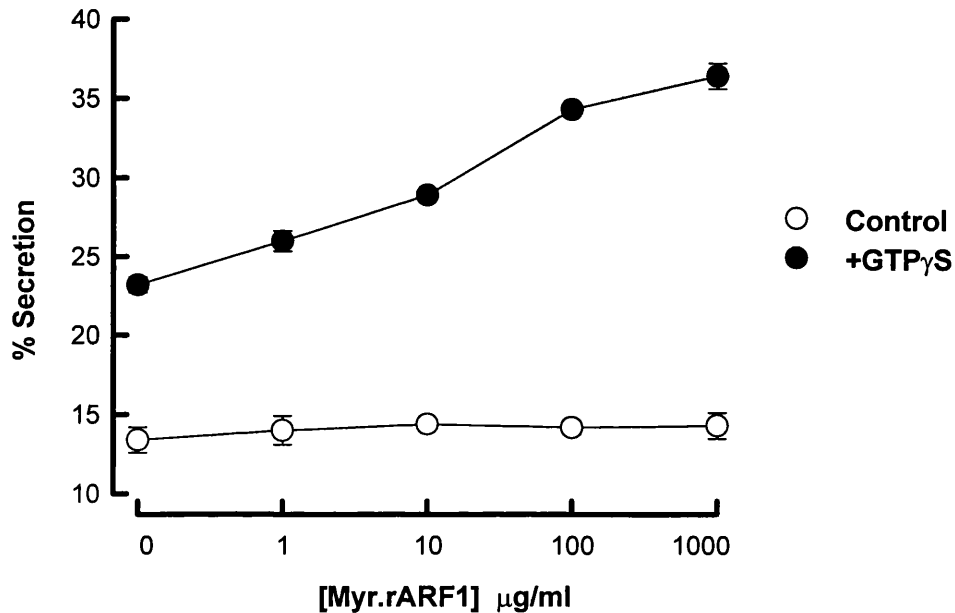
Figure 3.4.2. **Effect of myr.rARF-1 concentration on reconstituted, GTPγS-stimulated secretion from RBL-2H3 cells**

Cells were permeabilised for 10mins in PIPES buffer (pH 6.8) (according to the protocol described in section 2.3.5). Cells were assayed in the presence of 1mM MgATP, 2mM $MgCl_2$, 10μM Ca^{2+} buffered by 3mM EGTA, in the presence (closed symbols) or absence (open symbols) of 10μM GTPγS. Assays contained rising concentrations of myr.rARF-1, as indicated. Secretion was measured as described in section 2.4.

Figure 3.4.3. Effect of calcium concentration on myr.rARF-1 reconstituted, GTPγS-stimulated secretion from RBL-2H3 cells

Cells were permeabilised for 10mins in PIPES (pH 6.8) (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 1mM MgATP and 2mM $MgCl_2$ with rising concentrations of Ca^{2+} (buffered by 3mM EGTA). Cells assayed in the presence (closed symbols) or absence (open symbols) of 10μM GTPγS, and the presence (triangles) or absence (circles) of 100μg/ml myr.rARF-1. Secretion was measured as described in section 2.4.

ARF-reconstituted GTPγS-stimulated secretion was potentiated by MgATP. In order to demonstrate a true dependence on MgATP, the cells should have been assayed in the absence of glucose, so that they could not recover their endogenous ATP levels prior to assay. Unfortunately, these RBL-2H3 cells were highly sensitive to prolonged ATP-deprivation. In each attempt, metabolic inhibition followed by extended permeabilisation and assay in the absence of glucose, resulted in spontaneous lysis of cells. Therefore the assay buffer was supplemented with glucose, and only the effect of added MgATP was measured. The addition of 1mM MgATP alone to cytosol-depleted cells caused no significant increase in secretion. However when both myr.rARF-1 and GTPγS were added, MgATP's effect was significant: in the typical experiment shown (Fig.3.4.4) 40% secretion was measured under these conditions, in comparison with 20% measured in the absence of added MgATP. The sensitivity of the myr.rARF-1-reconstituted response is similar to that demonstrated by cytosol-depleted HL60 cells (161), and acutely permeabilised RBL-2H3 cells (see Fig.3.2.3). The dependence on this concentration of MgATP is thought to indicate the importance of a phosphorylation event, catalysed by the polyphosphoinositide kinases. GTP was also found to be an effective stimulus of myr.rARF-1-reconstituted secretion (see Fig.3.4.5). However as shown, the effect of even the highest concentrations of GTP is modest in comparison with GTPγS.

3.5 Reconstitution of PLD activity with myr.rARF-1

RBL-2H3 cells retain a partial secretory response to GTPγS even when cytosol-depleted. This partial response (see Fig.3.4.1) was mirrored by a partial PLD activation in response to GTPγS. In one respect these responses were different: myr.rARF-1-reconstituted PLD activity was stimulated by micromolar calcium alone, and secretion was not. However myr.rARF-1 sensitized PLD activity to lower concentrations of calcium, just as it did secretion. In the presence of calcium concentrations above 100nM, myr.rARF1- reconstituted PLD activity increased, whereas PLD activity measured in the absence of myr.rARF-1 increased only at ten-times this concentration. Myr.rARF-1-reconstituted PLD was sensitive to the same range of calcium concentrations as PLD activity from acutely permeabilised cells.
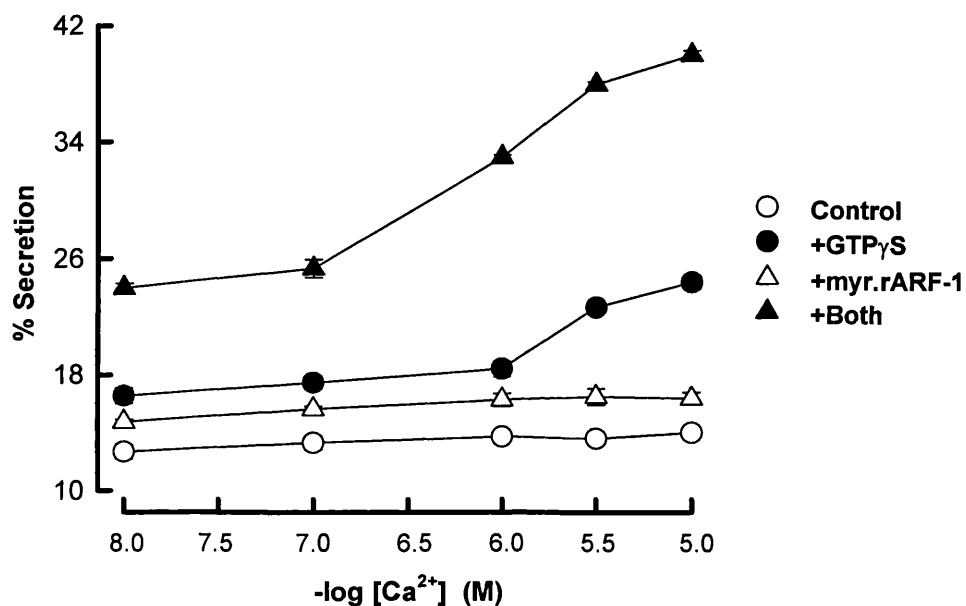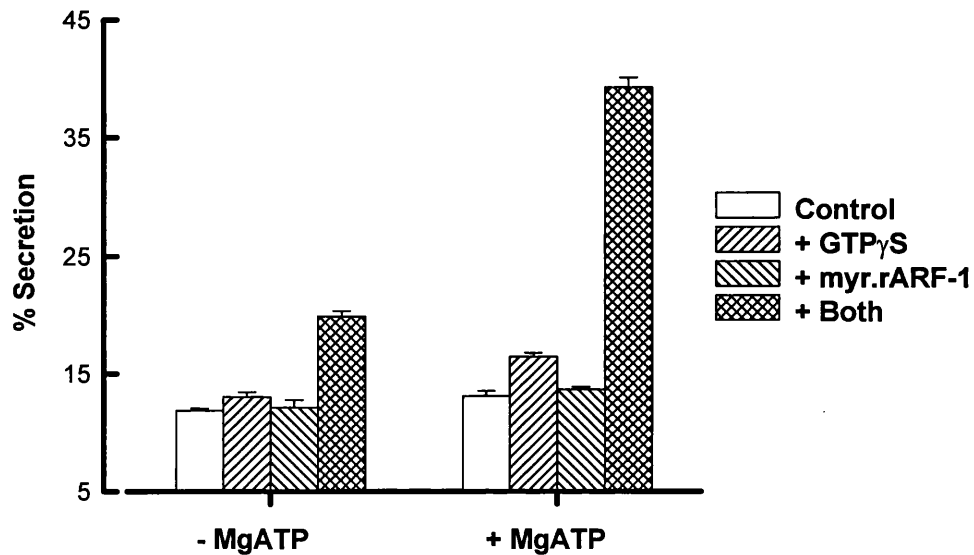
Figure 3.4.4. Effect of MgATP on myr.rARF-1 reconstituted, GTPγS-stimulated secretion from RBL-2H3 cells

Cells were metabolically inhibited before use (see section 2.3.6). Cells were permeabilised for 10mins in PIPES (pH 6.8) (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 2mM $MgCl_2$ and 10μM Ca^{2+} (buffered by 3mM EGTA). Assays carried out in the presence or absence of 10μM GTPγS, 100μg/ml myr.rARF-1 and 1mM MgATP as indicated. Secretion was measured as described in section 2.4.
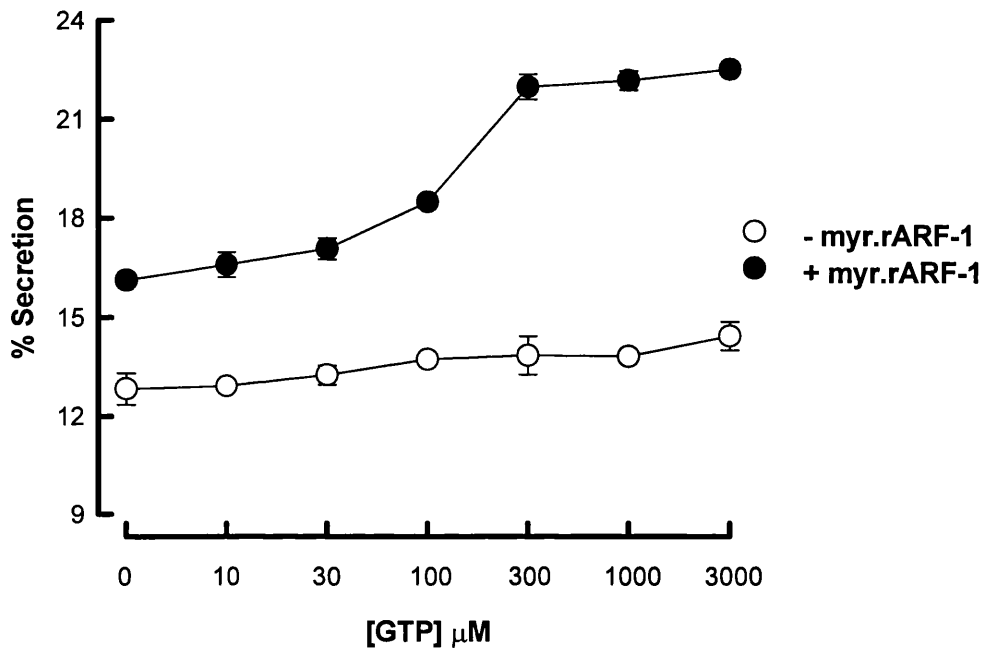
Figure 3.4.5. Effect of GTP concentration on myr.rARF-1 reconstituted secretion from RBL-2H3 cells

Cells were permeabilised for 10mins in PIPES (pH 6.8) (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 1mM MgATP, 2mM MgCl$_2$ and 10µM Ca^{2+} (buffered by 3mM EGTA). Assays carried out with rising concentrations of GTP as indicated, in the presence (closed symbols) or absence (open symbols) of 100µg/ml myr.rARF-1. Secretion was measured as described in section 2.4.
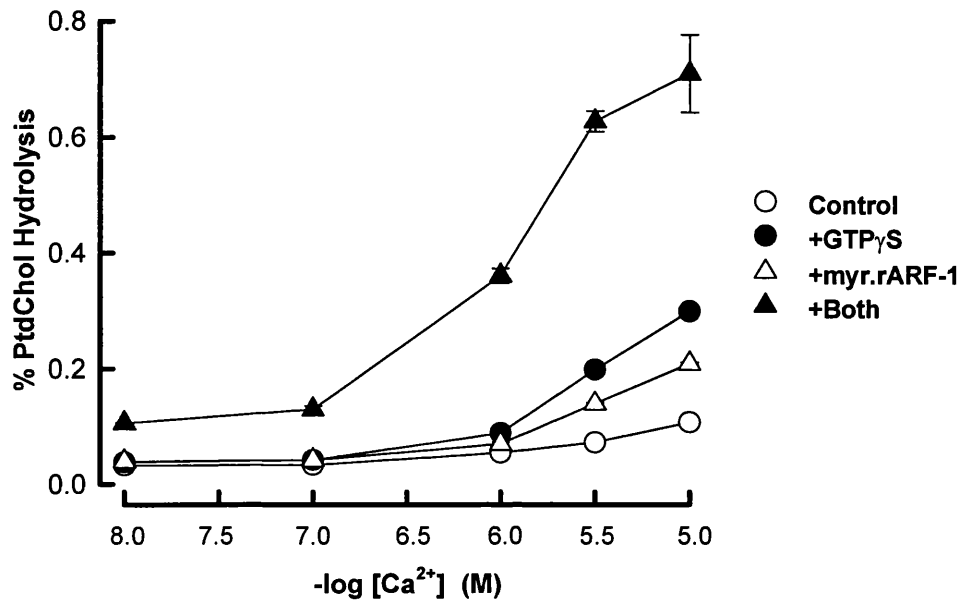
Figure 3.5.1. Effect of calcium concentration on myr.rARF-1 reconstituted, GTPγS-stimulated PLD activity in RBL-2H3 cells

[3H]-Choline-labelled cells were permeabilised for 10mins in PIPES buffer (pH 6.8) (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 1mM MgATP and 2mM $MgCl_2$ with rising concentrations of Ca^{2+} as indicated (buffered by 3mM EGTA). Cells assayed in the presence (closed symbols) or absence (open symbols) of 10μM GTPγS, and the presence (triangles) or absence (circles) of 100μg/ml myr.rARF-1. % PC hydrolysis was measured as described in section 2.5

The effect of MgATP on myr.rARF-1-reconstituted RBL-2H3 cells revealed a possible divergence between secretion and PLD activity. Unlike the secretory responses measured in metabolically inhibited cells, levels of PLD activity were high even in the absence of added MgATP. However, like secretion only responses measured in the presence of both GTPγS and myr.rARF-1 were significantly above control levels. MgATP potentiated the vestigial response to GTPγS. This potentiation was of a similar magnitude to the increase effected by myr.rARF-1. Therefore secretion was more dependent on MgATP than PLD activity. If Fig.3.4.4 and Fig.3.5.2 are compared: secretion doubled when MgATP was added in the presence of GTPγS and myr.rARF-1, however PLD activity increase by 50%.

3.6 Reconstitution of secretion with rPI-TPα

Like myr.rARF-1, rPI-TPα can recover secretion lost from permeabilised cells if applied within ten minutes of permeabilisation (see Fig.3.6.1). This activity "window" closed after fifteen minutes, thus reconstitution of secretion with rPI-TPα showed a greater dependence on time than secretion reconstituted with myr.rARF-1. Reconstitution by rPI-TPα was seen only when the protein was applied at concentrations above 3μg/ml. As shown in Fig.3.6.2, this response became saturated at concentrations above 30μg/ml rPI-TPα. (A concentration of 50μg/ml rPI-TPα was used in experiments unless otherwise indicated.) rPI-TPα was unable to reconstitute secretion in the absence of calcium, even in the presence of high concentrations of GTPγS. As shown in Fig.3.6.3, the dependence that RBL-2H3 cells displayed on GTPγS concentration when reconstituted with rPI-TPα was equivalent to that seen when they were acutely permeabilised. In both cases secretion increased in the presence of GTPγS concentrations as low as 1μM, becoming maximal in the presence of 10μM GTPγS.

The rPI-TPα-reconstituted secretory response displayed a dependence on a different range of calcium concentrations to that seen in both myr.rARF-1-reconstituted secretion, and secretion measured in acutely permeabilised cells (see Fig.3.6.4). Under these conditions, a response to GTPγS was measured
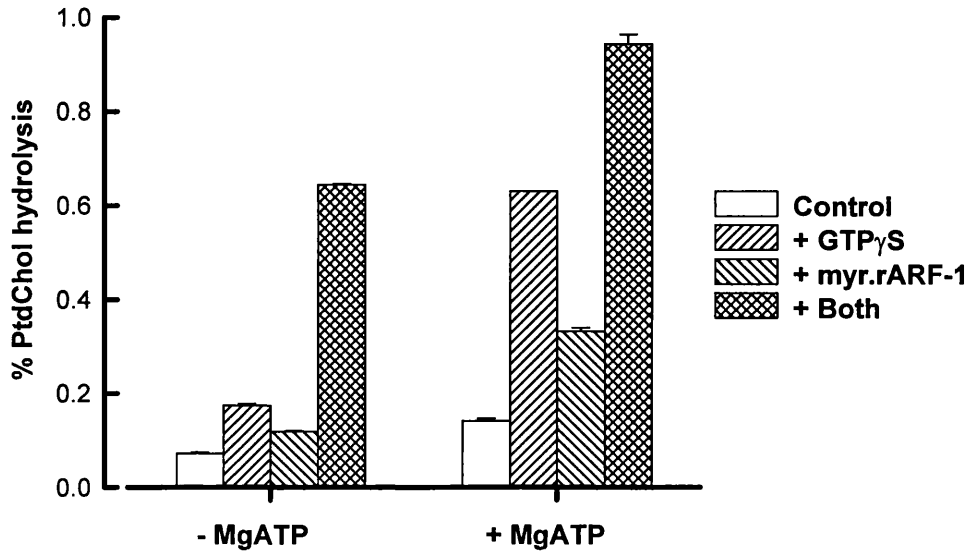
Figure 3.5.2. Effect of MgATP on myr.rARF-1 reconstituted, GTPγS-stimulated PLD activity in RBL-2H3 cells

[3H]-Choline-labelled cells were metabolically inhibited before use (see section 2.3.6). Cells were permeabilised for 10mins in PIPES buffer (pH 6.8) (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 2mM $MgCl_2$ and 10μM Ca^{2+} (buffered by 3mM EGTA). Assays carried out in the presence or absence of 10μM GTPγS, 100μg/ml myr.rARF-1 and 1mM MgATP as indicated. % PC hydrolysis was measured as described in section 2.5.
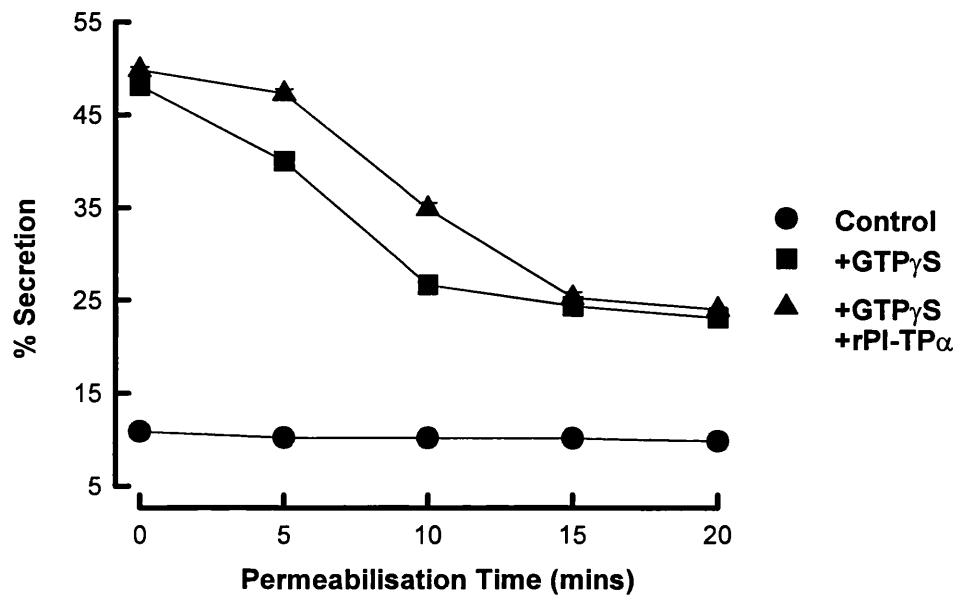
Figure 3.6.1. **Effect of permeabilisation time on rPI-TPα reconstituted, GTPγS-stimulated secretion from RBL-2H3 cells**

Cells were permeabilised in PIPES buffer (pH 6.8) in the presence of 0.4iu/ml SLO, 1mM MgATP, 2mM MgCl$_2$ and 100nM Ca^{2+}, buffered by 0.3mM EGTA. At stated time-points aliquots were removed and assayed in the presence of 1mM MgATP, 2mM MgCl$_2$ and 10μM Ca^{2+}, buffered by 3mM EGTA (circles), with 10μM GTPγS (squares), or 10μM GTPγS + 50μg/ml rPI-TPα (triangles). Secretion was measured as described in section 2.4.

even in the effective absence of calcium (see Figs.3.2.2 and 3.4.3). rPI-TPα-reconstituted secretion rose above background levels only at calcium concentrations above 100nM. Also unlike myr.rARF-1, rPI-TPα was unable to reconstitute GTPγS-stimulated secretion in the absence of added MgATP. As shown in Fig.3.6.5, the presence of MgATP alone did not potentiate rPI-TPα-reconstituted secretion, however in the presence of GTPγS, MgATP caused significant increases in secretion.

3.7 Reconstitution of secretion with rPI-TPβ

As shown in Fig.3.7.1, rPI-TPβ was also an effective reconstituting agent of cytosol-depleted RBL-2H3 cells. rPI-TPβ caused similar increases in secretion to both rPI-TPα and myr.rARF-1. When these proteins were added in combination: any two or all three together, a small additive effect was seen. Despite this increase, no combination of two reconstituting proteins, or even a combination of all three proteins together, could fully recover the response to GTPγS measured in acutely permeabilised cells. (In the typical experiment shown, the maximum reconstituted response was 41% secretion, whereas the acutely permeabilised response was 50%).

As shown in Fig.3.7.2, reconstitution of the secretory response from these cytosol-depleted cells was measured when rPI-TPβ was supplied to the cells at concentrations above 3μg/ml. At concentrations of rPI-TPβ above 30μg/ml, secretion was not significantly increased. Thus the range of concentrations over which rPI-TPβ was effective was exactly the same as the active range of rPI-TPα. (rPI-TPβ was used in reconstitution experiments at a concentration of 50μg/ml unless otherwise indicated.) Like rPI-TPα-reconstituted secretion, rPI-TPβ-reconstituted secretion was entirely dependent on the presence of added MgATP, whether assayed in the presence or absence of calcium or GTPγS (see Fig.3.7.3). Also, like rPI-TPα, rPI-TPβ did not reconstitute secretion in response to MgATP alone, however its activity in the presence of GTPγS and calcium was potentiated.
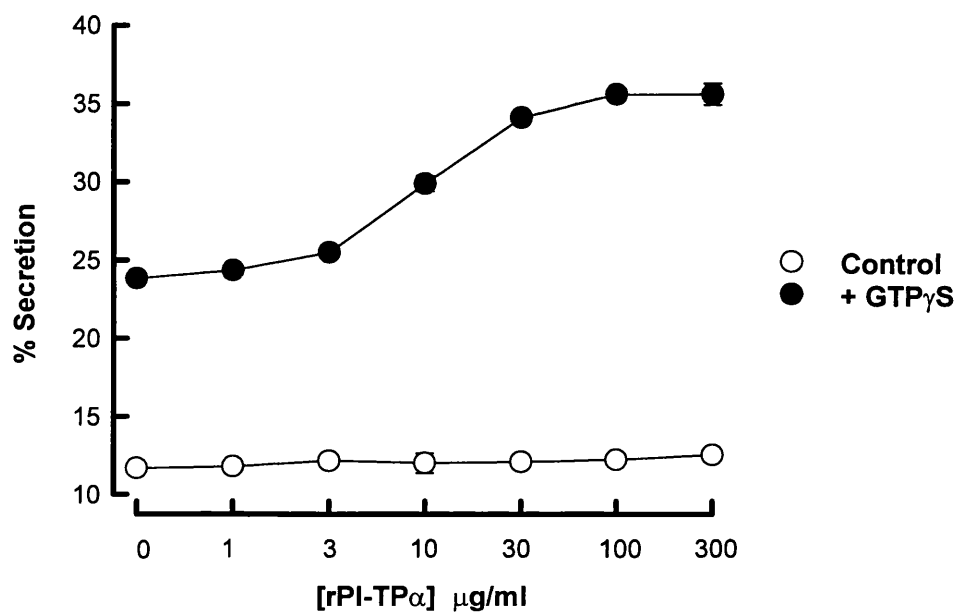
Figure 3.6.2. **Effect of rPI-TPα concentration on reconstituted, GTPγS-stimulated secretion from RBL-2H3 cells**

Cells were permeabilised for 10mins in PIPES buffer (pH 6.8) (according to the protocol described in section 2.3.5). Cells were assayed in the presence of 1mM MgATP, 2mM MgCl$_2$ and 10μM Ca^{2+} buffered by 3mM EGTA, in the presence (closed symbols) or absence (open symbols) of 10μM GTPγS. Assays contained rising concentrations of rPI-TPα, as indicated. Secretion was measured as described in section 2.4.
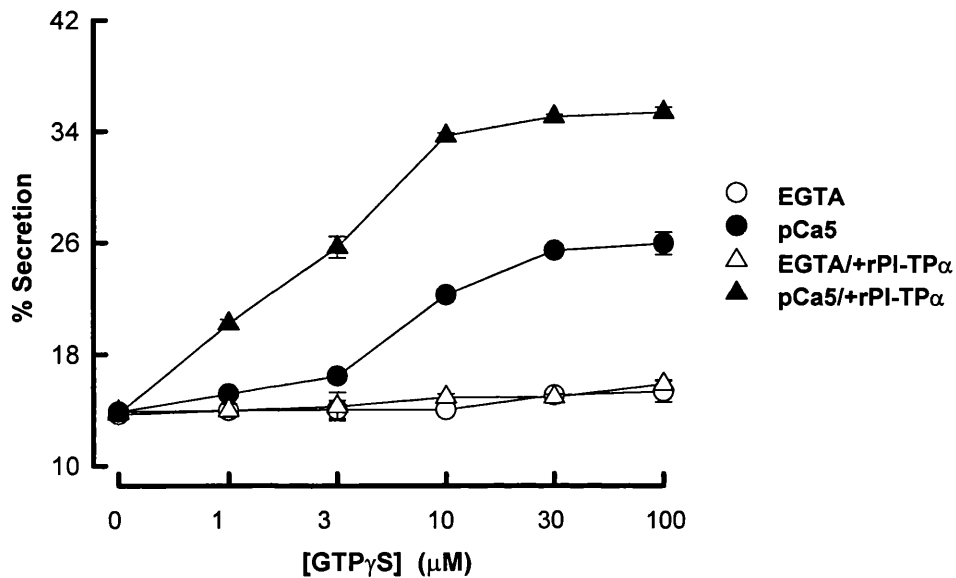
Figure 3.6.3. Effect of GTPγS concentration on rPI-TPα reconstituted secretion from RBL-2H3 cells

Cells were permeabilised for 10mins in PIPES buffer (pH 6.8) (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 1mM MgATP and 2mM MgCl$_2$ with rising concentrations of GTPγS. Cells assayed with 3mM EGTA in the presence (triangles) or absence (circles) of 10μM Ca^{2+}, and the presence (closed symbols) or absence (open symbols) of 50μg/ml rPI-TPα. Secretion was measured as described in section 2.4.

Figure 3.6.4. Effect of calcium concentration on rPI-TPα reconstituted, GTPγS-stimulated secretion from RBL-2H3 cells

Cells were permeabilised for 10mins in PIPES buffer (pH 6.8) (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 1mM MgATP and 2mM $MgCl_2$ with rising concentrations of Ca^{2+} (buffered by 3mM EGTA). Cells assayed in the presence (closed symbols) or absence (open symbols) of 10μM GTPγS, and the presence (triangles) or absence (circles) of 50μg/ml rPI-TPα. Secretion was measured as described in section 2.4.

Figure 3.6.5. Effect of MgATP on rPI-TPα reconstituted, GTPγS-stimulated secretion from RBL-2H3 cells

Cells were metabolically inhibited before use (see section 2.3.6). Cells were permeabilised for 10mins in PIPES (pH 6.8) (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 2mM $MgCl_2$ and 10μM Ca^{2+} (buffered by 3mM EGTA). Assays carried out in the presence or absence of 10μM GTPγS, 50μg/ml rPI-TPα and 1mM MgATP as indicated. Secretion was measured as described in section 2.4.

3.8 Discussion

The purpose of the experiments described in the first sections of this chapter was to characterise the responses of RBL-2H3 cells to GTPγS. Two responses were measured: PLD activity and the release of secretory granule contents. These experiments indicate that secretion from RBL-2H3 cells is dependent on the concentration of GTPγS, calcium and MgATP. A correlation between PLD and secretion has been noted in fMLP-stimulated (281) and GTPγS-stimulated HL60 cells (161). This correlation has been cited as evidence for the importance of PLD activity in secretion. In RBL-2H3 cells some correlation between the two responses was seen, for example the dependence of both responses on GTPγS-concentration is similar. However, these cells do not display the same degree of close correlation as has been demonstrated in HL60 cells.

The dependence of secretion and PLD activation on MgATP and calcium are different. In both cases where divergence is measured between PLD activity and the secretory response to GTPγS, secretion is maximal when PLD activity is approximately 50% of maximal. Thus it is possible that in RBL-2H3 cells, and in HL60 cells also, a "sufficient" level of PLD activity is required in order for secretion to proceed. Secretion and PLD activity in HL60 cells and RBL-2H3 cells may show different levels of correlation because they have different "sufficient" levels. In RBL-2H3 cells perhaps only quite a low level of PLD activity is sufficient. The level of PLD activation via the receptor is low (see chapter 4), and stimulated by higher concentrations of antigen than secretion in intact cells, suggesting that PLD activation *in vivo* may be quite modest response.

The responsiveness to calcium and GTPγS of RBL-2H3 cells is similar to that seen in mast cells (189). However one striking difference between mast cells and RBL-2H3 cells is their relative dependences on MgATP. Mast cells supplied with GTPγS and 10μM calcium are fully responsive (~100% release) in the absence of MgATP. RBL-2H3 cells are dependent on MgATP concentrations in the millimolar range. The exact reason for this difference is not clear - however it is likely that such a fundamental difference reflects a
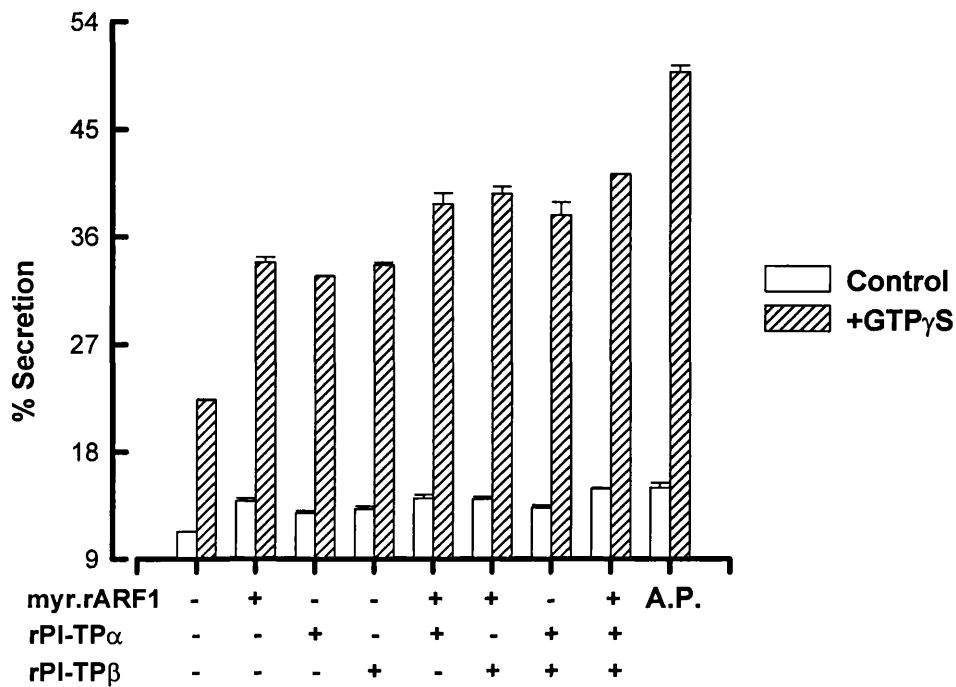
Figure 3.7.1. Effect of rPI-TPα, rPI-TPβ and myr.rARF-1 as reconstitutors of GTPγS-stimulated secretion in RBL-2H3 cells

Cells were permeabilised for 10mins in PIPES buffer (pH 6.8), with an aliquot of cells retained for measurement of the A.P. (acutely permeabilised) cell responses (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 1mM MgATP, 2mM $MgCl_2$ and 10μM Ca^{2+} (buffered by 3mM EGTA). Assays carried out in the presence or absence of 10μM GTPγS, 50μg/ml myr.rARF-1, 50μg/ml rPI-TPα and 50μg/ml rPI-TPβ as indicated. Secretion was measured as described in section 2.4.
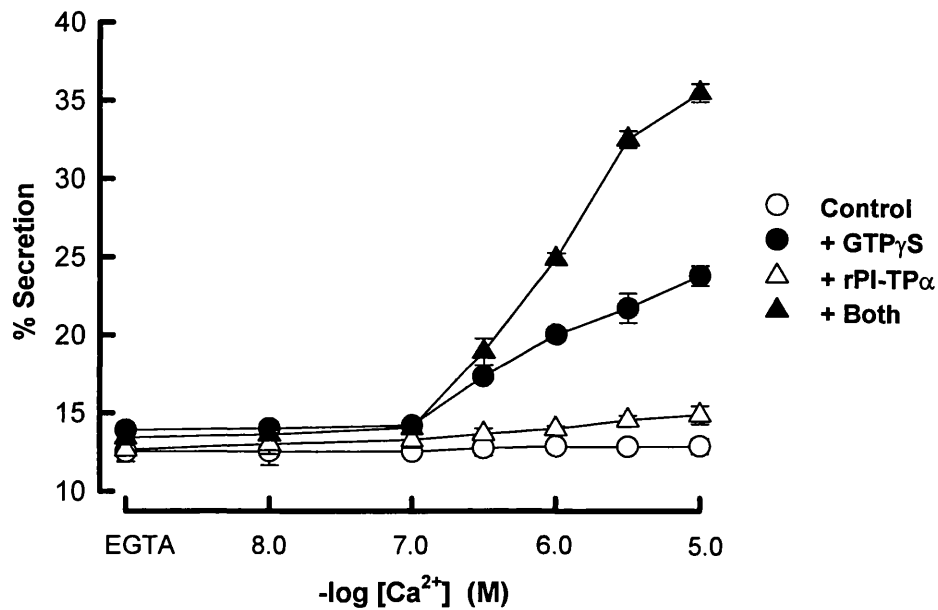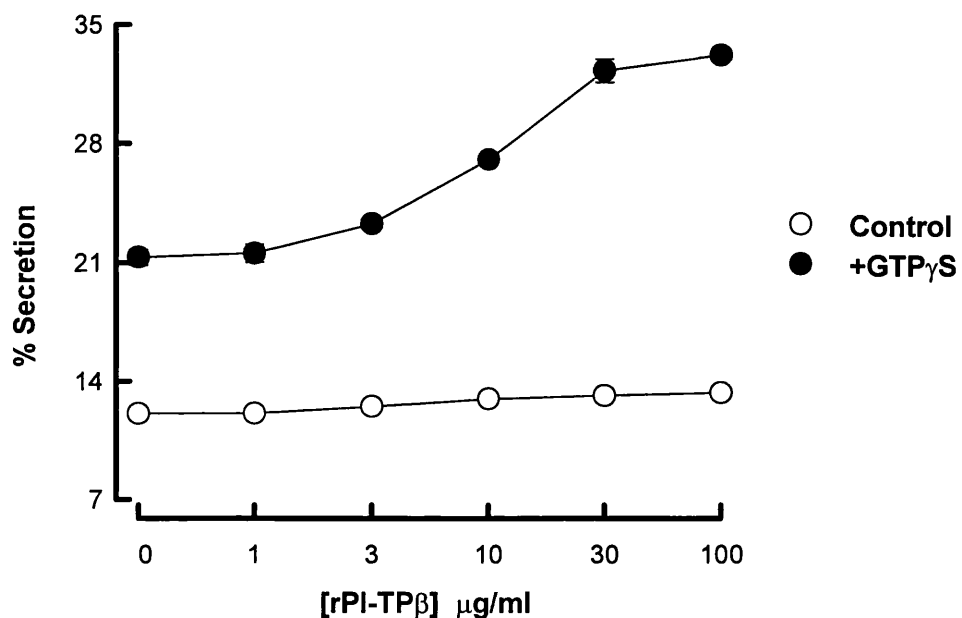
Figure 3.7.2. Effect of rPI-TPβ concentration on reconstituted, GTPγS-stimulated secretion from RBL-2H3 cells

Cells were permeabilised for 10mins in PIPES buffer (pH 6.8) (according to the protocol described in section 2.3.5). Cells were assayed in the presence of 1mM MgATP, 2mM MgCl$_2$ and 10μM Ca^{2+} buffered by 3mM EGTA, in the presence (closed symbols) or absence (open symbols) of 10μM GTPγS. Assays contained rising concentrations of rPI-TPβ, as indicated. Secretion was measured as described in section 2.4.

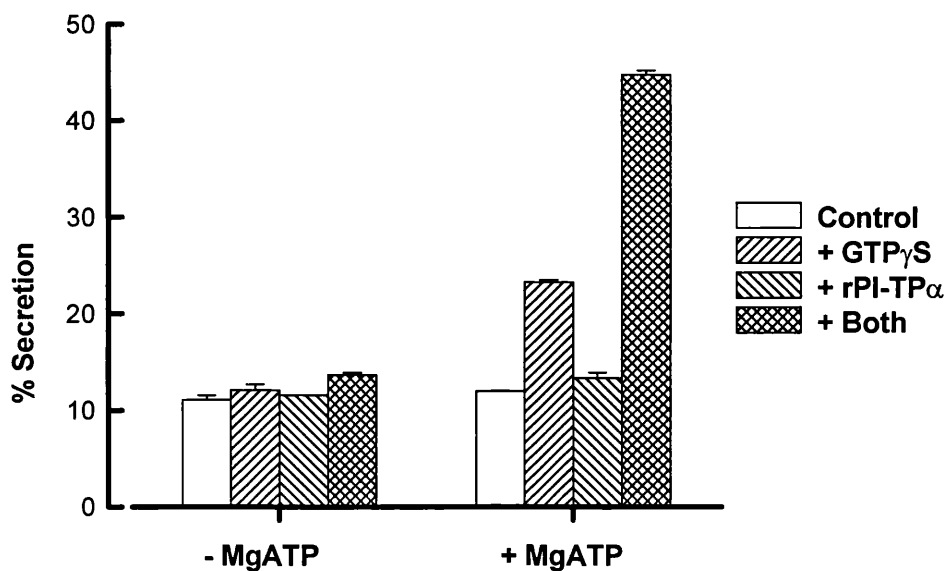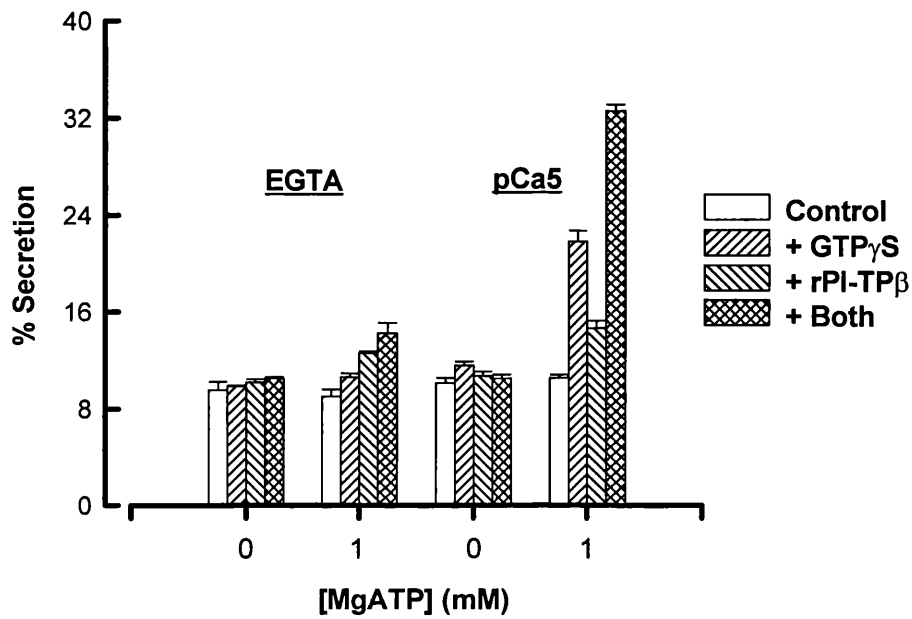**Figure 3.7.3.** **Effect of calcium and MgATP on rPI-TPβ reconstituted, GTPγS-stimulated secretion from RBL-2H3 cells**

Cells were metabolically inhibited before use (see section 2.3.6). Cells were permeabilised for 10mins in PIPES buffer (pH 6.8) (as described in section 2.3.5). Permeabilised cells were assayed with 2mM $MgCl_2$, in the presence or absence of 10μM GTPγS, 50μg/ml rPI-TPβ, 1mM MgATP and 3mM EGTA +/- 10μM Ca^{2+} as indicated. Secretion was measured as described in section 2.4

difference in the regulation of exocytosis from these related cells. The inhibition of RBL-2H3 cell secretion by high concentrations of MgATP has been reported before in eosinophils (282). The effect was attributed in this study to a non-specific effect of magnesium, but its mechanism remains obscure.

The importance of cytosolic proteins is clear from the decline in responsiveness resulting from extended permeabilisation. Three proteins that have been implicated in secretion were examined in particular - ARF, and the α and isoforms β isoforms of PI-TP. These proteins leak from the cells rapidly after permeabilisation, with a significant proportion of each protein leaked from cells after just ten minutes. The decline in GTPγS-stimulated secretion is slow however, retained after the majority of ARF and rPI-TPα/β has leaked. This suggests that a component necessary for GTPγS-stimulated secretion and PLD remains in cells, and can be activated directly by GTPγS.

RhoA is a good candidate for this component - a G-protein other than ARF that activates PLD activity. Certainly RhoA leaks more slowly than ARF (data not shown), implying that it could be responsible for the residual response to GTPγS. However rac and Cdc42 and heterotrimeric G-proteins - particularly G_{i3} have also been implicated. Rac and Cdc42 leak from permeabilised mast cells and support secretion when applied back to cells ((164) and Brown,A. and Gomperts,B. - personal communication). It is not clear whether rac or Cdc42 are retained in RBL-2H3 cells. The rate of mast cell run-down appears to be more rapid than RBL-2H3 run-down, a difference that is likely to be at least partly due to a difference in protocol. Thus rates of rac leakage from mast cells may not be compared with rates of leakage from RBL-2H3 cells, unless identical protocols are employed.

GTPγS-stimulated secretion and PLD activity decline at similar rates (see Fig.3.3.1). When myr.rARF-1 is used to reconstitute secretion and PLD, the resulting responses show parallel dependences on calcium and MgATP concentration. These reconstituted responses show similar responses to manipulation of these modulators as acutely permeabilised cells. These

findings support the hypothesis that the decline in PLD activity seen in permeabilised cells is due to the loss of ARF-1, and that the loss of PLD activity is at least partly responsible for the loss of secretory competence. The ability of myr.rARF-1 to reconstitute secretion and PLD activity has been demonstrated previously (161); in this report also the correlation between PLD activity and secretion was proposed to arise from the importance of PLD activity in regulated secretion.

The correlation between myr.rARF-1-reconstituted secretion and PLD activity is closer than the correlation seen in GTPγS-stimulated acutely permeabilised cells. This may indicate that modulators other than ARF-1 are lost during the 10min permeabilisation, and that this loss causes secretion and PLD activity to assume the same profile of responses to calcium and MgATP. That other modulators of secretion and PLD activity exist is clearly implied by the inability of myr.rARF-1, rPI-TPα and rPI-TPβ to fully reconstitute secretory competence.

The secretory responses reconstituted by myr.rARF-1 and the PI-TP's show similar dependences on calcium and MgATP - however there are differences. The calcium-independent response seen in myr.rARF-1-reconstituted PLD and secretion is not seen in either rPI-TPβ- or rPI-TPα-reconstituted secretion. Therefore it seems unlikely that the response measured in acutely-permeabilised cells in the absence of calcium arises from any activity of PI-TP. The effects of high concentrations of calcium are approximately equal however, whatever the reconstituting protein used. Similarly, the effect of MgATP on myr.rARF-1- and PI-TP-reconstituted secretion are approximately equal. This dependence on MgATP, proposed to be related to the production of PIP_2 would be predicted according to the hypothesized shared mechanism of ARF's and PI-TP's roles in secretion (see chapters 5 and 6).

The concentrations at which myr.rARF-1 and rPI-TPα/β are applied are appropriate - the total PI-TP concentration in HL60 cells has been estimated at 100-300μg/ml (283), and this is assumed to be similar to the concentration in RBL-2H3 cells. The concentration of recombinant myr.rARF-1 used is higher

than the concentration of native ARF in intact HL60 cells, however myr.rARF-1 is incompletely myristoylated in comparison to native protein, and this modification is known to increase the potency of the protein. When compared in HL60 cells, the effective concentrations of native ARF used to reconstitute PLD activity was found to be no more than 20% of the concentration of ARF present in intact HL60 cells (119).

The concentration of rPI-TPα that is effective in reconstituting secretion from HL60 cells is effective also in RBL-2H3 cells. However in HL60 cells, rPI-TPβ is effective at lower concentrations than rPI-TPα; this difference is not noted in RBL-2H3 cells. In HL60 cells, rPI-TPβ is the predominant isoform, in RBL-2H3 cells the two isoforms are expressed at approximately equal concentrations (283). It could be argued therefore that HL60 cells are sensitive to lower concentrations of PI-TPβ, hence the ability of small amounts of the protein to reconstitute secretion. The higher concentrations of native rPI-TPβ in RBL-2H3 cells indicate an increased dependence on this particular isoform - reflected in the higher concentrations required in reconstitution experiments. Despite the ability of these proteins to reconstitute secretion, rPI-TPα and rPI-TPβ do not reconstitute PLD activity (data not shown). This indicates that the effect of myr.rARF-1 on PLD activity is specific - and not an general artefact of recombinant proteins.

In myr.rARF-1-reconstituted secretion the effect of GTPγS may be explained by its activation of myr.rARF-1. However, the role of GTPγS in stimulating rPI-TPα- and rPI-TPβ-reconstituted secretion - proteins that do not bind GTPγS, is not so clear. The functional importance of two GTP-binding proteins in mast cell secretion has been suggested (190). The first, relatively up-stream G-protein regulates PLC in this system, and the second "G_E" regulates some unidentified down-stream effect. This downstream G-protein is presumed to be the GTP-dependent factor in PI-TP-reconstituted secretion - and is also assumed to be equally important in myr.rARF-1-reconstituted secretion.

CHAPTER FOUR

CHARACTERISATION OF ANTIGEN-STIMULATED SECRETION

AND PLD ACTIVITY FROM RBL-2H3 CELLS

Chapter 4 Characterlsation of RBL-2H3 cell secretion and PLD activity

4.1 Introduction

The physiological stimulus of RBL-2H3 mast cells is the cross-linking of the IgE-receptor by antigen. The experiments described in this chapter were designed primarily to examine the responsiveness of RBL-2H3 cells to this stimulus following permeabilisation. Having established an acutely permeabilised cell response to antigen-stimulus, the importance of other calcium and ATP as modulators of secretion was examined, and effective concentrations of antigen and optimal incubation times have been defined.

The effect of SLO permeabilisation over an extended interval on antigen-stimulated secretion was also examined. This response showed a marked dependence on cytosolic proteins, implied by the decline in secretion and PLD assayed following permeabilisation. As shown in chapter 3, GTPγS can be used to stimulate permeabilised cells, and cytosol-depleted cells when they are reconstituted with myr.rARF-1, rPI-TPα and rPI-TPβ. The purpose of the experiments described in later sections of this chapter was to attempt reconstitution of the antigen-stimulated system. The response to antigen stimulation declines very rapidly after permeabilisation, with approximately 40% of the maximal response lost within two minutes. Thus only a very short permeabilisation interval may be used if the receptor-driven response is to remain measurable. Yet the permeabilisation interval must be sufficiently long, so that a significant decline in responsiveness should occur, and meaningful levels of reconstitution established.

Thus initial experiments were primarily concerned with defining optimal conditions for reconstituting the fragile antigen-stimulated secretory response. As before, the concentrations of important modulatory factors, such as calcium and MgATP were manipulated, to assess their importance in this system. In addition, the ability of myr.rARF-1 to reconstitute receptor activated phospholipase D was examined.

4.2 Antigen-stimulated secretion

As shown in Fig.4.2.1a, the dependence of the antigen-stimulated response on pH is clear: secretion at pH 7.4 is twice that measured at pH 6.8. However the EGTA-based calcium buffers, used in all permeabilised cell experiments are constructed for use at pH 6.8. These buffers are pH sensitive, and a change from pH 6.8 to pH 7.2 will cause free calcium to be held at a different concentration from that intended. As a different type of calcium buffer may have introduced variation, it was decided to continue using the EGTA-based solutions. Thus it was vital to ascertain the variation in free calcium caused by the change in pH. The buffers were therefore diluted in HEPES buffer (pH 7.2) to a final concentration of 3mM, as in an experiment. These samples were mixed with a fluorescent indicator - Fura2 and the free calcium measured by comparison with a series of calibration measurements. The alteration of free calcium noted using the EGTA buffer constructed at 10μM was not considered to be significant enough to justify preparation of new buffers (see Fig.4.2.1b). The calculations were checked using the computer program "CHelate", to ensure that at this pH the EGTA provided adequate buffering.

The secretory response was acutely sensitive to DNP-HSA, stimulated by just 1ng/ml (see Fig.4.2.2). The intact cells were activated by lower concentrations than permeabilised cells although the differences were small and both responses were maximal at 30ng/ml. Therefore permeabilisation alone did not compromise antigen-stimulated responses significantly. The antigen-stimulated response was dependent on calcium; however GTPγS-stimulated cells responded 100nM calcium, and the response was maximal at 3μM, whereas the antigen-stimulated cells responded to calcium concentrations of 1μM and above (see Fig.4.2.3), and at 10μM the response is not yet maximal. Thus the antigen-stimulated response required higher concentrations of calcium. Antigen-stimulated secretion was also dependent on MgATP; with little secretion measured at concentrations below 300μM. As shown in Fig.4.2.4, MgATP enhanced secretion at concentrations between 300μM and 3mM. 10mM MgATP had an inhibitory effect, similar to that seen in GTPγS-stimulated cells.
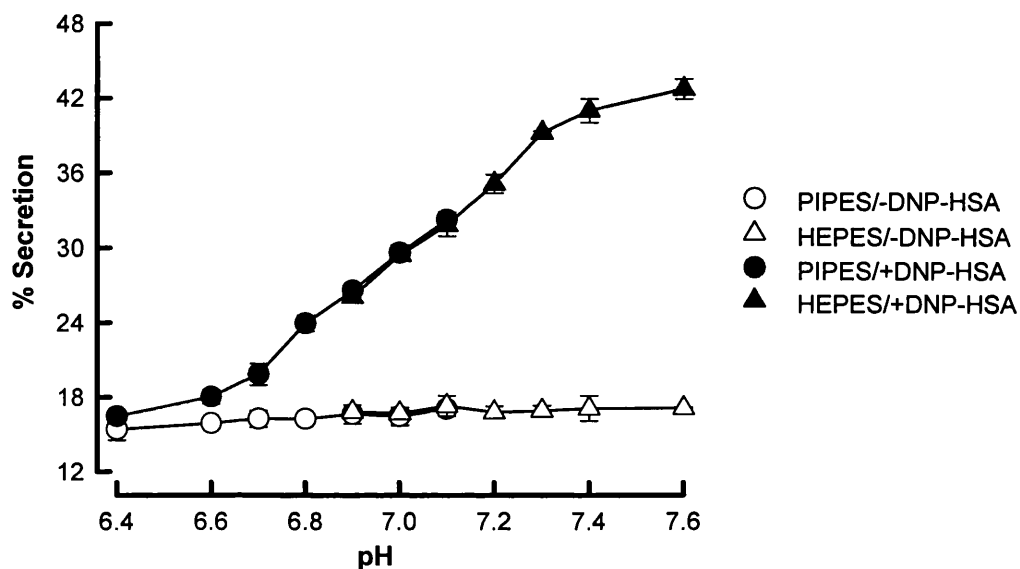
Figure 4.2.1.a **Effect of pH on antigen-stimulated secretion, measured from intact RBL-2H3 cells**

A variety of buffers was made, from both HEPES (triangles) and PIPES (circles), over an overlapping range of pH's. All buffers made for use with intact cells (see section 2.3.11). Cells were pre-treated with IgEαDNP then incubated, while still intact in the presence (closed symbols) and absence (open symbols) of 40ng/ml DNP-HSA. Secretion was measured as described in section 2.5
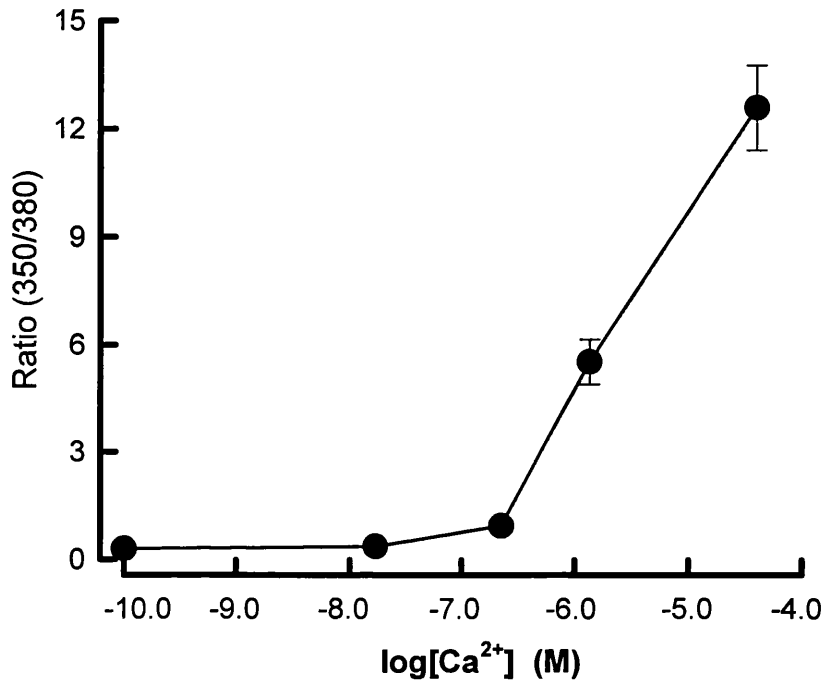
$$10nM = pCa8 = 0.358064 = pCa7.80 = 16.0nM$$
$$1\mu M = pCa6 = 1.467722 = pCa6.56 = 275.4nM$$
$$10\mu M = pCa5 = 10.26686 = pCa4.95 = 11.2\mu M$$

Figure 4.2.1.b **Calibration curve for free calcium concentration using Fura2**

A variety of calcium standards were mixed with Fura2. The fluorescent emission from these samples at 350nm and 380nm was measured and a ratio calculated for each. These values were used to construct a calcium standard curve. Samples of the EGTA-based calcium buffers were diluted to a final concentration of 3mM in HEPES buffer pH7.2, and mixed with Fura2. The ratio of fluorescence at 350nm and 380nm was calculated for each, and the free calcium measured by comparison with the calibration curve. (Samples analysed by A.Fleet)

(a) Effect of DNP-HSA concentration on secretion from intact RBL cells

Y-axis: % Secretion (0, 8, 16, 24, 32)
X-axis: [DNP-HSA] (ng/ml) (0, 1, 3, 10, 30, 100, 300, 1000)

(b) Effect of DNP-HSA concentration on secretion from acutely permeabilised cells

Y-axis: % Secretion (5, 10, 15, 20, 25, 30, 35)
X-axis: [DNP-HSA] (ng/ml) (0, 1, 3, 10, 30, 100, 300, 1000)

○ EGTA
● pCa5
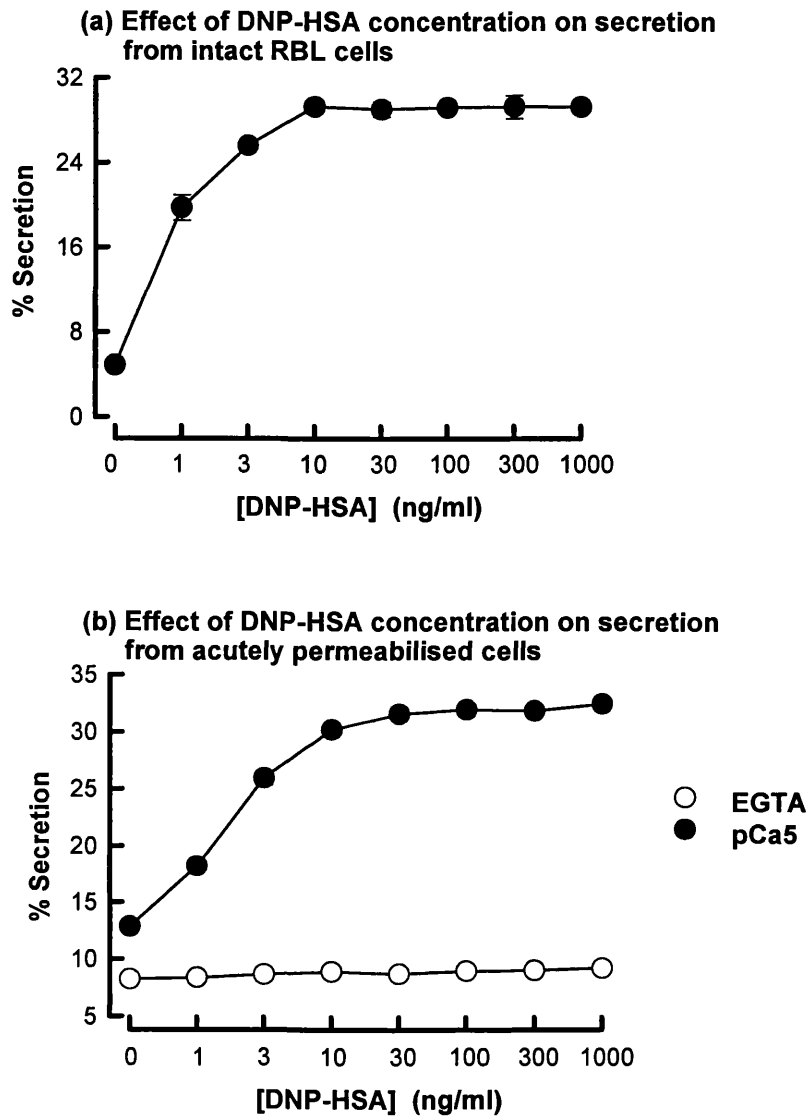
Figure 4.2.2. Effect of DNP-HSA on secretion from intact or acutely permeabilised RBL-2H3 cells

All cells were pretreated with IgEαDNP before use. Graph (a): the cells were stimulated while intact (see section 2.3.2), with rising concentrations of DNP-HSA. All solutions made using HEPES buffer for intact cells (see section 2.3.11). Graph (b): cells were assayed in standard HEPES buffer pH 7.2 in a variety of DNP-HSA concentrations. The standard acutely permeabilised cell protocol was used. All assays included 1mM MgATP, 2mM $MgCl_2$ in the absence (open symbols) and presence (closed symbols) of 10μM Ca^{2+}, buffered by 3mM EGTA.
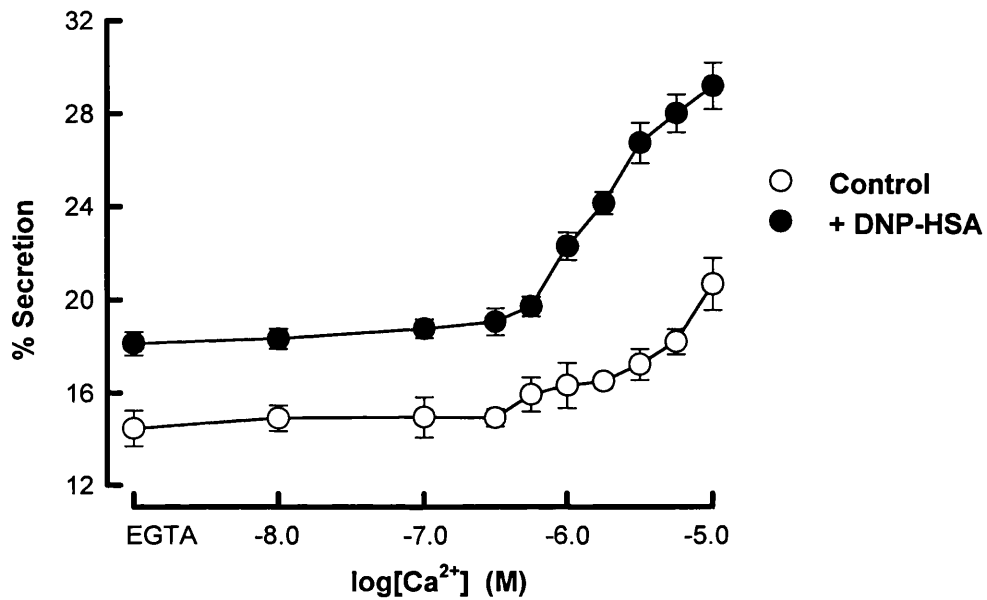
Figure 4.2.3 **Effect of Calcium concentration on antigen-stimulated secretion in acutely permeabilised RBL-2H3 cells**

Cells were pretreated with IgEαDNP then assayed in HEPES buffer (pH 7.2) with rising Ca^{2+} concentrations, buffered by 3mM EGTA. In the presence (closed symbols) and absence (open symbols) of 40ng/ml DNP-HSA. The standard acutely permeabilised protocol was used (see section 2.3.3). Secretion was measured as described in section 2.4.
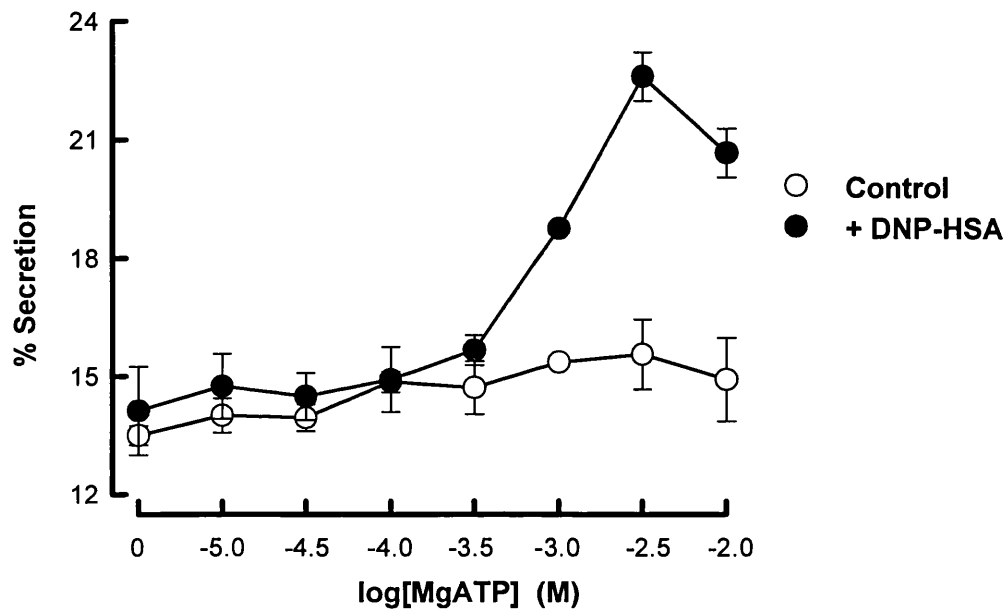
Figure 4.2.4 **Effect of MgATP concentration on antigen-stimulated secretion from acutely permeabilised RBL-2H3 cells**

Cells were pretreated with IgEαDNP and metabolically inhibited prior to use, and cells and all reagents prepared in glucose-free HEPES buffer (pH 7.2). Cells were assayed with rising MgATP concentrations In the presence (closed symbols) and absence (open symbols) of 40ng/ml DNP-HSA. All assays included 10μM Ca^{2+} buffered by 3mM EGTA and 2mM $MgCl_2$. The standard acutely permeabilised protocol was used (see section 2.3.3). Secretion was measured as described in section 2.4.

RBL-2H3 cells could be stimulated while attached, and while in suspension. The responses measured in suspended and attached cells were similar in extent and rate, with maxima measured approximately 20mins after stimulation. In the typical experiment shown in Fig.4.2.5, secretion measured from unstimulated suspended cells was 4%. This artefact - thought to arise from cell preparation may account partially for the apparently faster rate of secretion measured in suspended cells.

4.3 Antigen-stimulated PLD activity

Phospholipase D activity was stimulated by DNP-HSA both in intact and permeabilised cells, as shown in Fig.4.3. While both of these responses showed a similar dependence on DNP-HSA concentration, the response from acutely permeabilised cells was smaller, against a higher background. This background rate was thought to be caused by the $10\mu M$ calcium included in the assay. Secretion stimulated by DNP-HSA was saturated by DNP-HSA concentrations above 10ng/ml, however the PLD response was not saturated by 100ng/ml DNP-HSA.

4.4 Dependence of secretion and PLD activity on cytosolic proteins

In contrast to the decline in responsiveness to GTPγS seen in Fig.3.3.1, the response to DNP-HSA falls very rapidly after permeabilsation. As shown in Fig.4.4, the PLD and secretory responses declined at different rates. The secretory response fell very rapidly for two minutes after permeabilisation, then the rate of decline slowed. After 20mins, no secretory response to DNP-HSA was measured. PLD activity fell more slowly over the entire time-course, however, again no PLD response to stimulus was measured after 20mins permeabilisation.

4.5 Reconstitution of secretion with myr.rARF-1

The response to antigen stimulation fell very rapidly, with half of the total response lost during the first two minutes of permeabilisation. As shown in Fig.4.5.1, addition of myr.rARF-1 to the cells after 2mins of permeabilisation restored the declined secretory response, however even after this short

(a) Effect of time on secretion from intact suspended cells

% Secretion

Time (min)

○ Control
● + DNP-HSA

(b) Effect of time on secretion from intact attached cells

% Secretion

Time (min)
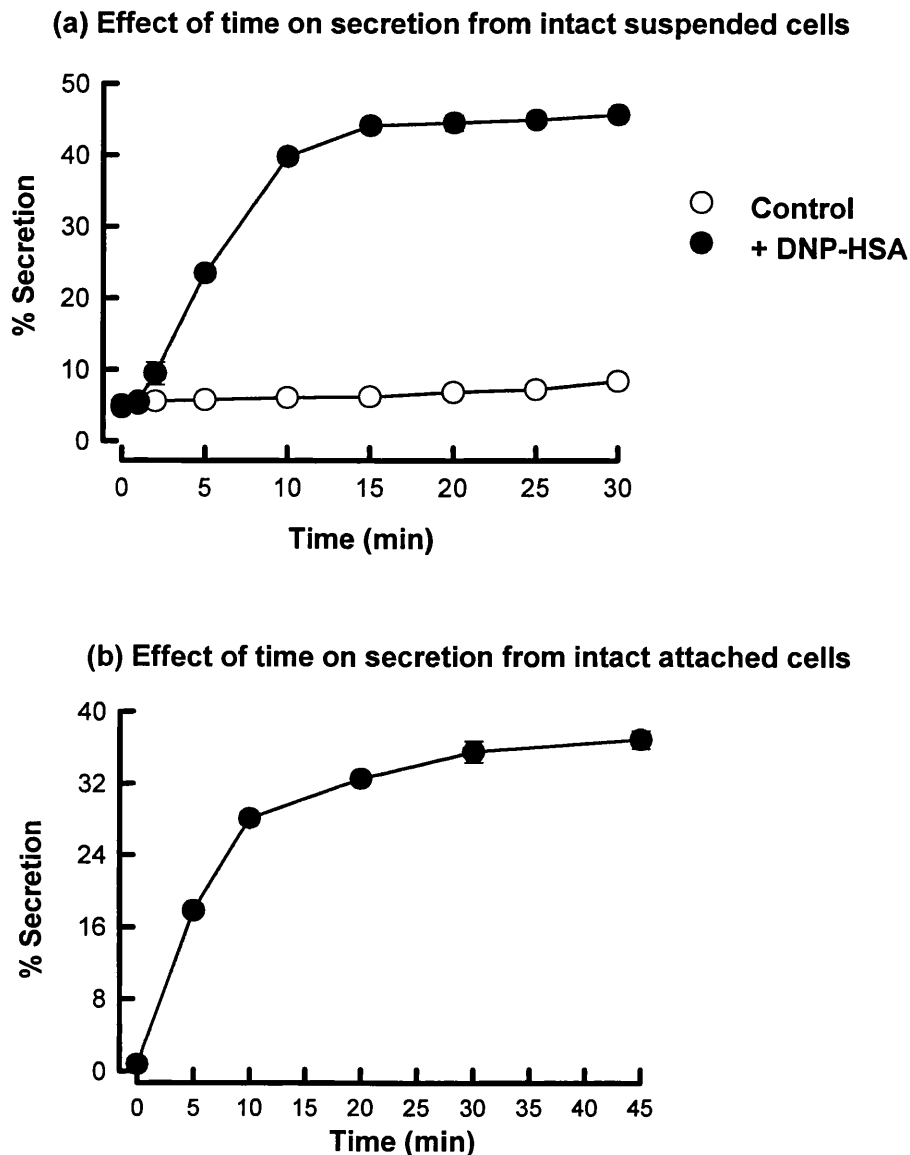
Figure 4.2.5. Time course of antigen-stimulated secretion, measured in acutely permeabilised suspended cells or intact attached RBL-2H3 cells

Cells were pretreated with IgEαDNP then assayed in HEPES buffer (pH 7.2) modified for use with intact cells (see section 2.3.12), in the absence (open symbols) and presence (closed symbols) of 40ng/ml DNP-HSA. Aliquots were removed at the stated time points. The standard acutely permeabilised protocol was used for graph (a) (see section 2.3.3), for graph (b) a modified protocol was used (see section 2.3.12). Secretion was measured as described in section 2.4.

(a) Effect of DNP-HSA concentration on PLD activity in intact RBL cells

(b) Effect of DNP-HSA concentration on PLD activity from acutely permeabilised cells
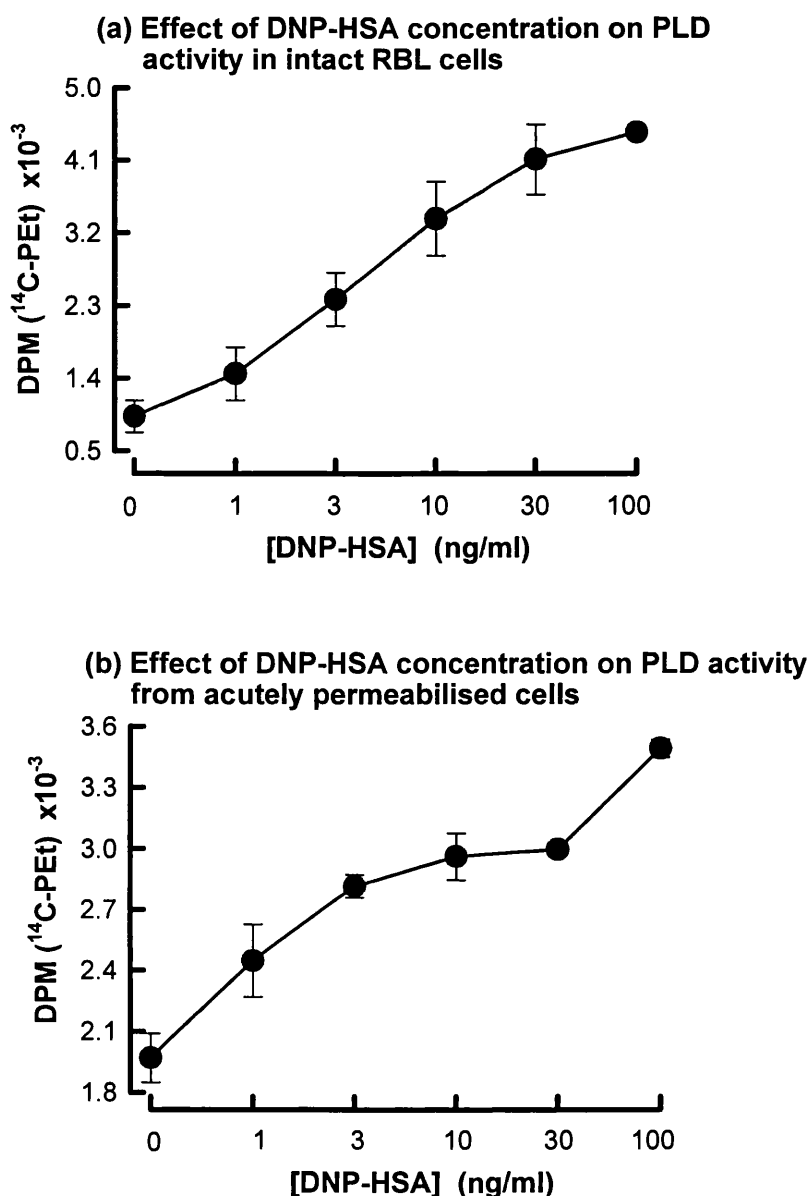
Figure 4.3. **Effect of DNP-HSA concentration on antigen-stimulated PLD activity in intact and acutely permeabilised RBL-2H3 cells.**

All cells were pretreated with IgEαDNP before use. Graph(a): Cells assayed in HEPES (pH 7.2) for intact cells, with rising concentrations of DNP-HSA. The standard intact cell protocol (see section 2.3.3) was modified to assay PLD activity via PEt formation (see section 2.6). Graph (b) Cells assayed using modified acutely permeabilised cell protocol in the presence of either 10μM or 10nM Ca2+ buffered by 3mM EGTA and rising concentrations of DNP-HSA.

(a) Effect of permeabilisation time on secretion

(b) Effect of permeabilisation time on PLD activity
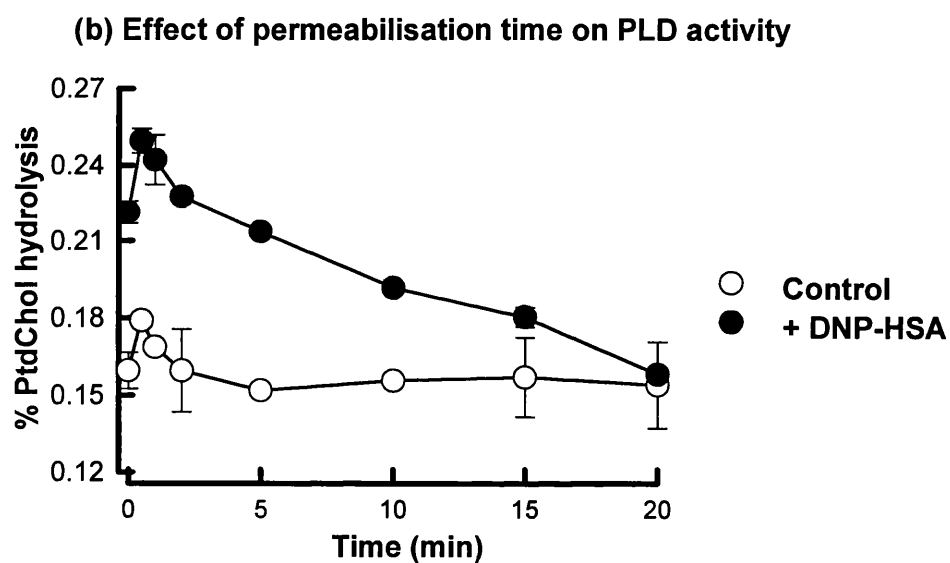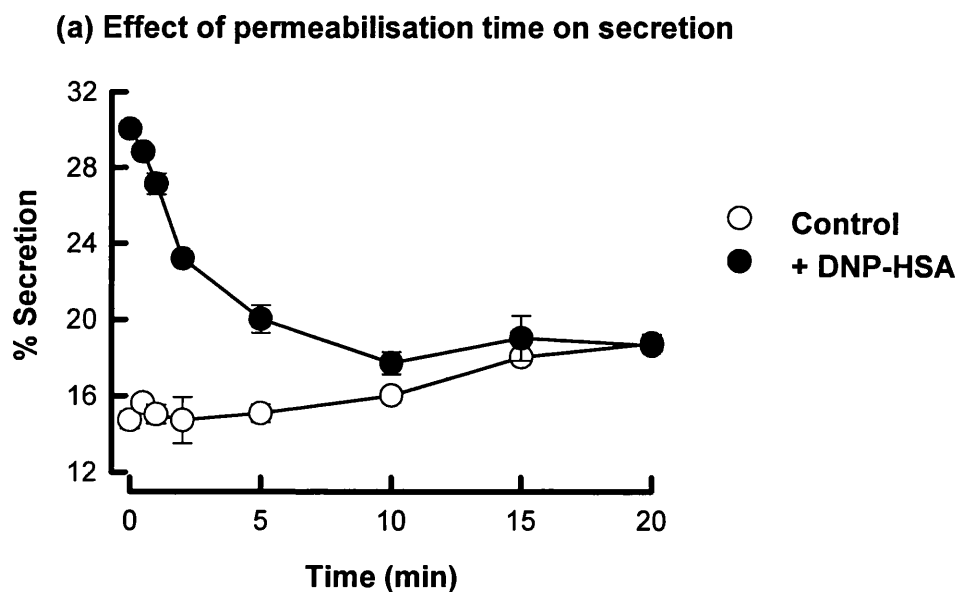
Figure 4.4. Effect of permeabilisation time on antigen-stimulated secretion and PLD activity

Cells were pretreated with IgEαDNP then assayed in HEPES buffer (pH7.2), in the presence (closed symbols) and absence (open symbols) of 40ng/ml DNP-HSA. All assays include 1mM MgATP, 2mM $MgCl_2$ and 10μM Ca^{2+} buffered b 3mM EGTA. Samples were assayed after various permeabilisation times (see section 2.3.4). Choline release and secretion were measured from the same samples, as described in section 2.5.
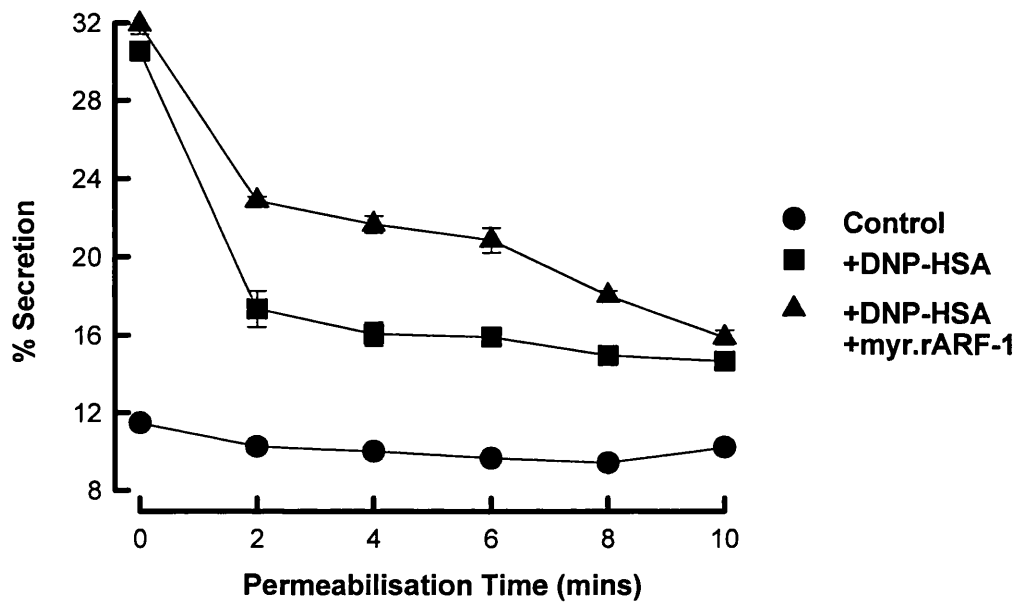
Figure 4.5.1. Effect of permeabilisation time on myr.rARF-1 reconstituted, antigen-stimulated secretion from RBL-2H3 cells

Cells were pre-treated with IgEαDNP, and permeabilised in HEPES buffer (pH 7.2) in the presence of 0.4iu/ml SLO, 1mM MgATP, 2mM $MgCl_2$ and 100nM Ca^{2+}, buffered by 0.3mM EGTA. At stated time-points aliquots were removed and assayed in the presence of 1mM MgATP, 2mM $MgCl_2$ and 10μM Ca^{2+}, buffered by 3mM EGTA (circles), with 40ng/ml DNP-HSA (squares), or 40ng/ml DNP-HSA + 100μg/ml myr.rARF-1 (triangles). Secretion was measured as described in section 2.4.

permeabilisation, myr.rARF-1 was unable to completely restore secretion. Partial restoration by myr.rARF-1 was possible in cells permeabilised for four and for six minutes. However after ten minutes, antigen stimulated secretion was not restored at all by the addition of myr.rARF-1.

Reconstitution of DNP-HSA-stimulated secretion by myr.rARF-1 was completely dependent on calcium (see Fig.4.5.2), and did not increase above background levels at calcium concentrations below $1\mu M$. This dependence was more pronounced than that displayed by acutely permeabilised receptor-stimulated secretion, where some response to antigen-stimulus was seen even in the effective absence of calcium. In contrast to GTPγS-stimulated response, the receptor-driven system was not sensitized to lower calcium concentrations by myr.rARF-1. However the response of this system to MgATP was equivalent to the response noted in the GTPγS-stimulated system (see Fig.4.5.3). In the absence of added MgATP RBL-2H3 cells did not respond significantly to the addition of either myr.rARF-1 or DNP-HSA. In the presence of MgATP, the response to antigen-stimulation was increased significantly, a response that was strongly enhanced by myr.rARF-1.

As shown in Fig.4.3, antigen-stimulated PLD activity was a small response, even before cytosol depletion. Thus it was necessary to examine reconstitution with myr.rARF-1 in the presence of 1% ethanol in order to exploit PEt formation in a more sensitive assay. In cytosol-depleted cells, stimulation of PLD activity by the MgATP and calcium included in the assay produced a significant background level, even in the absence DNP-HSA or myr.rARF-1. As shown in Fig. 4.5.4, there was a small response to antigen, and PLD activity was increased in response to myr.rARF-1 alone. This response, though significant may appear exaggerated because of the low level of PLD activity in these cells. When the cells were stimulated by DNP-HSA in the presence of myr.rARF-1, a clear reconstitution effect was measured.

Figure 4.5.2. Effect of calcium concentration on myr.rARF-1 reconstituted, antigen-stimulated secretion from RBL-2H3 cells

Cells were pre-treated with IgEαDNP, and permeabilised for 5mins in HEPES buffer (pH 7.2) (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 1mM MgATP and 2mM $MgCl_2$ with rising concentrations of Ca^{2+} (buffered by 3mM EGTA). Cells were assayed in the presence (closed symbols) or absence (open symbols) of 40ng/ml DNP-HSA, and the presence (triangles) or absence (circles) of 100μg/ml myr.rARF-1. Secretion was measured as described in section 2.4.
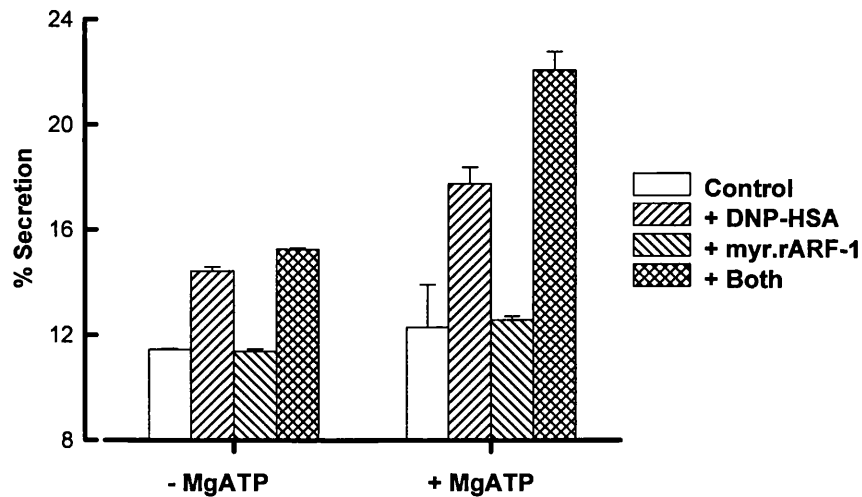
Figure 4.5.3. Effect of MgATP on myr.rARF-1 reconstituted, antigen-stimulated secretion from RBL-2H3 cells

Cells were pre-treated with IgEαDNP and metabolically inhibited before use (see section 2.3.6). Cells were permeabilised for 5mins in HEPES buffer (pH 7.2) (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 2mM $MgCl_2$ and 10μM Ca^{2+} (buffered by 3mM EGTA). Assays were carried out in the presence or absence of 40ng/ml DNP-HSA, 100μg/ml myr.rARF-1 and 1mM MgATP as indicated. Secretion was measured as described in section 2.4.
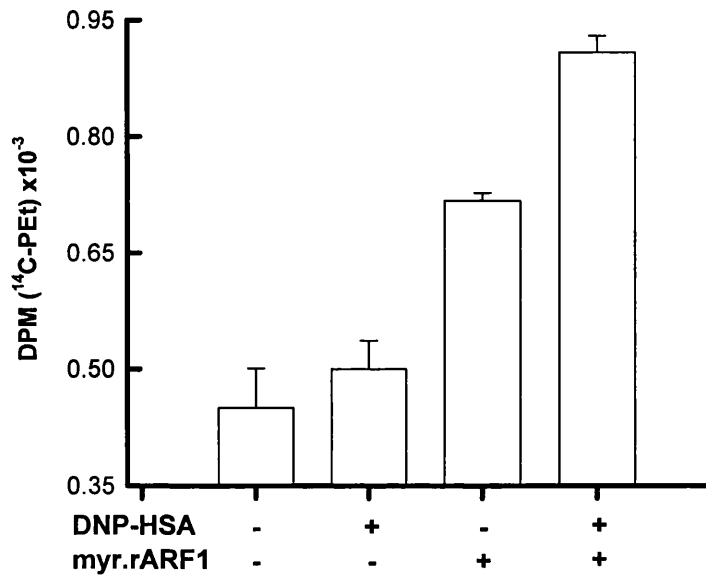
Figure 4.5.4. Reconstitution of antigen-stimulated Phospholipase D activity with myr.rARF-1

[^{14}C]-sodium acetate-labelled cells were pre-treated with IgEαDNP and permeabilised for 5mins in HEPES buffer (pH 7.2) (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 1mM MgATP, 2mM MgCl$_2$ and 10μM Ca^{2+} (buffered by 3mM EGTA). Cells assayed in the presence or absence of 40ng/ml DNP-HSA and 100μg/ml myr.rARF-1 as indicated. PEt formation was measured as described in section 2.6.

4.6 Reconstitution of secretion with rPI-TPα

The reconstitution of antigen-stimulated secretion by rPI-TPα was only evident at rPI-TPα concentrations above 3μg/ml. As shown in Fig.4.6.1, this response was saturated at concentrations above 30μg/ml. Therefore the receptor-activated system was sensitive to the same range of rPI-TPα concentrations as that activated by GTPγS. The reconstitution was dependent on calcium concentrations above 1μM, a dependence similar to that displayed by the myr.rARF-1-reconstituted responses. Cytosol depletion increased the dependence of receptor-stimulated secretion on calcium, as shown in Fig.4.6.2, either in the presence or absence of rPI-TPα, DNP-HSA-stimulated secretion increased above background levels only in the presence of calcium concentrations above 1μM. Thus receptor-activated rPI-TPα-reconstitution required ten times more calcium than the analogous GTPγS-stimulated response.

This response was also dependent on added MgATP. Metabolic inhibition prior to permeabilisation compromised the secretory response to DNP-HSA stimulation, whether the cells were provided with rPI-TPα or not. MgATP did not increase secretion alone, however when rPI-TPα was added to DNP-HSA-stimulated cells, the effect of adding 1mM MgATP was significant: in the typical experiment shown in Fig.4.6.3, 22% secretion was measured under these conditions, in comparison with 11% measured in the absence of added MgATP.

4.7 Reconstitution of secretion with rPI-TPβ

As shown in Fig.4.7.1, rPI-TPβ was effective at reconstituting antigen-stimulated secretion to a similar extent to myr.rARF-1 and rPI-TPα. The effect of adding combinations of recombinant proteins was examined: all combinations of the reconstituting proteins caused greater reconstitution than any used separately. However, even when all three proteins were used together, the maximal secretory response to DNP-HSA measured in acutely permeabilised cells was not recovered. In the typical experiment shown, the
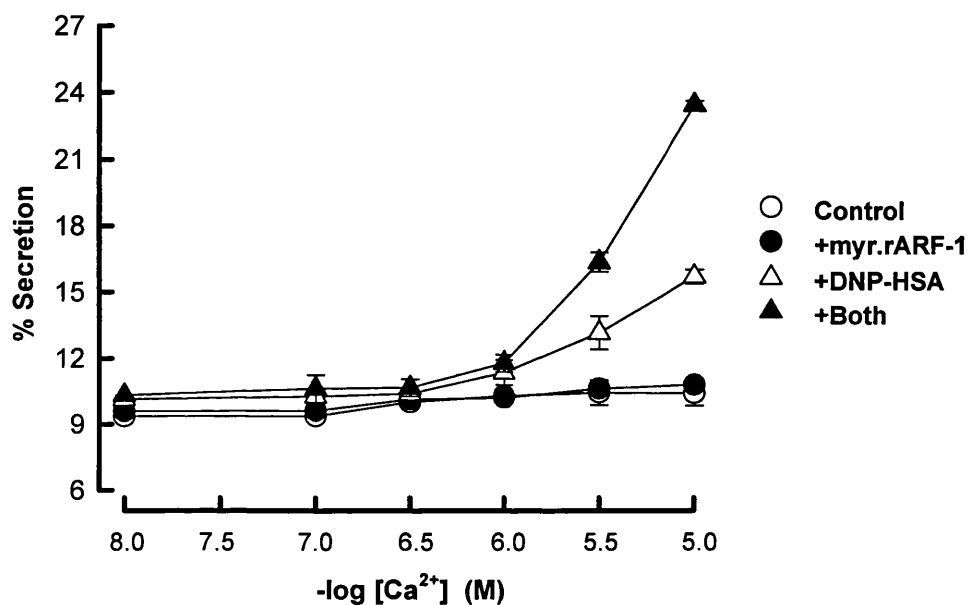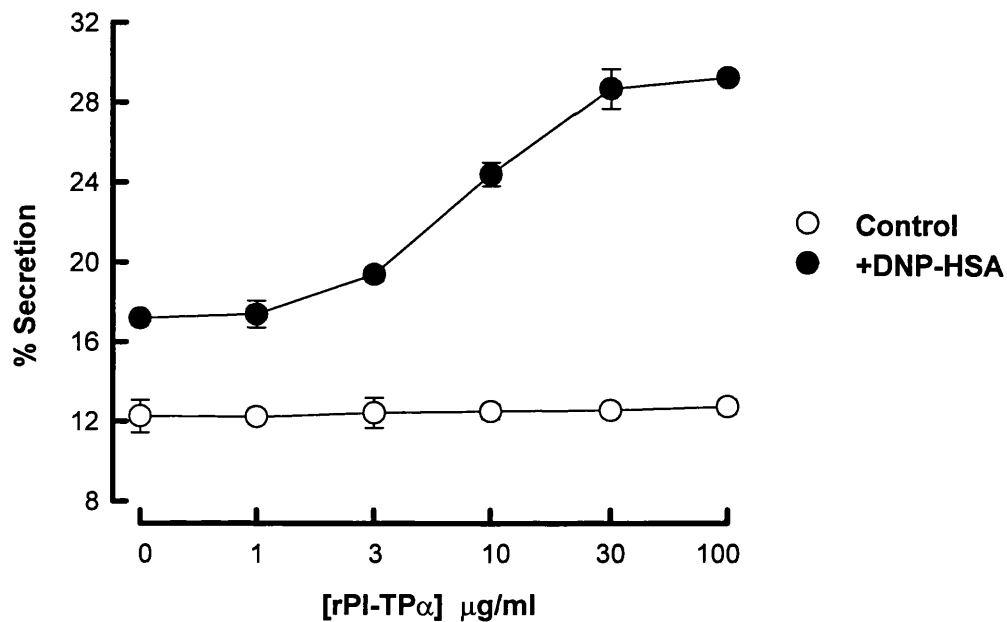
Figure 4.6.1. Effect of rPI-TPα concentration on reconstituted, antigen-stimulated secretion from RBL-2H3 cells

Cells were pre-treated with IgEαDNP, and permeabilised for 5mins in HEPES buffer (pH 7.2) (as described in section 2.3.5). Permeabilised cells were assayed with 1mM MgATP, 2mM MgCl$_2$ and 10μM Ca^{2+} (buffered by 3mM EGTA), in the presence (closed symbols) or absence (open symbols) of 40ng/ml DNP-HSA. Assays contained rising concentrations of rPI-TPα, as indicated. Secretion was measured as described in section 2.4.
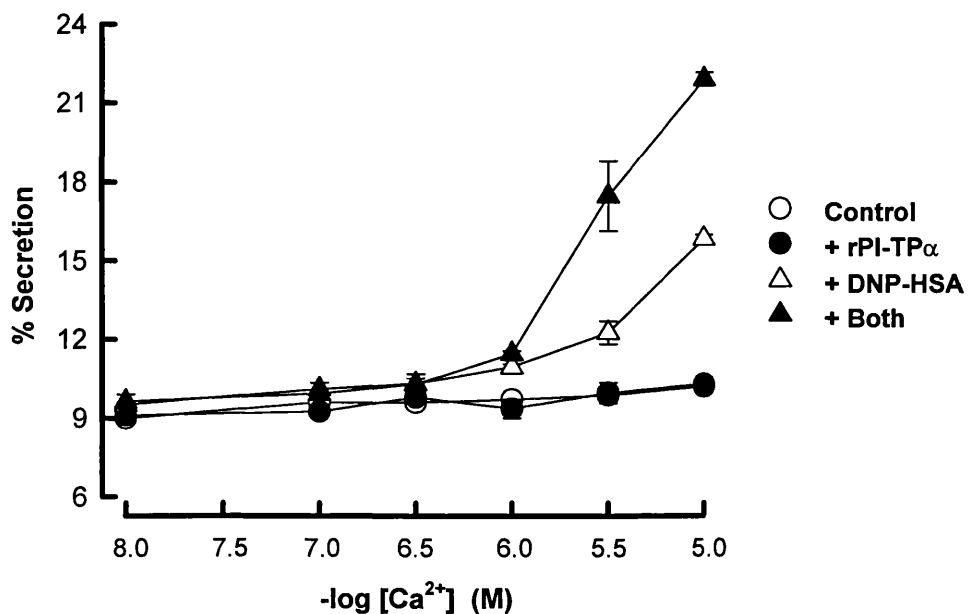
Figure 4.6.2. Effect of calcium concentration on rPI-TPα reconstituted, antigen-stimulated secretion from RBL-2H3 cells

Cells were pre-treated with IgEαDNP, and permeabilised for 5mins in HEPES buffer (pH 7.2) (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 1mM MgATP and 2mM $MgCl_2$ with rising concentrations of Ca^{2+} (buffered by 3mM EGTA). Cells assayed in the presence (closed symbols) or absence (open symbols) of 40ng/ml DNP-HSA, and the presence (triangles) or absence (circles) of 50µg/ml rPI-TPα. Secretion was measured as described in section 2.4.
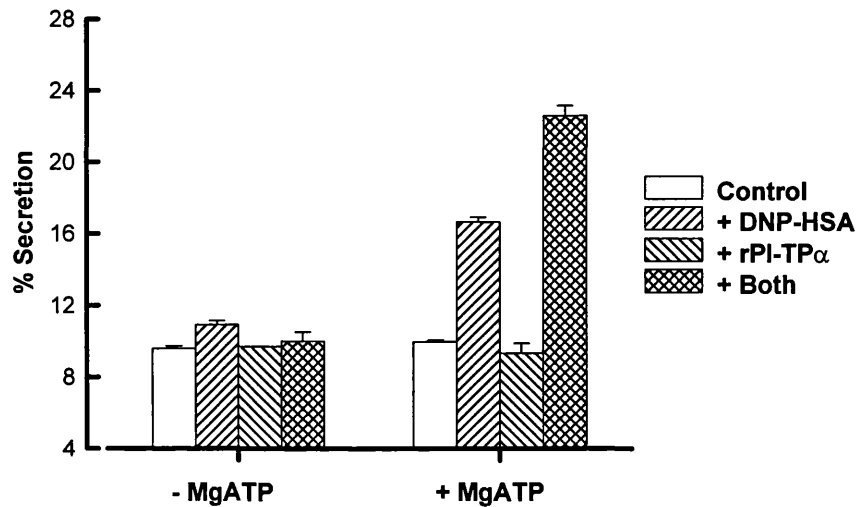
Figure 4.6.3. **Effect of MgATP on rPI-TPα reconstituted, antigen-stimulated secretion from RBL-2H3 cells**

Cells were pre-treated with IgEαDNP and metabolically inhibited before use (see section 2.3.6). Cells were permeabilised for 5mins in HEPES buffer (pH 7.2) (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 2mM $MgCl_2$ and 10μM Ca^{2+} (buffered by 3mM EGTA). Assays carried out in the presence or absence of 40ng/ml DNP, 50μg/ml rPI-TPα and 1mM MgATP as indicated. Secretion was measured as described in section 2.4.
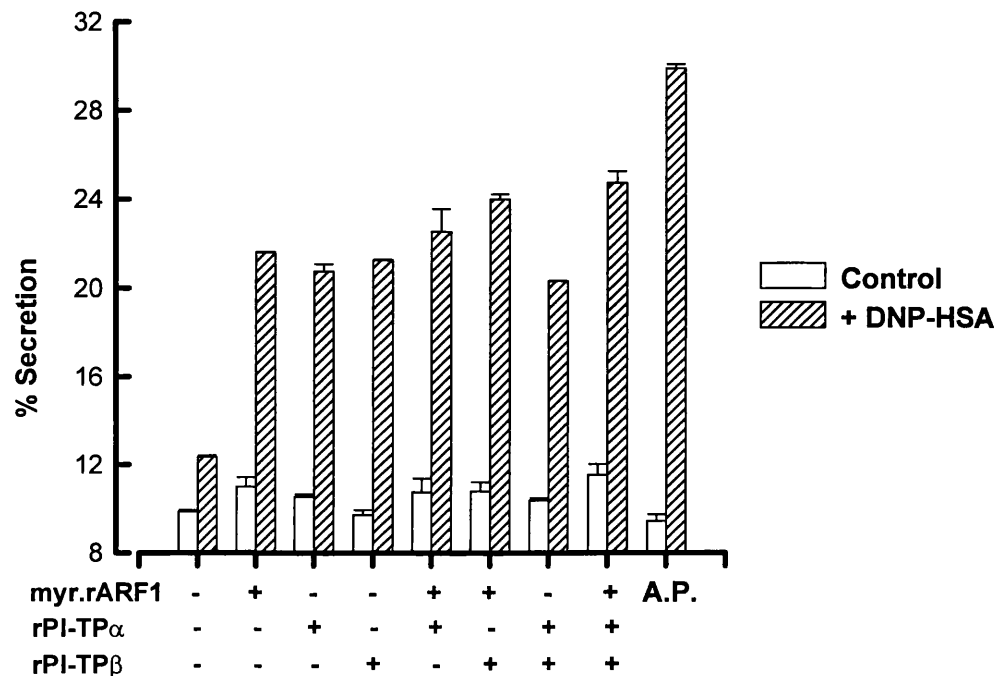
Figure 4.7.1. Effect of rPl-TPα, rPl-TPβ and myr.rARF-1 as reconstitutors of antigen-stimulated secretion in RBL-2H3 cells

Cells were pre-treated with IgEαDNP, and permeabilised for 5mins in HEPES buffer (pH 7.2), with an aliquot of cells retained for measurement of the A.P. (acutely permeabilised) cell responses (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 1mM MgATP, 2mM MgCl$_2$ and 10μM Ca^{2+} (buffered by 3mM EGTA). Assays carried out in the presence or absence of 40ng/ml DNP-HSA, 50μg/ml myr.rARF-1, 50μg/ml rPl-TPα and 50μg/ml rPl-TPβ as indicated. Secretion was measured as described in section 2.4.

maximal reconstituted response was 25%, while the acutely permeabilised response was 31%.

rPI-TPβ was an effective reconstituting agent at concentrations above 3μg/ml, maximal at 30μg/ml. No increase was noted at rPI-TPβ concentrations above 30μg/ml, therefore in the receptor-activated system, as in the GTPγS-stimulated system, rPI-TPβ was active over the same range of concentrations as rPI-TPα. This reconstitution was dependent on MgATP; cytosol-depleted cells secreted at low levels in the absence of added MgATP. MgATP did not increase secretion alone, but potentiated the effects of calcium, DNP-HSA and rPI-TPβ. When MgATP, calcium and DNP-HSA are all added together - in the typical experiment shown in Fig.4.7.3 secretion measured in the presence of rPI-TPβ was 23%, an increase of 12% above levels measured in the absence of added MgATP.

4.8 Discussion

Experiments in this chapter demonstrate that antigen-stimulated secretion and PLD activity may be measured in permeabilised cells. Thus permeabilisation itself does not compromise the reactivity of the cells to their physiological stimulus. The permeabilised cell assay has allowed the effects of calcium and MgATP on the antigen-driven responses to be examined. The sensitivity of this antigen-stimulated response to calcium is different to that displayed by the GTPγS-stimulated response - with antigen-stimulated secretion dependent on a higher concentration of calcium. In mast cells it has been hypothesised that GTPγS and GTP may enhance the secretory response's sensitivity to calcium (195). The nature of GTPγS as a stimulus, in that it permanently activates GTP-binding proteins, means that GTPγS may produce an "exaggerated" version of the GTP response. It is possible that this could explain the increased sensitivity of GTPγS-stimulated secretion to lower concentrations of calcium.

The importance of cytosolic proteins in the secretory response is clear from the decline in responsiveness that results from the loss of cytosol from the cells.
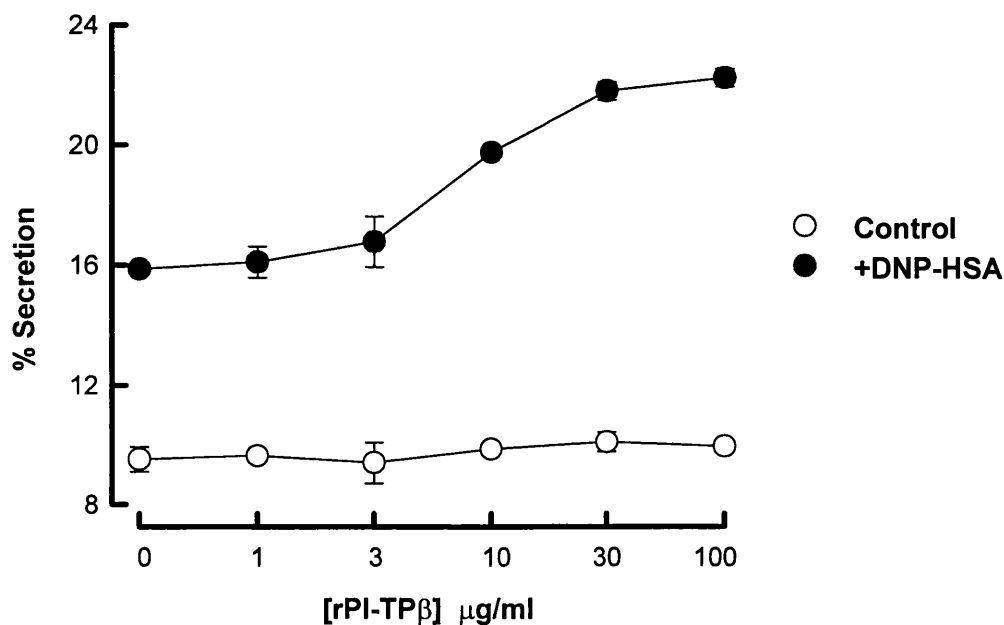
Figure 4.7.2. Effect of rPI-TPβ concentration on reconstituted, antigen-stimulated secretion from RBL-2H3 cells

Cells were pre-treated with IgEαDNP, and permeabilised for 5mins in HEPES buffer (pH 7.2) (as described in section 2.3.5). Permeabilised cells were assayed with 1mM MgATP, 2mM $MgCl_2$ and 10μM Ca^{2+} (buffered by 3mM EGTA), in the presence (closed symbols) or absence (open symbols) of 40ng/ml DNP-HSA. Assays contained rising concentrations of rPI-TPβ, as indicated. Secretion was measured as described in section 2.4.
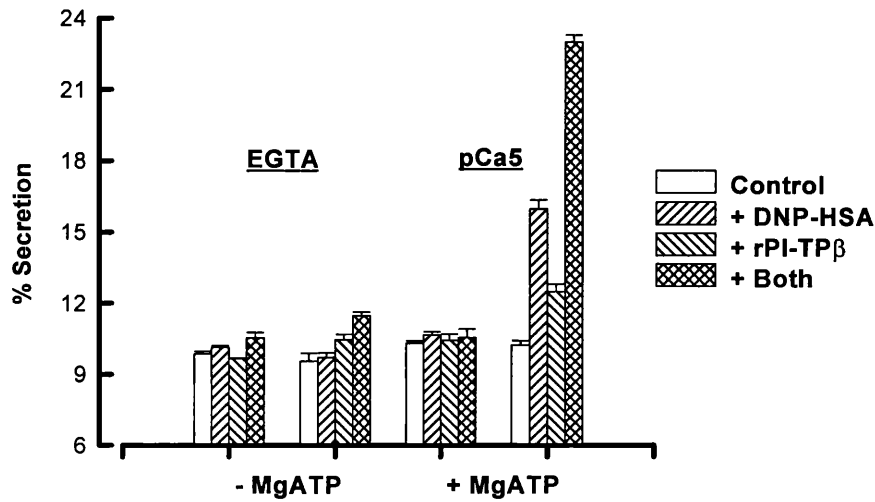
Figure 4.7.3. Effect of calcium and MgATP on rPI-TPβ reconstituted, antigen-stimulated secretion from RBL-2H3 cells

Cells were pre-treated with IgEαDNP and metabolically inhibited before use (see section 2.3.6). Cells were permeabilised for 5mins in HEPES buffer (pH 7.2) (as described in section 2.3.5). Permeabilised cells were assayed with 2mM $MgCl_2$, in the presence or absence of 40ng/ml DNP, 50μg/ml rPI-TPβ, 1mM MgATP and 3mM EGTA +/- 10μM Ca^{2+} as indicated. Secretion was measured as described in section 2.4.

The decline in antigen-stimulated secretion and PLD is more rapid than the decline in the GTPγS-stimulated responses, suggesting that an essential component of the antigen-stimulated pathways leaks from the cells rapidly. This decline in responsiveness correlates well with the leakage of ARF-1, PI-TPα and PI-TPβ described in the previous chapter. Data presented in this chapter demonstrates the ability of each of these proteins to partially restore responsiveness to the antigen-stimulated cells.

The correlation between myr.rARF-1-reconstituted secretion and PLD activity may not be proven by the data described in this chapter. Receptor-stimulated PLD activity is a modest response even in intact cells. In cells which have been permeabilised and partially cytosol-depleted the response is extremely "fragile". The response is sufficiently robust however to show a decline on permeabilisation, and for reconstitution by myr.rARF-1 to be demonstrated. Thus PLD activated via the receptor, is regulated by myr.rARF-1, just as GTPγS-stimulated PLD activity is. The importance of this result is that myr.rARF-1 may now be regarded as a component of the physiological cascade that leads to PLD activity *in vivo*. Other experiments described in this chapter demonstrate that myr.rARF-1 reconstitutes receptor-stimulated secretion from RBL-2H3 cells also. These experiments together indicate that myr.rARF-1-regulated PLD is of physiological importance in secretion from these cells.

The experiments in the later sections of this chapter also demonstrate that rPI-TPα and rPI-TPβ participate in the receptor-activated secretory response. Signalling via PLC in antigen-stimulated cells has already been demonstrated to be dependent on rPI-TPα and rPI-TPβ (and sec14) (245). The translocation and activation of PLC-γ by the FcεR1 receptor has been demonstrated, and PI-TP is known to modulate the activity of all the PLC's regardless of type (see chapter 1). Therefore some mechanism for the previous result could be predicted. The mechanism underlying the ability of rPI-TPα and rPI-TPβ to participate in the secretory cascade is not as clear. Just as in the GTPγS-stimulated secretory response however, rPI-TPα- and rPI-TPβ-reconstituted antigen-stimulated secretion is clearly dependent on calcium and MgATP.

The pattern of dependence of each of the reconstituted systems on MgATP and calcium is similar to that displayed by the responses measured in acutely permeabilised cells. However these responses - whether reconstituted by myr.rARF-1 or either of the rPI-TP's, appear to be far more dependent on the presence of high concentrations of calcium than the analogous GTPγS-stimulated responses. It is possible that this reflects the loss of a calcium-sensing component of the exocytic machinery, that can be directly activated by or sensitized in the presence of GTPγS. This highlights the different characteristics of GTPγS-stimulated reconstitution systems, and antigen-stimulated reconstitution; a difference also highlighted by the extreme dependence on permeabilisation time exhibited by myr.rARF-1-reconstituted secretion. The failure of myr.rARF-1 to reconstitute antigen-stimulated secretion from cells permeabilised for longer than ten minutes contrasts with the ability of myr.rARF-1 to reconstitute GTPγS-stimulated secretion under the same conditions.

The success of the GTPγS-stimulated response indicates that the down-stream exocytic machinery is still intact. Therefore the failure of the antigen-stimulated response is presumably due to the absence of some component of the up-stream signal transduction machinery. That GTPγS can still activate this system indicates the presence and functional importance of a GTP-binding protein in this machinery. Such a putative protein could be activated directly by GTPγS. In the presence of antigen, in five-minute permeabilised cells this protein is no longer activated. This is either because the protein itself may have leaked from cells, or another protein further upstream has leaked - or is otherwise unavailable to activate this GTP-binding protein.

Therefore this assay may be used, as in this chapter, to demonstrate the importance of proteins in secretion such as rPI-TPα, rPI-TPβ and myr.rARF-1. Application of this assay does not suggest that these three proteins, or other proteins such as PLD that are associated with them, represent the full exocytic machinery. Application of this assay instead reveals - or at least implies the complexity of this response, and the potential variety of proteins that may be involved in regulating it.

CHAPTER FIVE

THE MECHANISMS OF ARF AND PI-TP EFFECTS ON SECRETION

Chapter 5   The mechanisms of ARF and PI-TP effects on secretion

5.1 Introduction

Experiments described in the previous chapters demonstrate the ability of myr.rARF-1, rPI-TPα and rPI-TPβ to reconstitute secretion from permeabilised cells, and of myr.rARF-1 to reconstitute PLD activity.   These proteins are equally active in their restoration of secretion, and all three are active in both GTPγS- and DNP-HSA-stimulated secretion.

The structure and known biological functions of ARF and the PI-TP's are strikingly different however (see chapter one), therefore it would be difficult to imagine a single activity that could be modulated by all three.   However, previous work carried out in GTPγS-stimulated HL60 cells suggest that these proteins do in fact share a common mechanism in secretion. This mechanism involves the effect of these proteins on the synthesis of PIP_2. Details of this hypothesised mechanism, and the possible implications of increased PIP_2 synthesis are examined in chapter six.

The purpose of the experiments in this chapter is firstly to examine the importance of PLD in secretion, in order possibly to distinguish between the effects of ARF and those of the PI-TP's.   Secondly experiments are described that directly examine the hypothesised function of PI-TPα and myr.rARF-1 in PIP_2 synthesis, in order to test this hypothesis in a new cell-type.   Also the consequences of PIP_2 sequestration and hydrolysis by PLC-δ, on reconstituted exocytosis are demonstrated.

Finally, data are presented that represent the beginnings of an attempt to elucidate the spacial aspects of PI-TPα's role in exocytosis.   It has been proposed that PI-TP is essential because of its ability to potentiate the synthesis of PIP_2. Clearly a lipid second messenger such as PIP_2 must exert its effect(s) in a membrane. Thus it must be considered of critical importance to discover whether PI-TPα's effects on PIP_2 are reflected in global changes in membrane compartments throughout the cell, or whether only precisely defined domains are modified.   If the latter is true, then clearly it would be of great interest to discover the precise nature of these domains.

5.2 Inhibition of secretion by ethanol

As shown in Fig.5.2.1, ethanol inhibited GTPγS-stimulated secretion, however this effect was only evident in the presence of fairly high concentrations of ethanol - between 2% and 5%. At very high concentrations, for example 10% ethanol, "secretion" increased (to 28% in the experiment shown) both in the presence and absence of GTPγS, (data not shown). This increase was thought to be due to lysis - or solubilisation of the cells, resulting in non-specific release of β-hexosaminidase. The lower range of concentrations used here was assumed not to cause this insult to the integrity of the membrane.

3% ethanol also inhibited myr.rARF-1-reconstituted, GTPγS-stimulated secretion. As shown in Fig 5.2.2, 3% ethanol abolished 68% of the increase in secretion due to myr.rARF-1. Thus it was inferred that whatever ARF's role is in secretion, it must participate in a reaction that is vulnerable to ethanol, and that this reaction is likely to involve PLD activity. However, as shown in Fig.5.2.3, in the presence of 3% ethanol, just 20% of the rPI-TPα-reconstituted, GTPγS-stimulated secretory response was inhibited by 3% ethanol. rPI-TPβ-reconstituted, GTPγS-stimulated secretion displayed the same resistance to inhibition by ethanol. In the typical experiment shown in Fig.6.2.4, 3% ethanol abolished 22% of the increase in secretion due to rPI-TPβ. While this experiment does not demonstrate that rPI-TPα and rPI-TPβ participate in the same reaction, it does imply that the PI-TPα- and PI-TPβ-driven steps in exocytosis are unlikely to involve - or be influenced by PLD activity.

5.3 Effect of reconstitution on polyphosphoinositide levels

The method of detection used in this assay measures the amount of PIP_2 synthesised during the course of the experiment. Thus increases in detected ^{32}P-PIP_2 do not indicate absolute increases in amounts of total PIP_2. Instead such an increase indicates that the rate of synthesis during the experiment has exceeded the rate at which newly-synthesised PIP_2 is hydrolysed. The introduction of GTPγS alone into permeabilised cells caused a sharp decline in ^{32}P-PIP_2 levels. This PIP_2 hydrolysis is thought to be catalysed by PLC-β,
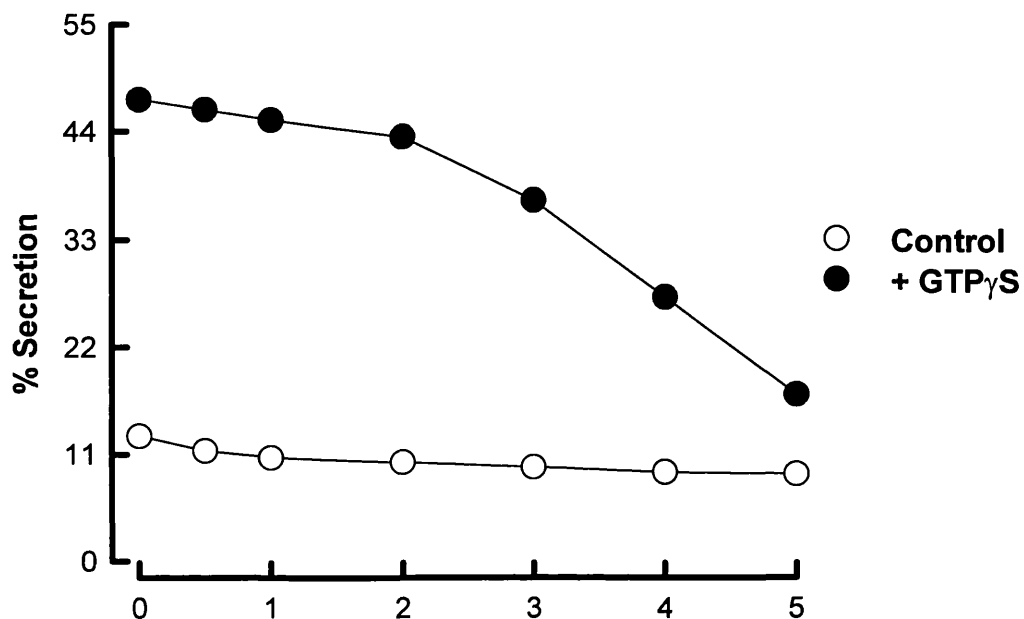
Figure 5.2.1. Effect of ethanol concentration on secretion, measured in acutely permeabilised RBL-2H3 cells

Cells were assayed in PIPES buffer (pH 6.8), in a variety of ethanol concentrations as shown; in the absence (open symbols) and presence (closed symbols) of $10\mu M$ GTPγS. The assay was carried out in the presence of 1mM MgATP, 2mM $MgCl_2$, 0.4 iu/ml SLO and $10\mu M$ calcium, buffered by 3mM EGTA. The standard acutely permeabilised cell protocol was used (see section 2.3.3). Secretion was measured as described in section 2.4.
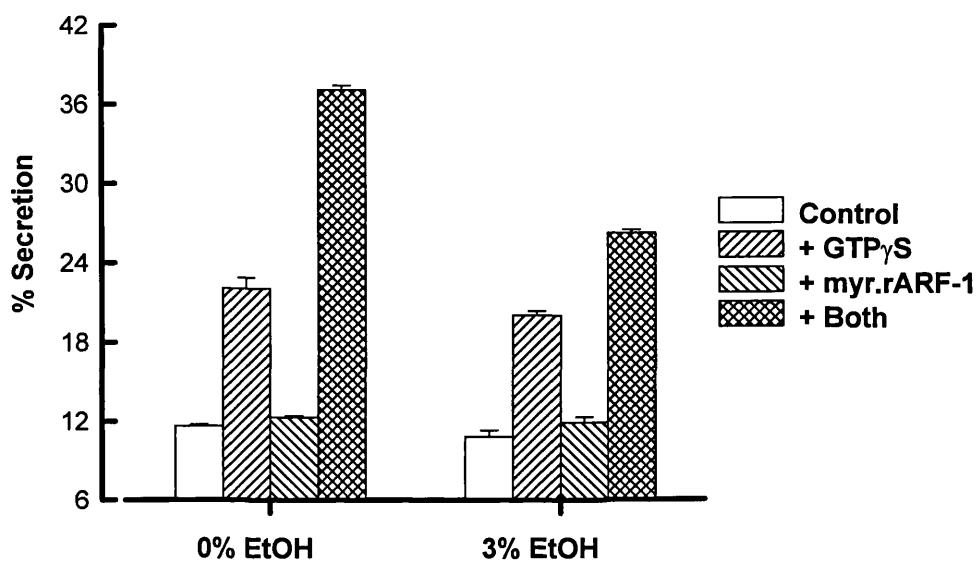
Figure 5.2.2. Effect of ethanol concentration on myr.rARF-1reconstituted, GTPγS-stimulated secretion from RBL-2H3 cells

Cells were permeabilised for 10 mins in PIPES buffer (pH 6.8), (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 1mM MgATP, 2mM $MgCl_2$ and 10μM calcium, buffered by 3mM EGTA. Assays carried out in the presence and absence of 10μM GTPγS, 100μg/ml myr.rARF-1 and 3% ethanol as indicated. Secretion was measured as described in section 2.4.

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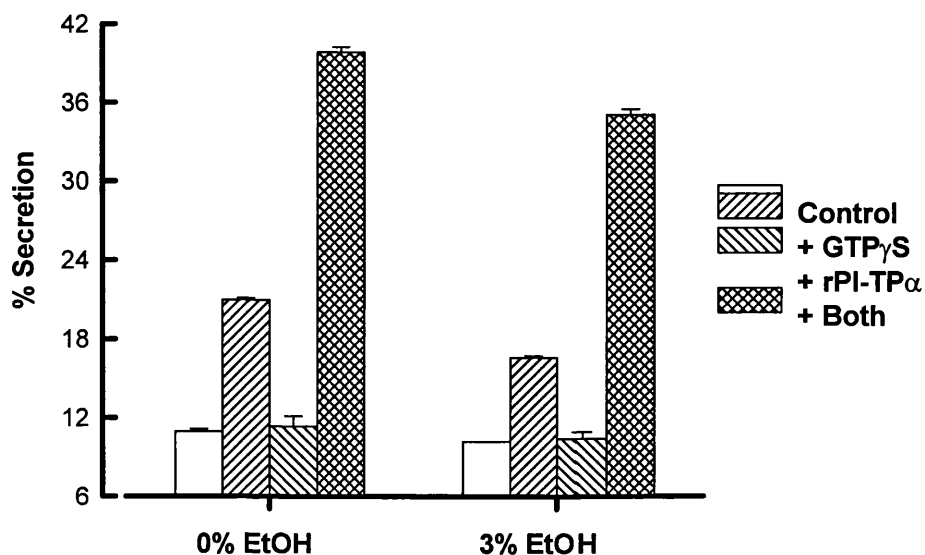
Figure 5.2.3. Effect of ethanol concentration on rPI-TPα reconstituted, GTPγS-stimulated secretion from RBL-2H3 cells

Cells were permeabilised for 10 mins in PIPES buffer (pH 6.8), (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 1mM MgATP, 2mM $MgCl_2$ and 10μM calcium, buffered by 3mM EGTA. Assays carried out in the presence and absence of 10μM GTPγS, 50μg/ml rPI-TPα and 3% ethanol as indicated. Secretion was measured as described in section 2.4.
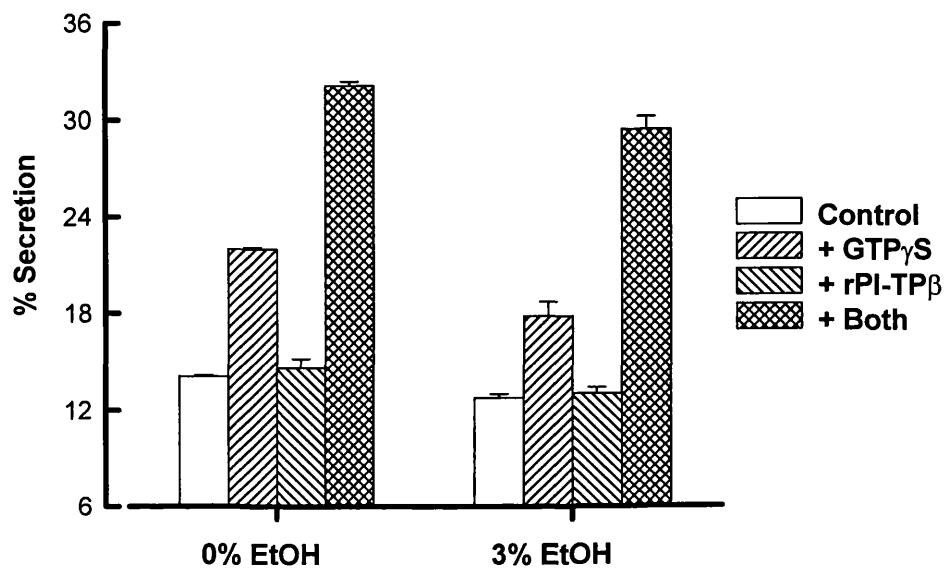
Figure 5.2.4. Effect of ethanol concentration on rPI-TPβ reconstituted, GTPγS-stimulated secretion from RBL-2H3 cells

Cells were permeabilised for 10 mins in PIPES buffer (pH 6.8), (as described in section 2.3.5). Permeabilised cells were assayed in the presence of 1mM MgATP, 2mM $MgCl_2$ and 10μM calcium, buffered by 3mM EGTA. Assays carried out in the presence and absence of 10μM GTPγS, 50μg/ml rPI-TPβ and 3% ethanol as indicated. Secretion was measured as described in section 2.4.

which following ten minutes of permeabilisation was probably still present in the cells. Leakage of PLC-β from HL60 cells occurs only after 15 mins of permeabilisation (284), and this is assumed to true also in RBL-2H3 cells. As shown in Fig.5.3.1, myr.rARF-1 caused an increase in ^{32}P-PIP$_2$, and a decrease in ^{32}P-PIP levels. Thus it was assumed that myr.rARF-1 increased PIP$_2$ by specifically driving the conversion of PIP to PIP$_2$. Like myr.rARF-1, rPI-TPα caused an increase in the levels of PIP$_2$. However while the myr.rARF-1 mediated increase in PIP$_2$ was accompanied by a decrease in PIP, rPI-TPα increased levels of both PIP$_2$ and PIP (see Fig.5.3.2). These data suggest that rPI-TPα increases PIP$_2$ synthesis by increasing flux through both PI 4-kinase and PI4P 5-kinase. This supports the idea that myr.rARF-1 and rPI-TPα share a common aim - the synthesis of PIP$_2$, but the two proteins operate via different mechanisms.

The effect of PIP$_2$ hydrolysis by PLC-δ1 on secretion was examined (see Fig.5.3.3). PLC-δ1 hydrolyses PIP$_2$, in the presence of calcium (72). Addition of rPLC-δ1 inhibited secretion in all conditions studied, completely abolishing both myr.rARF-1 and rPI-TPα-reconstituted secretion. Increases in PLC-δ1-catalysed IP$_3$ production confirm that the enzyme hydrolysed PIP$_2$, this hydrolysis increased in the presence of rPI-TPα. The PH domain of PLC-δ1 is known to bind to PIP$_2$ with high affinity (285). Therefore it is possible that PLC-δ1 inhibited secretion by sequestering PIP$_2$ also. Studies using the recombinant PLC-δ1 PH domain to inhibit reconstitution from HL60 cells (161), indicate that at the concentration used in this experiment, sequestration effects are likely to be trivial.

5.4 Subcellular location of ARF and PI-TP following stimulation

This assay was carried out by stimulating intact cells, then permeabilising and allowing cytosolic proteins to leak from the cells. Therefore increased signal in "membrane" extracts, corresponds to retention of the relevant protein inside the cell - i.e. prevention of leakage. As shown in Fig.5.4, both ARF-1 and PI-TPα show increased signal in the "membrane" fraction following stimulation. The proteins were assumed to translocate to some membranous area on

	PIP	PIP$_2$
Control	2800	1913
+ myr.rARF-1	1191	3535
% Change	-43%	+85%
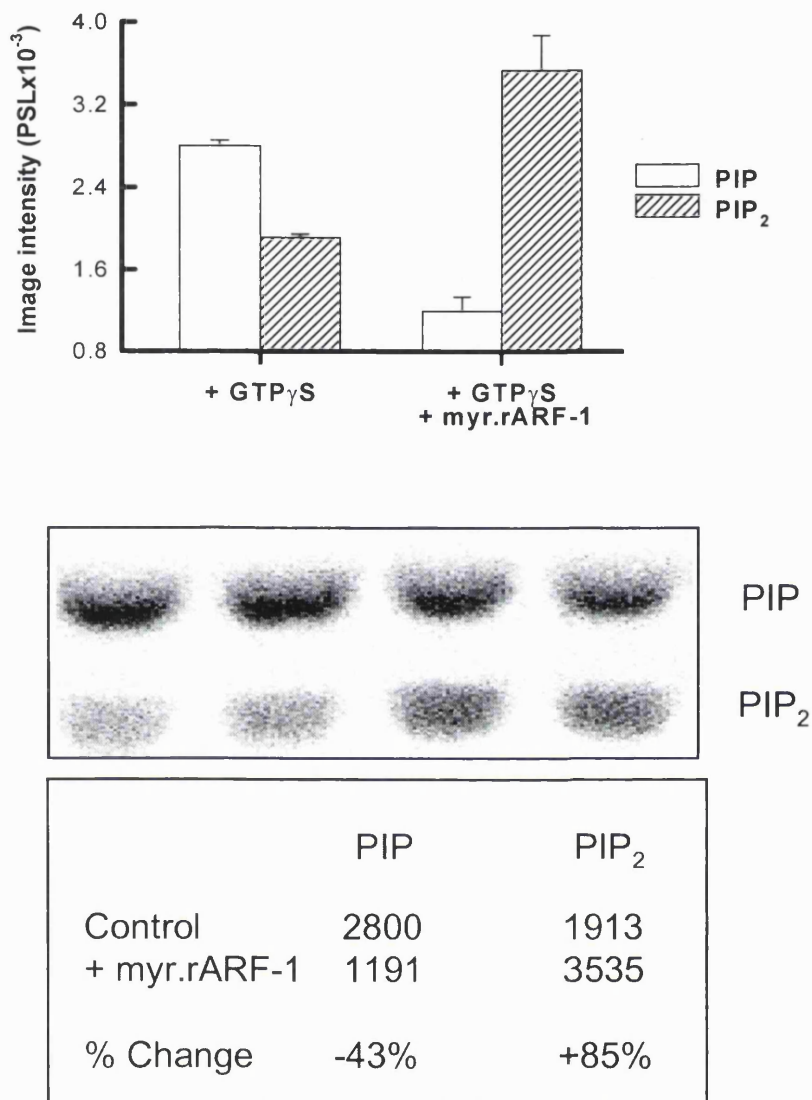
Figure 5.3.1. **Effect of myr.rARF-1 on PIP$_2$ levels in GTPγS-stimulated RBL-2H3 cells**

Cells were permeabilised for 10 mins in PIPES buffer (pH 6.8) as described in section 2.3.5. Immediately before assay, the cells were labelled with 20μCi/ml ^{32}P-ATP. Cells were assayed in the presence of 1mM MgATP, 2mM MgCl$_2$, 10μM GTPγS and 10μM calcium buffered by 3mM EGTA. Assays carried out in the presence and absence of 100μg/ml myr.rARF-1. Lipids were extracted and quantified as described in section 2.10.
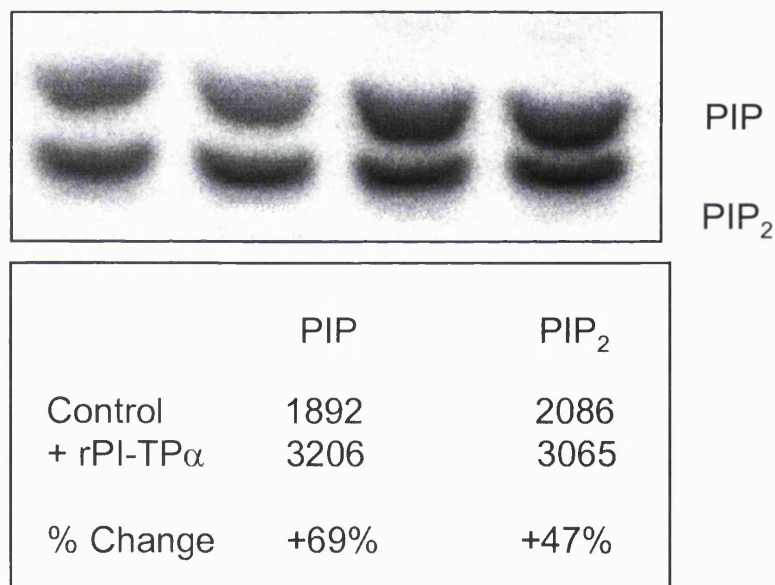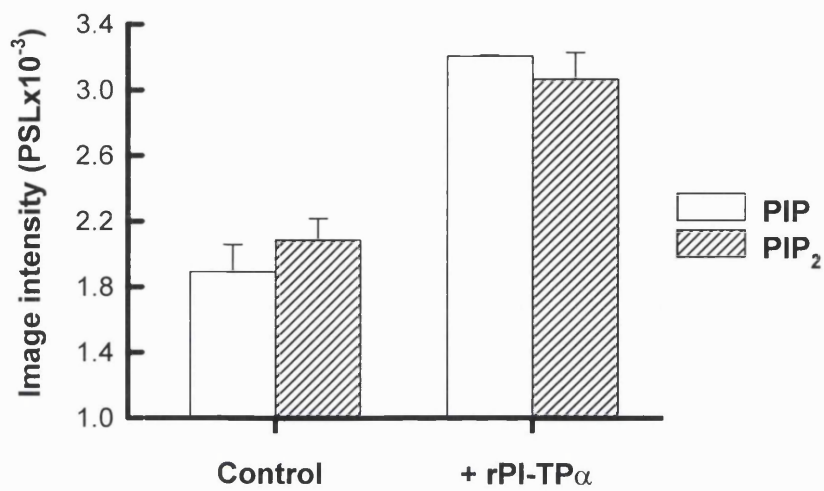
Figure 5.3.2. Effect of rPI-TPα on PIP$_2$ levels in permeabilised RBL-2H3 cells

Cells were permeabilised for 10 mins in PIPES buffer (pH 6.8) as described in section 2.3.5. Immediately before assay, the cells were labelled with 20μCi/ml ^{32}P-ATP. Cells were assayed in the presence of 1mM MgATP, 2mM MgCl$_2$ and 10μM calcium buffered by 3mM EGTA. Assays carried out in the presence and absence of 50μg/ml rPI-TPα. Lipids were extracted and quantified as described in section 2.10.

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(a) Effect of rPLC-δ1 on reconstituted secretion

DNP-HSA	-	+	-	-	+	+
myr.rARF1	-	-	+	-	+	-
rPI-TPα	-	-	-	+	-	+
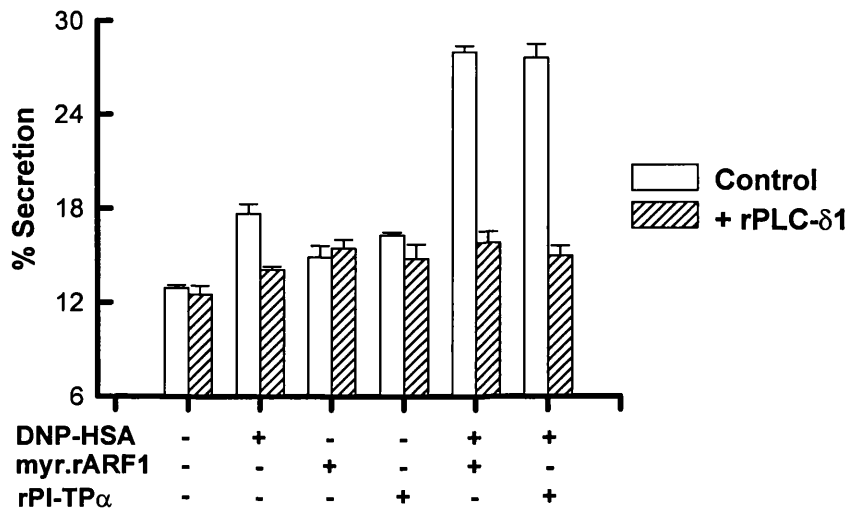
(b) Effect of rPLC-δ1 on reconstituted PLC activity

DNP-HSA	-	+	-	-	+	+
myr.rARF1	-	-	+	-	+	-
rPI-TPα	-	-	-	+	-	+
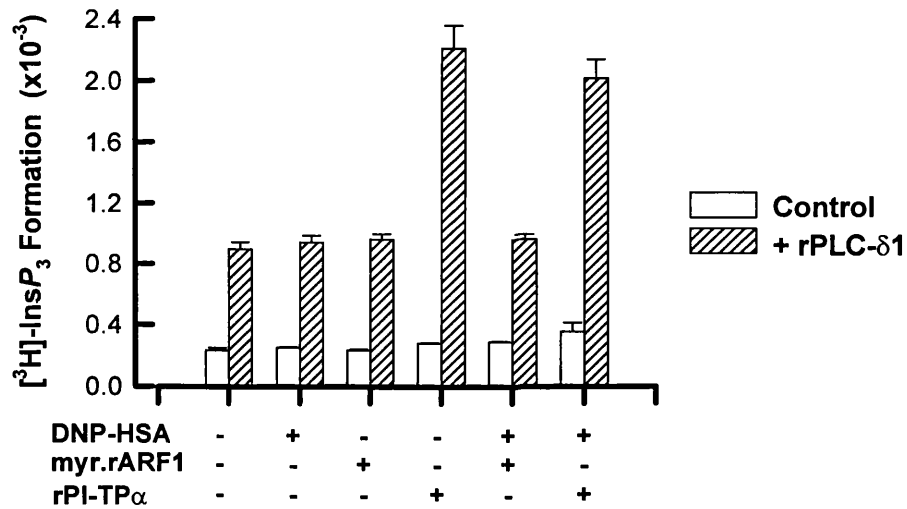
Figure 5.3.3. Effect of rPLC-δ1 on rPI-TPα- and myr.rARF-1-reconstituted antigen-stimulated secretion from RBL-2H3 cells

Cells were permeabilised for 5mins in HEPES buffer (pH 7.2) as described in section 2.3.5. Cells were assayed in the presence of 1mM MgATP, 2mM $MgCl_2$, and 1µM calcium buffered by 3mM EGTA. Assays carried out in the presence and absence of 50µg/ml rPI-TPα, 100 µg/ml myr.rARF-1, 40ng/ml DNP-HSA and 10µg/ml rPLC-δ1. Secretion was measured as described in section 2.4.

stimulation, rendering them unlikely to leak from the permeabilised cells. PI-TPβ displayed a different leakage pattern before and after antigen stimulation. More PI-TPβ was retained in quiescent cells than was free to leak out. Following stimulation, more PI-TPβ leaked than was retained - though still a large proportion was found associated with the cell.

5.5 Time-course of PI-TP translocation following antigen stimulation

(See Fig.5.5). In quiescent cells, PI-TPα was seen throughout the cytosol, and in the nucleus. This pattern was the same as that seen previously in Swiss 3T3 fibroblasts (286). Throughout the experiment, PI-TPα was detected in the cytosol at approximately the same level. This level of staining was not a non-specific effect of the secondary antibody-fluorophore complex, which as seen in the final panel produced only a very low signal. Shortly after stimulation (5mins) the yellow colour - which corresponded to increased signal (larger amounts of specifically detected PI-TPα) was seen at increased levels around the outside of the cell - including the edges of the cells' processes. This shift in staining - corresponding to a translocation of PI-TPα to the cells' cortex increased throughout the time-course of the experiment. Twenty minutes after stimulation, PI-TPα staining formed a complete ring around the outside of the cell.

5.6 Discussion

Data in the previous chapters suggest that PLD activity is important in secretion. This suggestion is made on the basis of the correlation between PLD activity and secretion when concentrations of modulators such as calcium are varied, when cytosolic proteins are depleted from cells, and when myr.rARF-1 is restored to cytosol-depleted cells. These data show that when PLD activity is stimulated - secretion is stimulated also, and when PLD activity is compromised, the secretory response is diminished. This correlation could be strengthened for example if a specific inhibitor of PLD activity abolished secretion. Unfortunately no specific inhibitor has been identified. Ethanol, while not an inhibitor, does partly abolish the effects of PLD activity, by causing PEt to be produced in place of PLD's usual product - PA. Thus if PLD activity is important in secretion, ethanol would be predicted to inhibit
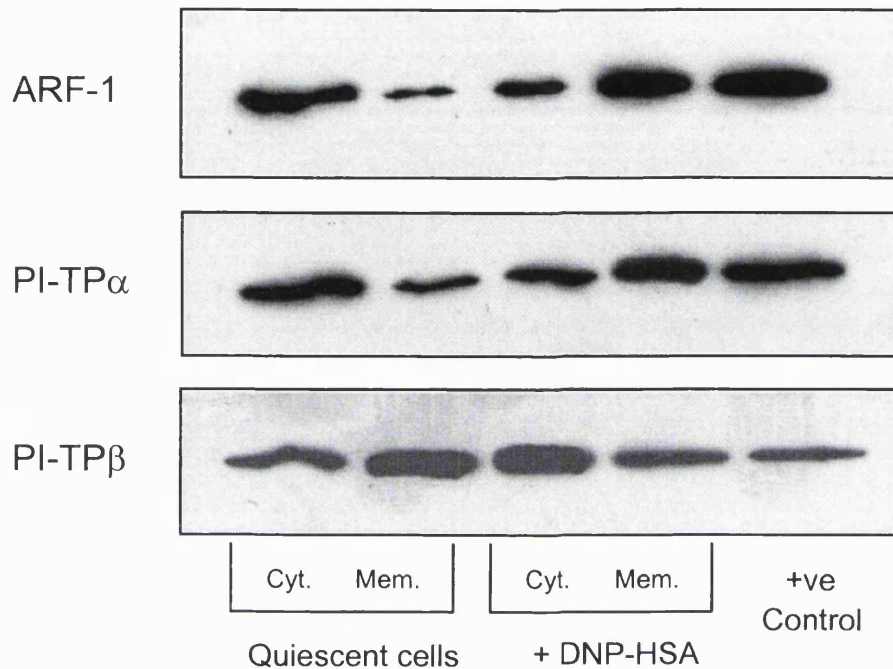
**Figure 5.4 Effect of antigen stimulation on the location of ARF-1, PI-TPα
and PI-TPβ in RBL-2H3 cells**

Two equal samples of cells were pre-treated with IgEαDNP as described in section
2.9.2. One sample was stimulated with 40ng/ml DNP-HSA in HEPES buffer (pH 7.2)
as described in section 2.3.2 for 10mins. A permeabilisation buffer was added to both
samples containing 0.4 iu/ml SLO and 100nM calcium, buffered by 3mM EGTA.
Following 10mins of permeabilisation, cell membranes from both samples were
separated from their leaked cytosolic contents as described in sections 2.9.3/4. The
leaked material and membranes were prepared as samples, and probed by Western
blot with monoclonal antibodies directed against ARF-1, PI-TPα and PI-TPβ as
described in section 2.9.3-8

secretion. In the experiments shown in this chapter, ethanol does indeed inhibit GTPγS-stimulated secretion from acutely-permeabilised cells. The effective concentrations of ethanol are high however, raising the possibility of pleiotropic effects.

However this finding does strengthen the case for PLD's role in secretion. Ethanol inhibition of cytosol-depleted cells strengthens this case even further. Myr.rARF-1-reconstituted secretion is particularly vulnerable to inhibition, when compared to rPI-TPα- or rPI-TPβ-reconstituted secretion. If ARF-1 and PI-TP are essential in secretion, because of their ability to increase PIP_2 synthesis by different mechanisms, this pattern would be expected. ARF-stimulated PLD activity is proposed to exert its effect on PIP_2 synthesis via PA's effect on type 1 PI4P 5-kinase (see chapter 6). Therefore reduced PA production in the presence of ethanol would be predicted to interfere particularly with myr.rARF-1-reconstituted secretion. PI-TPα and PI-TPβ are proposed to increase PIP_2 levels by increasing flux through the PI → PIP → PIP_2 pathway directly, via a cofactor role (see chapter 1). Therefore decreased PA would not be expected to profoundly affect this process - and as seen in section 5.2, it does not.

The proposed model for ARF and PI-TP effects on secretion are also supported by the data in section three of this chapter. Both myr.rARF-1 and rPI-TPα were shown to increase levels of PIP_2. However, examination of PIP levels in the presence of these two reconstituting proteins reveal a difference in the mechanisms used. Myr.rARF-1 caused an increase in PIP_2, but a decrease in PIP. Thus in the presence of myr.rARF-1, the rate of PIP conversion to PIP_2 is increased, suggesting that ARF-stimulated PLD activity stimulates the enzyme responsible for this conversion - PI4P 5-kinase. As stated above, the product of PLD activity - PA, has been shown to stimulate one of this class of enzyme. PI-TPα however, which, it has been suggested acts as an "escort" for PI through its sequential phosphorylation by PI 4-kinase and PI4P 5-kinase, causes an increase in both PIP and PIP_2. This finding supports the suggestion that PI-TP increases PIP_2 production via potentiation of both of these lipid kinases.

Thus data in the first part of this chapter supports the hypothesis regarding ARF's and PI-TP's roles in secretion, that will be presented and discussed in the next chapter.

The proteins studied in this work have been implicated in pathways that involve the modification of lipids. Lipids are clearly not soluble messengers. By their nature, they must exert their effects on membranes. These signalling mechanisms are likely therefore to be highly spatially defined. The work initiated here represents an attempt to fully elucidate this aspect of ARF and PI-TP signalling in exocytosis.

Translocation of ARF from cytosol to Golgi membranes in a PKC-dependent step has been demonstrated previously (287). The data presented here are consistent with this finding - with increased retention of ARF detected in cells following stimulation. This pattern was found for PI-TPα also, suggesting that this protein may be translocated to some membrane on stimulation.

PI-TPβ shows a different pattern of distribution. A large proportion of PI-TPβ is seen to be membrane associated in quiescent RBL-2H3 cells, and in stimulated cells also. However the protein leaks more from stimulated cells than from quiescent cells, suggesting that PI-TPβ is released from a membrane compartment on stimulation, rather than recruited to one. PI-TPβ is found associated with Golgi membranes in quiescent Swiss 3T3 fibroblasts (286). It is possible that this pattern of increased leakage in the presence of stimulus (seen also in HL60 cells (283)) arises from increased translocation from one membrane compartment to another. The increased amounts of PI-TPβ "in transit", that are cytosolic and therefore likely to leak from cells, would produce this pattern.

Finally, the translocation of PI-TPα that is implied by the Western blot (illustrated by Fig.5.4) was examined more closely using immunofluorescence. This technique allows cells to be stimulated by antigen then fixed. These fixed cells can be probed in a manner exactly analogous to Western blotting -

allowing the sub-cellular localisation of proteins to be examined. This process reveals that following antigen stimulation PI-TPα translocates from the cytosol to the cell cortex. It is not thought likely that this translocation is to the plasma membrane itself. However the region that becomes so enriched in PI-TPα is clearly proximal to the plasma membrane. Possible candidates include the cortical actin cytoskeleton, secretory vesicles that have docked but not fused, or possibly endocytic vesicles containing proteins recovered from the plasma membrane. At present however, no information is available to distinguish between these or other possibilities.

Quiescent cells
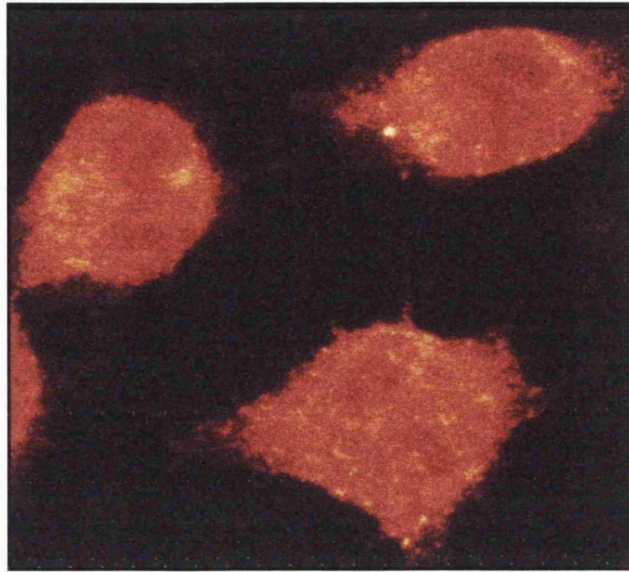
5 mins
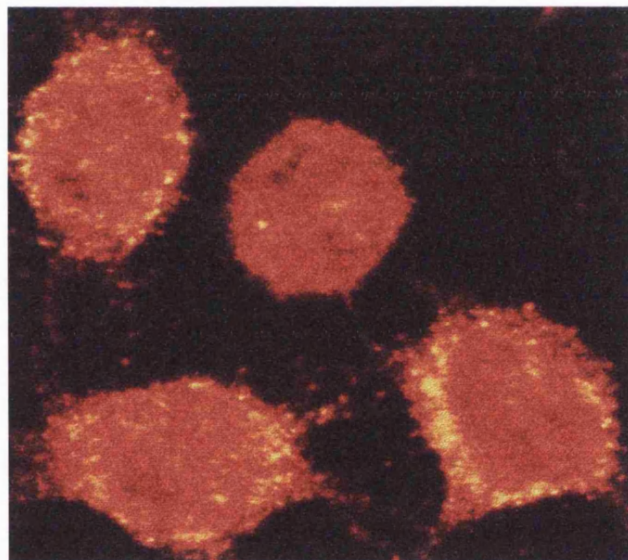
Figure 5.5a Time-course of PI-TPα translocation in RBL-2H3 cells

Cells are grown on glass slides for 48hrs before use and sensitized to DNP-HSA with IgEαDNP as described in section 2.11.1. The attached cells were stimulated with DNP-HSA over an increasing time-course, as indicated. The cells were fixed with paraformaldehyde, and probed using a primary monoclonal antibody directed against PI-TPα and a secondary antibody conjugated to the fluorescent CY3 dye. The slides were mounted using Mowiol as described in section 2.11.2. Images were gathered using a confocal microscope, and visualised using a colour look-up table.

10 mins
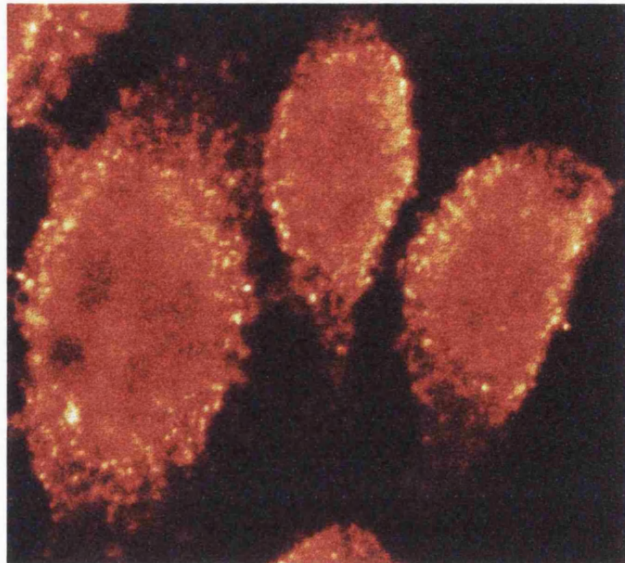
15 mins
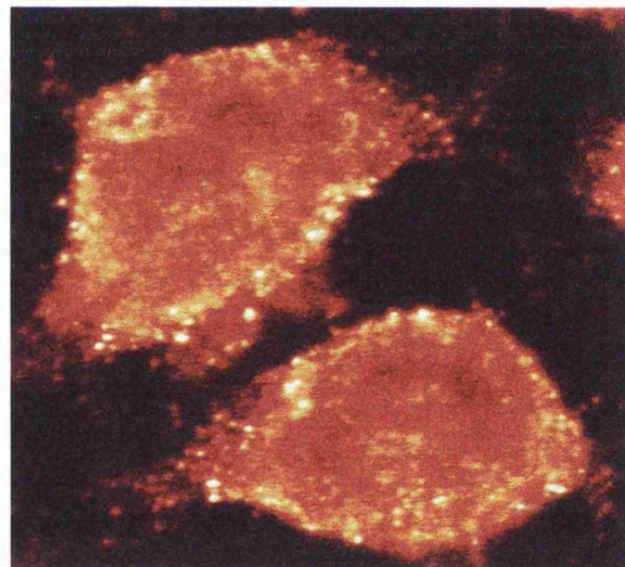
Figure 5.5b Time-course of PI-TPα translocation in RBL-2H3 cells (con.)

20 mins
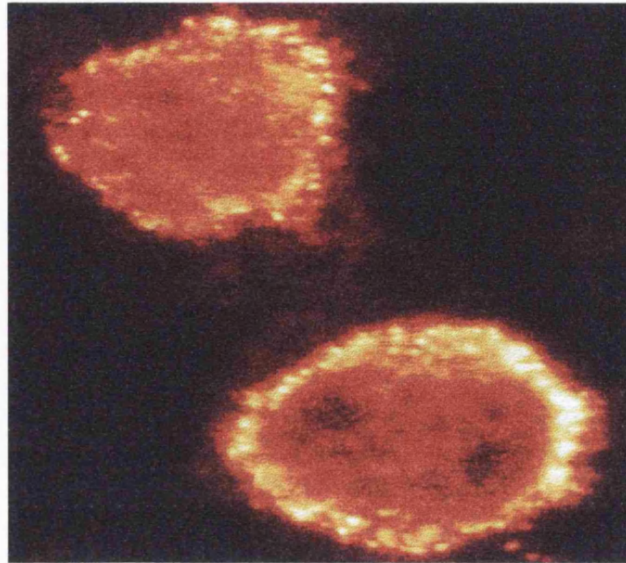
Secondary ab. control
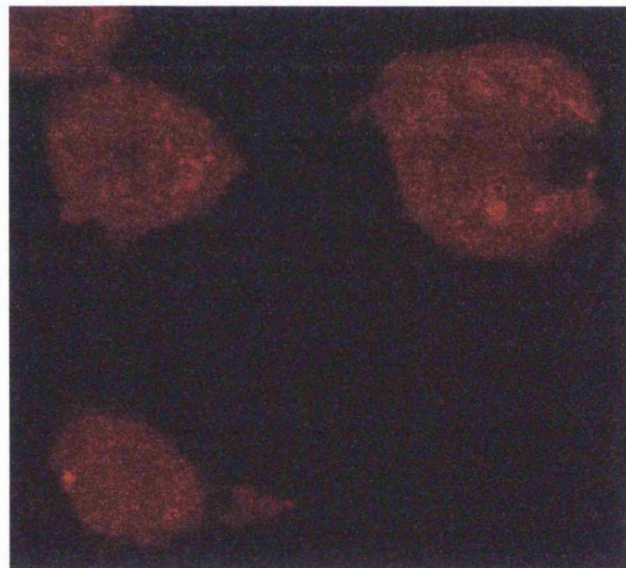
<u>Figure 5.5c</u> Time-course of PI-TPα translocation in RBL-2H3 cells (con)
Cells shown in lower panel were probed only with the secondary antibody-CY3 conjugate, to determine the level of non-specific interaction.

CHAPTER SIX

DISCUSSION

Chapter 6 Discussion

6.1. Introduction

In previous work carried out in differentiated HL60 cells stimulated with fMLP and GTPγS, a close correlation between PLD activity and secretion was noted (281). This correlation is good evidence that PLD activation is linked to the initiation of secretion. Later studies revealed that G-protein stimulated PLD activity was dependent on the presence of cytosolic factor(s) (117). These factors were identified as ARF1/3 (119). Later ARF was established as a potent reconstitution agent, not only of PLD activity but of secretion also (217), thus strengthening further the hypothesis that PLD activity is essential in secretion.

A similar approach was adopted in HL60 cells, to purify a cytosolic factor essential to sustained PLC activity - a protein identified as PI-TP (243). When both isoforms of this protein were introduced into permeabilised cells, they too were found to reconstitute GTPγS-stimulated secretion (161).

The aims of the work presented in this thesis were to ascertain whether ARF could reconstitute GTPγS-stimulated PLD activity and secretion, and whether PI-TP could reconstitute GTPγS-stimulated secretion in RBL-2H3 cells, as in HL60 cells. In addition, the proteins were applied to antigen-stimulated cells to test their ability to reconstitute this system. If ARF and PI-TP are components of the secretory machinery in mast cells *in vivo*, they must be effective under physiological stimulus. Another aim was to determine the role of these proteins in secretion, to ascertain for example if ARF mediates its effects via PLD or another effector, and if these proteins operate in different pathways, or in a common mechanism.

Firstly, ARF and both forms of PI-TP have been used successfully to reconstitute GTPγS-stimulated secretion - confirming and extending the primary observation made in HL60 cells. ARF has also been shown to be an effective reconstituting agent of PLD activity from these cells (see chapter 3). Secondly, these proteins were found to be effective at reconstituting secretion from partially cytosol-depleted cells, activated via the receptor (see chapter 4).

These results not only extend the general finding: the ability of ARF and the PI-TP's to reconstitute antigen-stimulated secretion demonstrates that these proteins are of physiological importance - proteins that are functionally important in an essential cellular process.

6.2 The mechanism of ARF's effects in secretion.

Thus ARF is effective in reconstituting secretion and PLD activity; but does ARF exert its effect in secretion by activating PLD, or by another mechanism? The parallel loss of PLD activity and secretory competence from permeabilised cells supports the possibility that ARF's primary effects are on PLD. Some correlation is also noted when the concentrations of calcium and MgATP are altered. However the correlation between PLD activity and secretion was not as exact as in HL60 cells. Therefore, although it appears that ARF's effects in secretion are mediated by ARF's effects on PLD activity, other possible mechanisms may be suggested. ARF has a well-established role in vesicular budding from the Golgi, thus it is possible that ARF increases formation of nascent secretory vesicles following stimulation. Thus ARF may increase the extent of secretion in cytosol-depleted cells by increasing the size of the available population of vesicles, rather than potentiating their immediate release. The importance of PLD in ARF's effects in vesicular budding have been suggested, but not conclusively demonstrated (see section 1.2.4.5).

6.3 The mechanism of PI-TP's effects in secretion.

The inhibition of rPI-TPα- and rPI-TPβ-reconstituted secretion by ethanol is modest by comparison. This result indicates that PI-TP reconstitution is not dependent on PLD activity. This idea is also supported by the inability of PI-TP to reconstitute PLD activity. Experiments carried out in HL60 cells however, do indicate that at some level, ARF and PI-TP participate in a common mechanism. This is concluded because of the lack of synergy seen when ARF and PI-TP are supplied together to permeabilised cells. When used at sub-optimal concentrations, PI-TP and ARF have only additive effects on the reconstituted system (161).

6.4 A common mechanism for ARF and PI-TP's effects in secretion.

PI-TP has been shown to be an essential cofactor for PLC signalling (243). There is a simple explanation for PI-TP's effect in PLC signalling. PLC activation causes PIP_2 hydrolysis. Therefore sustained PLC activity would be predicted to deplete the enzyme's local areas of membrane of PI. PI-TP's *in vitro* activity is to shuttle between membranes maintaining PI and PC concentrations in the correct ratio (234). Thus it is possible that PI-TP supports PLC signalling just by correcting the low local concentrations of PI in areas where PLC has depleted it. This idea has been refuted however. Deletion of five residues from the carboxy-terminus of PI-TPα diminishes PI-transfer activity by 30%, but completely abolishes the proteins ability to reconstitute PLC signalling (240). In addition, nsL-TP, a protein that has an equivalent PI-transfer activity to PI-TP is not at all effective at reconstituting PLC activity (248). This suggests that PI-TP's effect on PLC signalling does not only arise from its ability to transfer PI.

Double reconstitution experiments in HL60 cells, where PLC-β and rPI-TPα were added back together to cytosol-depleted cells, have been used to probe this process further (247). PI-TP was found to be essential in maintaining levels of PIP_2 following stimulation with GTPγS. The data suggested that the PIP_2 hydrolysed by PLC in stimulated cells is predominantly newly synthesised PIP_2. It was concluded that PLC preferentially hydrolyses PIP_2 that is presented by PI-TP. Thus PI is thought to be carried by PI-TP through its sequential phosphorylations, remaining associated with the lipid throughout. In support of this model: PI-TP has been shown to have high affinity for PIP_2 (288), but it cannot transfer the lipid between membranes (289). Thus PI-TP potentiates the formation of PIP_2.

The products of ARF-activated PLD are PA and choline. As previously explained, while no second-messenger effects have been attributed to choline, PA's importance as a second messenger has become established. PA activates type 1 PI4P 5-kinase (151), the enzyme that catalyses the phosphorylation of PIP forming PIP_2. Therefore ARF also, indirectly potentiates the formation of PIP_2. Potentially ARF/PLD-regulated PIP_2
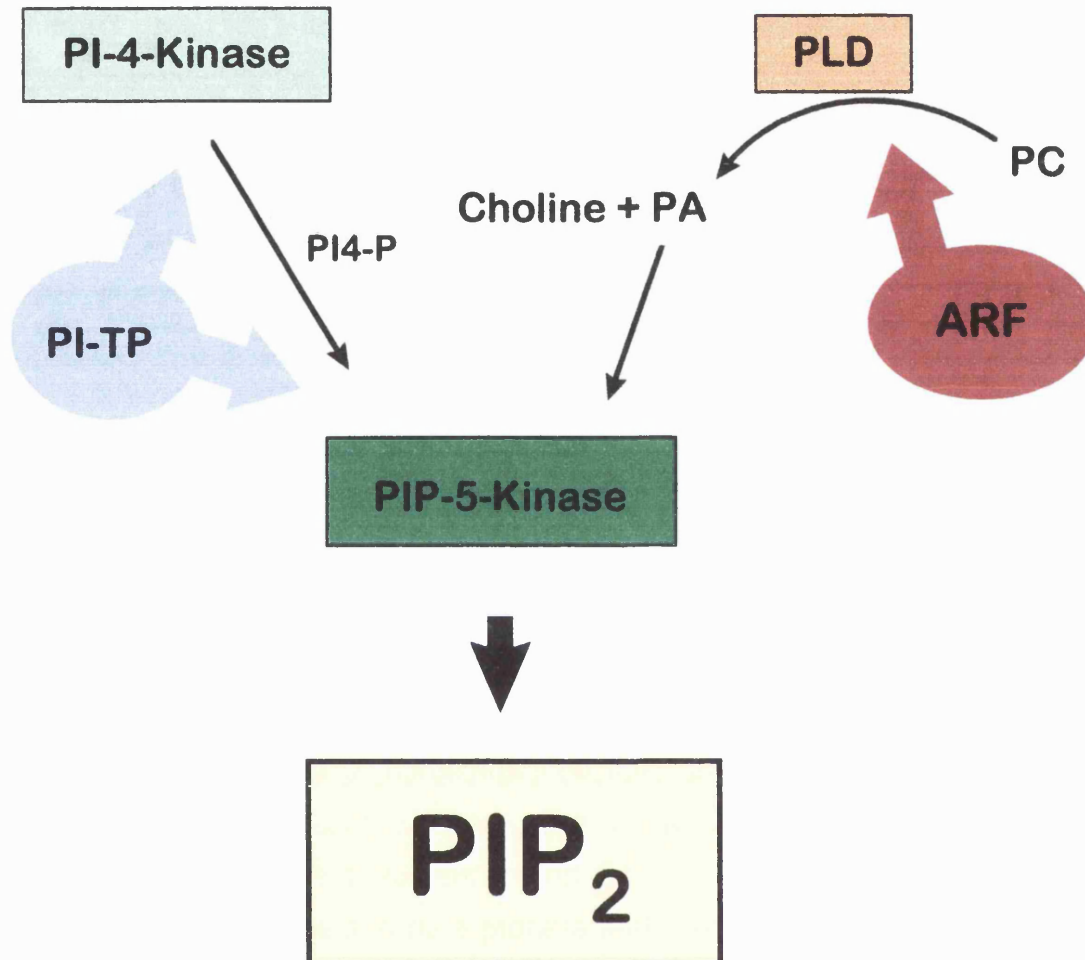
Fig 6.1 The proposed common mechanism for ARF and PI-TP's effect on
secretion

formation occurs via a positive feed-back loop: ARF stimulates PLD activity, leading to increased PIP_2 formation. PIP_2 activates PLD activity leading to increased formation of PA. PA activates PI4P 5-kinase activity, leading to increased PIP_2 formation. PIP_2 increases PLD activity *in vitro* (290), and also in intact cells (131), leading to increased PA formation etc. This mechanism could theoretically lead to very rapid accumulation of PIP_2. Positive feedback mechanisms such as this must be tightly regulated. In this case the "brake" is likely to be the inactivation of ARF-stimulated PLD activity. ARF is a G-protein with little intrinsic GTP-ase activity. Inactivation requires a GAP protein. ARF-GAP is activated by PIP_2 in the presence of PA (291), thus the positive feedback loop is terminated following accumulation of its products.

The potential for ARF and PI-TP to potentiate PIP_2 formation by the mechanisms described above have been confirmed in HL60 cells, and here also in RBL-2H3 cells. PI-TP and ARF are thought therefore to play their essential role in secretion, by potentiating the regulated formation of PIP_2.

6.5 The importance of PIP_2 in the priming of exocytosis

In PC12 cells release of norepinephrine has been dissected, revealing two sequential stages (232). These two stages are distinguishable by a dependence on different proteins and on different dependence on MgATP and calcium. Priming is said to be a process that involves the MgATP-dependent accumulation of an intermediate, triggering involves the calcium-dependent consumption of this intermediate. In PC12 cells three cytosolic proteins are thought to be involved in priming. The two such proteins that have been identified so far - PI-TP and PI4P 5-kinase both participate in the formation of the phospholipid PIP_2. It seems likely therefore that appropriate synthesis of this lipid represents an essential step in a cell's preparation for exocytosis.

With regard to priming, mast cells appear to be anomalous, in that they do not display a true MgATP dependence in secretion. This suggests that in some way, these cells do not require priming - or at least that priming is not rate-limiting. This anomaly is also suggested by the pattern of these cells' dependence on heterotrimeric G-proteins. G_o and G_{i3} are implicated in
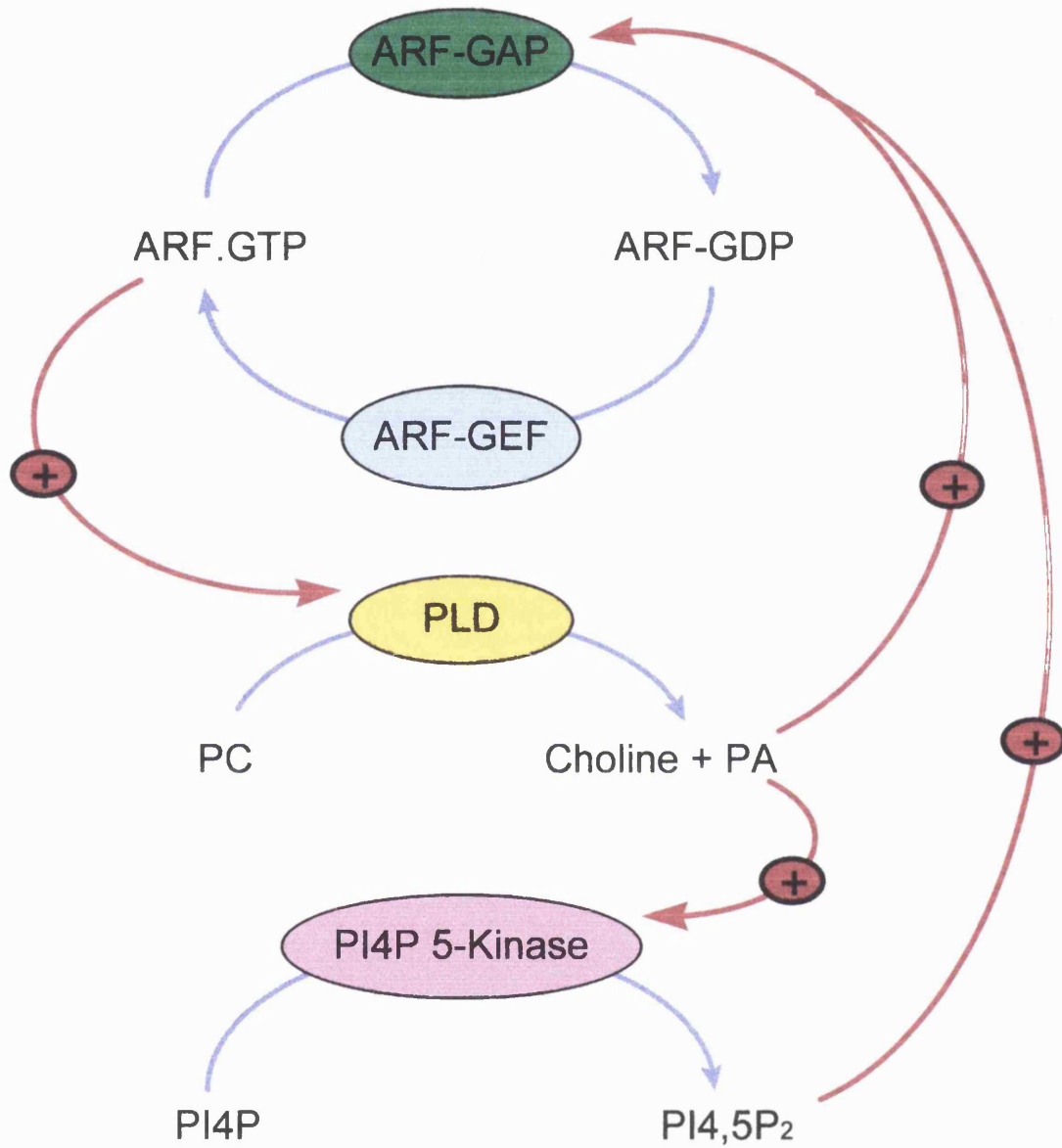
Fig 6.2 Termination of PIP$_2$ formation by PLD inactivation

secretion from adrenal chromaffin cells. Further work has suggested that G_o is involved specifically in priming and G_{i3} in triggering (202). Though mast cells do display dependence on G_{i3} (203), no dependence on G_o - the priming G-protein has been demonstrated. The fact that RBL-2H3 cells do display a dependence on MgATP also suggests a fundamental difference in the regulation of secretion from these cells. This is also suggested by the reported lack of effect seen when PI-TP - a protein implicated specifically in priming (233), is added to permeabilised mast cells (164). More recent work has demonstrated that PI-TP is effective in reconstituting secretion from mast cells, provided the cells are metabolically inhibited ("de-primed") over a prolonged period before reconstitution (A.Pendleton and A.Koffer - unpublished observation).

6.6 Localisation of signalling mechanisms

As discussed in chapter 5, signalling mechanisms involving membrane phospholipids have scope for strict compartmentalisation. Potentially such spacial regulation of signalling can confer great efficiency on these mechanisms. ARF-regulated PLD activity has been detected in cytosol and on the plasma membrane in HL60 cells (292). This activity is thought to carry out an essential role in PIP_2 synthesis - a function thought to be particularly important in priming. It is assumed that this modification in lipid composition would be concentrated at the plasma membrane: if priming is a preparative process in secretion, modification of the acceptor membrane for vesicles would be predicted. Clearly if ARF-stimulated PLD is responsible for increasing the PIP_2 content of the plasma membrane, the presence of PI 4-kinase and PIP 5-kinase at the plasma membrane would be essential. These enzymes have been detected at these sites in HL60 cells (292). Therefore in stimulated cells ARF-stimulated PLD could activate these enzymes in the appropriate location.

The presence of PI-TP at the plasma membrane would also be predicted, where it would be appropriately located to support receptor activated PLC signalling. However, in quiescent Swiss 3T3 cells, PI-TPα has been detected in the cytosol and nucleus, and PI-TPβ detected at the Golgi (286). The data

presented here represents the first report of PI-TPα translocation in stimulated cells.

As shown in Fig.5.5, receptor stimulation leads to the translocation of PI-TPα to an area proximal to the plasma membrane. However the pattern of staining does not suggest that PI-TPα translocates to the plasma membrane itself. It is possible that the punctate staining at the cells' cortex represents staining of docked secretory vesicles. In Clara cells - secretory cells in lung tissue, PI-TPα is detected on the secretory granules, and on the apical membrane from which granules are released (293). In PC12 cells, it has been suggested that PI-TP may act co-operatively with a PI 4-kinase has been detected at secretory vesicles (294,295). The presence of PI 4-kinase on secretory vesicles from HL60 cells or RBL-2H3 cells has not been demonstrated. The enzyme is detected in semi-purified endo-membrane fractions from HL60 cells, which consist of Golgi, ER and azurophilic granules (292).

The time-course of PI-TPα translocation to the cortex is comparable to the time-course of secretion from antigen-stimulated secretion from attached cells (compare Figs.5.5 and 4.2.5). However if PI-TPα is a priming protein, then this time-course would be expected to be much more rapid than exocytosis - to be complete before secretion starts. Clearly this result must be investigated further.

6.7 Regulation of PI-TP and ARF via the receptor

PLD is not thought to be regulated by direct receptor association, but by activation of intermediate regulators. There is no clear evidence of how receptor activation leads to ARF-regulated PLD activity, however a mechanism may be proposed. Stimulation of neutrophils with fMLP resulted in a recruitment of both ARF and Rho to cell membranes (296). This same pattern of ARF-recruitment following receptor activation is suggested in RBL-2H3 cells by Fig.5.4. In rat adipocytes also, ARF translocation has been noted, and PI 3-kinase activity has been implicated in this process (297). ARF-GEF proteins containing specific PH domains, such as cytohesin (139), have been identified. Thus the translocation noted here could form the basis of a mechanism of

receptor activated PLD activity. Modification of membrane lipids, via receptor-activated PI 3-kinase for example, could cause activation of ARF-GEF's in appropriate cellular locations. Activated ARF-GEF's could recruit ARF, which in turn would stimulate PLD activity. The different specificity of ARF-GEF PH domains, and the fact that ARF-GEF's have been identified that do not contain PH domains at all, indicates that different classes of ARF-GEF are probably activated by different mechanisms, indicating that PLD signalling may be complex.

The mechanism of the regulation of PI-TP via receptor signalling is equally unclear. As shown in Fig.5.5, PI-TPα translocates following stimulation. Stimulation of Swiss 3T3 cells by either bombesin or PMA was found to cause an increase in PI-TP's basal phosphorylation state (298). (PI-TP contains five PKC consensus sites (236)). Despite the increased phosphorylation noted in stimulated cells, no precise effect has been attributed to this modification. In this study, translocation was shown to be independent of phosphorylation. However, recruitment of PI-TPα - a cytosolic protein to membranous sites associated with PLC signalling seems likely. In fact, PI-TP has been detected in a signalling "complex" with PLC-γ and PI 4-kinase from stimulated A431 cells (244). PI-TPβ is known to have a different distribution to PI-TPα (286). This may suggest differences in function, however in the experiments presented in this thesis the ability of the two proteins to reconstitute GTPγS- and antigen-stimulated secretion were equal. Previous work has also indicated that the isoforms are equally able to reconstitute PLC signalling from permeabilised HL60 cells (245). These findings may indicate similarities in the mechanism of activation of these isoforms. However if PI-TPα and PI-TPβ do have different functions, these findings also demonstrate a limitation of the permeabilised cell assay.

6.8 Permeabilised cell assays

The permeabilised cell assay has been used successfully to identify many proteins implicated in cell responses, for example ARF in PLD activity (119) and PI-TP in PLC signalling (284). These assays were carried out by screening fractionated cytosol for reconstituting activities, on the basis of their

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ability to restore a declined response from permeabilised cells. This is a powerful technique, but it has limitations. Restoration of response may often depend on the presence of downstream targets of the restored protein. For example, PI-TP was identified as a co-factor of PLC-β signalling. These assays were possible because of the slow leakage of PLC-β. Later work has revealed that PI-TP also supports PLC-γ signalling (245). However PLC-γ leaks rapidly from cells, therefore had PLC-γ signalling been investigated first, it is unlikely that PI-TP's activity would have been detected.

In addition the physiological importance of proteins may not be truly reflected by these experiments. Recent work concerning the importance of annexin II in PC12 exocytosis raises an interesting possibility (299). Annexin II retards run-down from permeabilised adrenal chromaffin cells, therefore its importance in exocytosis had been suggested (300). However modification of expression levels of this protein did not affect exocytosis from the related PC12 cell-line. It seems likely therefore that in permeabilised, cytosol-depleted adrenal chromaffin cells, annexin II prevents the leakage of proteins that are active in exocytosis, rather than being active itself. Other drawbacks of the cytosol-depleted cell protocol is that assays may be carried out in the absence of specific protein inhibitors. In addition, the effects of reconstituting proteins that act in synergy with proteins that do not leak from cells, may appear exaggerated because of this synergy. For example, ARF leaks more quickly from human neutrophils than Rho (296). ARF and Rho appear to have synergistic effects on PLD activity. Therefore, in ARF-reconstitution experiments, where native Rho is still present, it may be difficult to distinguish the effects of ARF alone, from the effects of ARF on Rho-reconstituted activity.

Permeabilised cells assays must therefore be planned and interpreted carefully. Other techniques such as modulation of expression levels of proteins under investigation, or in some cases judicious use of *in vitro* assays to corroborate data garnered in this way, may be appropriate. However, in conclusion the permeabilised cell assay remains a powerful tool in the investigations of complex signalling pathways.

6.9 Summary

myr.rARF-1 has been demonstrated to be effective at reconstituting GTPγS- and antigen-stimulated secretion and PLD activity from permeabilised RBL-2H3 cells. rPI-TPα and rPI-TPβ have also been tested and found effective at reconstituting secretion, stimulated by GTPγS and via the receptor. The correlation between PLD activity and secretion noted in HL60 cells is noted here also, strengthening the hypothesis that PLD activation is important in secretion. Inhibition studies using ethanol indicate that myr.rARF-1 exerts its effect in secretion via PLD-mediated formation of PA.

The effect of both of these proteins in the promotion of PIP_2 synthesis was examined. Both rPI-TPα and myr.rARF-1 produce increases in PIP_2 levels. Examination of the effect of these proteins on PIP_2's precursor - PIP suggests that myr.rARF-1-stimulated PLD activity specifically promotes the conversion of PIP to PIP_2 via PA activation of PI4P 5-kinase, while PI-TP acts by promoting both PIP and PIP_2 synthesis, probably by acting as a cofactor to both PI 4-kinase and PI4P 5-kinase. This promotion of PIP_2 synthesis is assumed to occur at the plasma membrane, however localisation studies reveal that PI-TPα is not present at the plasma membrane - either before or after stimulation.

6.10 Further work

The most obvious suggestion for further work is to examine the effects of myr.rARF-1 and PI-TP in PIP_2 levels, in cells stimulated via the antigen. Data presented here concerning ARF-mediated increases in PIP_2 are complicated by the presence of GTPγS. In the permeabilised cells, PLC-β is assumed to be still present, and activated by GTPγS. Therefore addition of GTPγS causes a decrease in background PIP_2 levels, against which ARF effects must be examined. Addition of antigen-stimulus would not be complicated in this way, therefore effects of ARF and PI-TP could be more clearly seen and compared.

Secondly, the exact identity of the compartment that PI-TPα translocates to after stimulation must be determined. This may be achieved by double-labelling experiments. These involve ascertaining the position of various

compartments, by probing with antibodies directed at proteins of known location, for example anti-mast cell protease antibody to identify secretory vesicles. The distribution of the protein of interest - in this case PI-TPα can then be compared with the distribution of these markers. PI-TPα could also be localised using electron microscopy - as has been shown in Clara cells (293). The time-course of PI-TPα translocation matches the time-course of secretion. However the protocol used for stimulation in immunofluorescence is different to that used for stimulation otherwise. The time-course of secretion using the immunofluorescence protocol must therefore be determined to allow valid comparison. This may be achieved using markers directed against the interior of the vesicle - for example concanavalin-A. When fluorescently labelled this agent can identify secreting cells (163), and so be used to ascertain the kinetics of the response. The localisation of PI-TPβ is also of interest. Any difference in the localisation of these two very similar proteins could suggest a difference in their function.

A possible future development from the work presented here involves the localisation of PIP$_2$ formation. As stated, priming is likely to involve modification of the lipid content of some membrane compartment, probably by increasing PIP$_2$ synthesis at the plasma membrane. This must be tested experimentally. The PH domains already isolated with high affinities for PIP$_2$ - such as that from PLC-δ1 (285) could be exploited in this work. Introduced into permeabilised cells, peptides corresponding to these domains could be used in immunofluorescence studies, in an exactly analogous way to antibodies. This approach has been used to demonstrate spatial aspects of DAG signalling in RBL-2H3 cells, using a DAG-binding peptide from PKCγ coupled to GFP (301). If PH domains could be isolated or modified with sufficiently high affinity, or sufficient powers of discrimination, then different polyphosphoinositides could be localised using this technique. Using the permeabilised cell assay in antigen-stimulated cells, different localisation or kinetics of ARF - or PI-TP-mediated PIP$_2$ synthesis could be tested for. This method could potentially reveal more about the precise nature of the reactions that these proteins participate in.

Acknowledgements

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