REFERENCE ONLY

UNIVERSITY OF LONDON THESIS

Degree	Year	Name of Author
PhD	2005	HAMMOND, G. R. V.

The role of phosphoinositides in mast cell exocytosis

Gerald R. V. Hammond

Thesis submitted for the degree of Doctor of Philosophy, University of London

September, 2005

Molecular Neuropathobiology
Laboratory
Cancer Research UK London Research
Institute
44 Lincoln's Inn Fields
London
WC2A 3PX

Department of Biochemistry and
Molecular Biology
University College London
Gower Street
WC1E 6BT

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

The phosphoinositides (PIn) are a family of phospholipids that contain *myo*-inositol as their headgroup. Despite being present in eukaryotic membranes with low abundance, their high rates of metabolic turnover allow them to control a plethora of cellular functions. In particular, phosphatidylinositol 4,5-*bis*phosphate (PtdIns(4,5)P_2) regulates several processes, including preparing secretory organelles to undergo fusion with the plasma membrane in response to a stimulus (regulated exocytosis), budding and fission of vesicular cargo from the plasma membrane (endocytosis), and controlling the cortical actin cytoskeleton. In this thesis, the role of PIn in regulated exocytosis is examined in mast cells, since these undergo an acute, massive and rapid exocytosis, without any immediate endocytosis.

Using a reconstitution approach, it was not possible to define which PIn are involved in exocytosis, although it was concluded that at least one PIn that is not PtdIns(4,5)P_2 is required. In order to study PtdIns(4,5)P_2 dynamics in primary mast cells, a novel quantitative immunofluoescence technique for PIn was established. Using this technique, PtdIns(4,5)P_2 was identified at the plasma membrane of mast cells, but was depleted almost entirely during exocytosis; the latter observation was confirmed using biochemical approaches. This depletion was blocked by inhibitors of phospholipase C (PLC), an enzyme that breaks down PtdIns(4,5)P_2 into diacylglycerol (DAG) and the calcium mobilising messenger, inositol 1,4,5-*tris*phosphate (Ins(1,4,5)P_3). Although PLC activity was required for initiation of calcium signalling in mast cells, experiments whereby the Ins(1,4,5)P_3/calcium pathway was bypassed demonstrated further requirements for PLC activity. These were not precisely defined, but simple elimination of plasma membrane PtdIns(4,5)P_2 or production of DAG were not sufficient. Both events may be required in conjunction, however. A model is proposed whereby elimination of plasma membrane PtdIns(4,5)P_2 together with production of DAG may activate the protein machinery for membrane fusion during exocytosis.

Lay abstract

The various tissues in our bodies are made-up of tiny modules called cells. These cells release chemical messengers that coordinate the tissues, allowing the body to function as a whole. Failure to release these messengers, or their inappropriate release, can therefore cause disease. The outsides of cells are made of a vanishingly thin layer of fat. Opening of pores in this fatty layer allows release of the messengers from compartments inside cells.

The aim of this thesis was to better understand how these pores in the fatty layer are formed. The role of the fatty layer itself has been considered: in particular, a family of special fatty units called PIPS ("pips") were studied. Consistent with previous reports, one of the PIPS, PIP-2 ("pip-two") was observed within the fatty layer of mast cells. Mast cells release the messenger histamine, which causes allergic and inflammatory reactions in the body. Mast cells were studied because they release all their histamine extremely rapidly, so are easy to study. It was found that, whilst releasing histamine, all of the PIP-2 is destroyed. This process of destruction was essential for the release of histamine, although quite why was not clear. Mast cells destroy the PIP-2 by breaking it into two fragments. These fragments by themselves do not allow histamine release to occur, nor does manipulating the cells so that PIP-2 is destroyed in another fashion. From these observations, it is concluded that destruction of PIP-2 as well as one of the fragments produced are required to form the pores, allowing histamine release. In addition, it seemed that another type of PIP is required, but this has not been identified. Together, these results increase our understanding of how mast cells (and by extension, other tissues) release their chemical messengers.

Acknowledgements

This thesis is essentially all my fault, and I wouldn't want anyone else to be blamed for it. However, the damage was limited by many people, whom I express my gratitude to below:

Firstly, I am extremely grateful to my supervisor, Gipi Schiavo; without his unending support and enthusiasm, this work would simply not have been possible. Many thanks to Steve Dove, both for excellent collaboration and for many hours of stimulating discussion; these have been extremely formative for the ideas developed herein (but we're back to not blaming anyone else...). Also, to Frank Cooke for many helpful discussions, and for teaching me the essentials of lipid biochemistry. The work herein was greatly assisted by the CRUK Light Microscopy Laboratory, and particularly by collaboration with Alastair Nicol and Daniel Zicha; thanks guys. Thanks also to Steve Gschmeissner for his patience in the many attempts at electron microscopy on peritoneal mast cells, and to all at biological resources. I am very grateful to Tracey Hadfield for teaching me about the software used to prepare this thesis; it has certainly cut down on the amount of swearing that went into organsing it! I am indebted to my old teachers Anna Koffer, Jef Pinxteren, Peter Tatham and Geraint Thomas for many helpful discussions and for all their assistance over the years.

The Molecular Neuropathobiology Laboratory has been a stimulating, fun and interesting place to work over the years; a big thank-you to all members, past and present. In particular, I'm very grateful to Claire Thomas and Matthew Golding for all their assistance and the patience that came with it. Many thanks to Katrin Deinhardt for all the great advice, discussion, and for being such a good friend. Thank-you also to Steffi Bohnert and Choon-Ping Tan, for their friendship and for feeding me beer on those days when everything had turned to what one should not mention in a thesis. Thank-you to my parents and big sister; their support through the last few years has been amazing, as it always has.

And finally, to Claire Cheetham, for the fastidious task of proofreading this thesis. Without her, there would have been many more mitakes.

Table of contents

List of figures

List of tables

Abbreviations

48/80	compound 48/80
βARK	β-adrenergic receptor kinase
BMMC	cultured bone marrow mast cell
BSA	bovine serum albumin
CAPS	calcium-dependent activator protein for secretion
DAG	1,2-diacyl-*sn*-glycerol
DMEM	Dulbecco's minimum essential medium
EB	extracellular buffer
EGTA	ethylene glycol-*bis*(2-aminoethyl)-N,N,N',N'-tetraacetic acid
GFP	green fluorescent protein
β-GP	β-glycerophosphate
Gro*P*Ins(4,5)P_2	glycerophosphoinositol 4,5-*bis*phosphate
GST	glutathione S-transferase
GTPγS	guanosine-5'-O-(3-thiotriphosphate)
HEK	human embryonic kidney
β-hex	*N*-acetyl-β-D-glucosaminidase (β-hexosaminidase)
IB	intracellular buffer
Ins	D-*myo*-inositol
Ins(1,4,5)P_3	inositol 1,4,5-*tris*phosphate

InsP	inositol phosphate(s)
InsPP	inositol polyphosphate(s)
KGB	potassium glutamate buffer
Mints	Munc18 interacting proteins
NaGB	sodium glutamate buffer
LDCV	large dense-core vesicle
PAGE	polyacrylamide gel electrophoresis
PAO	phenyl arsine oxide
PBS	phosphate-buffered saline
PDBu	phorbol 12,13-dibutyrate
PH	pleckstrin homology
PI3K	PI 3-kinase (PtdIns 3-OH kinase)
PI4K	PI 4-kinase (PtdIns 4-OH kinase)
PIPK I	phosphatidylinositol 4-phosphate 5-OH kinase
PIPK II	phosphatidylinositol 5-phosphate 4-OH kinase
PIn	phosphoinositide(s)
PPIn	polyphosphoinositide(s)
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PtdCho	phosphatidylcholine
PtdIns	phosphatidylinositol

PtdIns4*P*	phosphatidylinositol 4-phosphate
PtdIns(4,5)P_2	phosphatidylinositol 4,5-*bis*phosphate
PtdOH	phosphatidic acid
RBL	rat basophilic leukaemia cell
RPMC	rat peritoneal mast cell
SDS	sodium dodecylsulphate
SNAP-23	synaptosomal-associated protein of 23 kDa
SNAP-25	synaptosomal-associated protein of 25 kDa
SNARE	soluble NSF attachment receptor
SL-O	streptolysin-O
SV	synaptic vesicle
VAMP	vesicle associated membrane protein

"The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka'! (I found it!) but 'That's funny ...'"

Isaac Asimov, US science fiction novelist & scholar (1920 - 1992)

Chapter 1: Introduction

This thesis is concerned with the regulation of exocytosis from rat peritoneal mast cells by phosphoinositides. Mast cells were chosen not for their biological properties *per se*, but as a model system that possesses certain features that are advantageous for dissecting regulated exocytosis. In this chapter, a brief introduction to the cell biology of exocytosis will be given, followed by an overview of the phosphoinositides. Next, the current knowledge concerning the control of regulated exocytosis by these molecules will be discussed. This background will then be placed in the context of the biology that underpins mast cell exocytosis. Finally, the aims of the work presented in this thesis will be laid out.

1.1. Exocytosis

Exocytosis is an eukaryotic process that may be defined as fusion of the limiting membrane of a cellular organelle with the plasma membrane. Traditionally, exocytosis is associated with the secretion of the organelle's lumenal content (Keller and Simons, 1997). However, there are several functions that are associated with exocytosis (Chieregatti and Meldolesi, 2005). Increases in cell surface area are mediated by exocytosis of excess membrane; a fundamental example is exocytosis of membrane required for increases in cell surface area during cytokinesis (Danilchik *et al.*, 2003). Deposition of membrane proteins at the cell surface also occurs by exocytosis; a prominent example of this is exocytosis of Glut4 transporters to enable uptake of glucose by cells (Watson *et al.*, 2004). Finally, membrane lesions may be repaired by the stimulated exocytosis of lysosomes (Reddy *et al.*, 2001). This may be associated with the secretion of lysosomal hydrolases in defence against microbial infection (Chakrabarti *et al.*, 2003). In this thesis, exocytic secretion is considered, so from here on exocytosis will be discussed in this context.

1.1.1. The secretory pathway

Before outlining the secretory pathway, it is important to distinguish between secretion and exocytosis; the latter is a cellular mechanism that can be used to achieve the former. Examples have been described above of non-secretory exocytosis. However, it is also important to note that secretion often occurs by

means distinct from exocytosis. For example, acinar cells of the salivary gland secrete amylase by exocytosis (Fujita_Yoshigaki *et al.*, 1996), whereas water is secreted by means of aquaporin channels (Krane *et al.*, 2001). Thus when the secretory pathway is discussed, it is actually the *exocytic* secretory pathway that is being referred to. Thus from hereon, where 'secretion' or 'secreted' is mentioned, the author is referring to exocytosis.

Secreted proteins are translocated co-translationally into the lumen of the endoplasmic reticulum (ER). As discussed above, membrane proteins also reach the cell surface by exocytosis, i.e. along the secretory pathway; these are inserted into the membrane of the ER co-translationally. Once at or in the lumen of the ER, the protein undergoes folding to reach the correct conformation. After satisfying a quality control mechanism, the protein is packaged into vesicular transporters at ER exit sites for transport to the Golgi (Lippincott-Schwartz *et al.*, 2000). Once the protein arrives at the Golgi, it is transported through the cisternae, receiving pertinent post-translational modification along the way. Once it reaches the trans-Golgi network, it is packaged into tubular-vesicular transport carriers; these pinch off and migrate along microtubules towards the plasma membrane, where they undergo exocytosis (Keller and Simons, 1997; Lippincott-Schwartz *et al.*, 2000).

In the case of regulated exocytosis, the transport organelles wait in the cytosol until they receive an appropriate stimulus to fuse at the plasma membrane, and this defines the difference between ***regulated*** and ***constitutive*** exocytosis. However, in the case of regulated exocytic secretion there is a second crucial difference: a process of vesicle maturation occurs before the secretory organelle is ready for exocytosis. This is exemplified by the formation of endocrine cell secretory granules, which involves the aggregation of the newly-budded, immature secretory granules (ISGs). The ISGs undergo a process of homotypic fusion, followed by removal of excess membrane and proteins that are no longer required by a clathrin-dependent mechanism. After acidification, the lumenal contents condense to form a dense core, thereby forming mature secretory granules (Tooze *et al.*, 2001).

Thus this pathway defines the first type of secretory organelle: the secretory granules or large dense-core vesicles (LDCV), so called due to their large size (200-800 nm) and electron-dense granule cores. Secretory granules are found ubiquitously in professional secretory cells throughout the body (Burgoyne and Morgan, 2003). The second class of organelle are smaller (~50 nm), and electron lucent: these are the small synaptic vesicles (SV), and predominate at the synapse although they do occur in neuroendocrine cells (Burgoyne and Morgan, 2003; Torrealba and Carrasco, 2004).

1.1.2. SNAREs and membrane fusion

Membrane fusion between secretory organelles and the plasma membrane is the defining feature of exocytosis. Membrane fusion between cellular organelles is believed to be controlled by Soluble NSF-Attachment Receptor proteins (SNAREs). The first clue to the role of SNARE proteins in membrane fusion came from studies on the clostridial neurotoxins, which cleave SNARE proteins and prevent neurotransmission, a process that relies on synaptic vesicle exocytosis (Schiavo *et al.*, 1992; Schiavo *et al.*, 2000). The SNAREs represent a large family of proteins containing a juxtamembrane heptad repeat, or SNARE motif, with either glutamine (Q) or arginine (R) residues at their core (Chen and Scheller, 2001). Four of these motifs (from 3 or 4 SNARE proteins) combine to form a four-helix bundle, with unique thermodynamic properties (Brunger, 2000). However, the Q/R core *per se* is not required for assembly, since functional SNARE complexes may be formed with normal kinetics after mutation of these residues; rather, they seem to function in efficient disassembly of the complex (Chen and Scheller, 2001).

Traditionally, SNARE proteins have been assigned as either target membrane (t-SNAREs) or vesicle membrane (v-SNAREs). Since each complex generally contains three Q-containing SNAREs on one membrane and a single, R-containing SNARE on the other membrane, the nomenclature has since been changed to R-SNAREs and Q-SNAREs. This is favoured, since it can be applied to SNARE-dependent homotypic fusion events (Chen and Scheller, 2001).

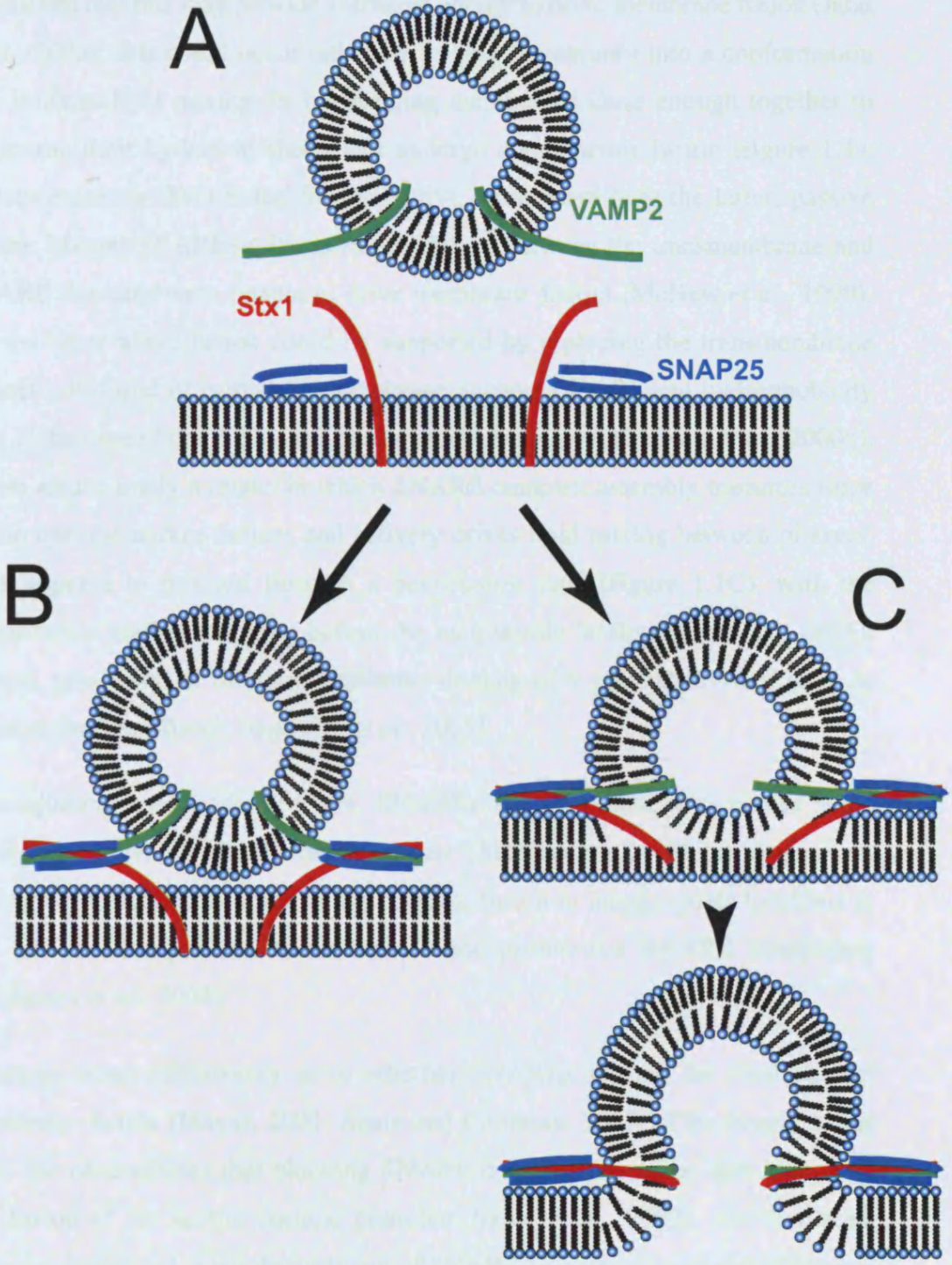

Figure 1.1: Two models of SNARE-mediated membrane fusion. (A) shows a vesicle before assembly of the 4-helix SNARE complex between the 2-Q SNARE SNAP-25, 1-Q Syntaxin 1 (Stx1) and 1-R VAMP 2. In (B), formation of the complex brings the membranes into close apposition, overcoming the hydration barrier. Fusion may then occur spontaneously, or be initiated by another factor. In (C), formation of the complex is sufficient to force the bilayers together and drive fusion, proceeding via a hemifusion state. See text for details.

The assembly of the four-helix SNARE complex is an exothermic reaction, and it is believed that this may provide sufficient energy to drive membrane fusion (Jahn *et al.*, 2003); this could occur either by driving membranes into a conformation that initiates lipid mixing, or by bringing membranes close enough together to overcome their hydration shells and undergo spontaneous fusion (figure 1.1). Recent evidence favours the former, active mechanism over the latter, passive model. Mutant SNAREs with extended linkers between the transmembrane and SNARE domains were unable to drive membrane fusion (McNew *et al.*, 1999). On the other hand, fusion could be supported by replacing the transmembrane helices with lipid or isoprenoid membrane anchors of sufficient hydrophobicity and, in the case of the R-SNARE, that spanned the bilayer (McNew *et al.*, 2000b). These results imply a model in which SNARE complex assembly transmits force to the transmembrane helices and actively drives lipid mixing between bilayers. This appears to proceed through a hemifusion state (figure 1.1C), with the cytoplasmic leaflets merging before the exoplasmic leaflets (Lu *et al.*, 2005). Indeed, truncation of the transmembrane domain of a yeast R-SNARE leads to arrest at the hemi-fusion stage (Xu *et al.*, 2005).

In conjunction with other factors, SNAREs are also thought to confer some specificity to which membranes may fuse (McNew *et al.*, 2000a). Interaction among SNAREs can also negatively regulate fusion in inappropriate locations in the cell by competitive formation of non-productive SNARE complexes (Varlamov *et al.*, 2004).

There is some controversy as to whether SNAREs initiate the final step of membrane fusion (Mayer, 2001; Szule and Coorssen, 2003). This largely arises from the observations that blocking SNARE complex formation does not block the fusion of sea urchin cortical granules (Szule *et al.*, 2003), and that yeast vacuolar fusion can occur downstream of SNARE complex disassembly (Peters *et al.*, 2001). In these models, SNARE complex assembly acts before fusion (figure 1.1B), with lipid mixing initiated by another protein such as the V_0 ATPase (Mayer, 2001). However, there is compelling evidence that SNAREs constitute the minimal machinery necessary for membrane fusion. SNAREs incorporated into the outer leaflet of the plasma membrane are sufficient to drive fusion of cells

(Hu *et al.*, 2003), and SNARE proteins incorporated into artificial membranes are sufficient to drive fusion (Weber *et al.*, 1998) at physiologically relevant rates (Fix *et al.*, 2004). Thus SNAREs certainly play a central role in controlling fusion at many stages of membrane traffic, including exocytosis. It should be noted, however, that there are examples of membrane fusion in eukaryotes that proceed independently of SNARE proteins, such as viral entry (Jahn *et al.*, 2003) and mitochondrial fusion (Mozdy and Shaw, 2003).

1.1.3. Regulated exocytosis

Regulated exocytosis occurs in response to an appropriate signal. This is usually relayed by an increase in cytosolic calcium concentration, as exemplified by exocytosis from chromaffin cells (Baker and Knight, 1978). However, certain cell types can employ other 2nd messengers in the absence of increases in cytosolic calcium, such as cAMP in the case of parotid acinar cells (Fujita-Yoshigaki, 1998) or GTP (presumably acting via G-proteins) in the case of gastric chief cells (Raffaniello and Raufman, 1993). No matter what the stimulus, the response will be to drive membrane fusion of the secretory organelle with the plasma membrane; since it is generally held that fusion is catalysed by SNARE proteins, SNARE complex assembly represents the ultimate point of regulation. Indeed, recent structural studies of the neuronal R-SNARE VAMP-2 show that the juxtamembrane region of the SNARE motif is buried in the membrane, and so unable to form a SNARE complex; therefore, triggering of exocytosis presumably requires induction of conformational changes in this region that, thereby permiting SNARE complex formation (Kweon *et al.*, 2003).

The molecular characterisation of regulated exocytosis is most advanced in the case of neuronal and neuroendocrine cells (Rettig and Neher, 2002; An and Zenisek, 2004), so these will be used to outline the major points of what is understood about regulated exocytosis. Figure 1.7 shows the major steps in the regulated exocytosis of SV, although these general concepts are also applicable to neuroendocrine cells. It should be noted that although Ca^{2+} plays the central role in triggering exocytosis in these cells, GTP is still required to activate small G-proteins of the Rab family, which co-ordinate multiple stages of membrane traffic (McBride *et al.*, 1999).

Two key regulators of neuronal exocytosis, Unc 13 and Unc 18, have been discovered in *Caenorhabditis elegans* and are conserved in mammalian cells (Richmond and Broadie, 2002). Mammalian Unc 18 (Munc18) binds to the plasma membrane Q-SNARE syntaxin 1A, holding it in a closed conformation and inhibiting exocytosis (Dulubova *et al.*, 1999). Unc 13 was shown to displace Unc 18 from syntaxin *in vitro* (Sassa *et al.*, 1999), and mutants of syntaxin that are held in open conformations are able to bypass the blockade of neurotransmission in an *Unc13* background (Richmond *et al.*, 2001). Therefore, it is believed that Munc18 acts as a chaperone, preventing non-productive SNARE complex formation until the appropriate time, when Munc13 displaces Munc18 and allows formation of productive SNARE complexes (Richmond and Broadie, 2002).

Calcium-binding proteins, or "calcium sensors" mediate the triggering of exocytosis in neurons and neuroendocrine cells. Calmodulin was the first such sensor proposed, although it now appears that it plays a modulatory rather than essential role in exocytosis; nevertheless, it may act as the predominant calcium sensor in other membrane fusion events such as yeast vacuole fusion (Burgoyne and Clague, 2003). One family of proteins involved in the coupling of calcium transients to SNARE complex formation in neurons are the synaptotagmins. These proteins are found in the secretory organelle or plasma membranes. They contain a transmembrane domain, a cytoplasmic linker region and two C2 domains; they are implicated in coupling the Ca^{2+} transient to exocytosis via their C2 domains (Bai and Chapman, 2004), though not all synaptotagmins bind Ca^{2+} (Sudhof, 2002). Synaptotagmins have been shown to oligomerise, and to bind to SNARE complexes as well as to phospholipids in a Ca^{2+}-dependent manner (Chapman, 2002), although recent studies imply that phospholipid binding activity is more important for exocytosis (Shin *et al.*, 2003). Whatever the relevant interaction, synaptotagmins play an important role in membrane fusion and are able to enhance SNARE-mediated fusion between artificial membranes, although whether this is Ca^{2+}-dependent is controversial (Mahal *et al.*, 2002; Tucker *et al.*, 2004).

Another class of calcium-sensitive proteins implicated in the regulation of SNARE complex assembly are the complexins. These proteins were shown to be essential for efficient coupling of calcium transients to exocytosis in neurons (Reim *et al.*, 2001). Squid complexin was found to form a helical interaction with the SNARE complex (Bracher *et al.*, 2002); these SNARE-complexin complexes are present at stoichiometric quatities in brain extracts, and cause a shift in the migration of the core complex of full length SNAREs. This is interpreted as efficient coupling of Q- and R-SNARE transmembrane domains (Hu *et al.*, 2002). These observations indicate that complexins could in principle couple calcium influx to SNARE complex assembly, although a precise mechanism for how this occurs is not yet clear.

Many of the molecular interactions described above, particularly in terms of the SNARE complex, are required for membrane fusion outside the nervous system. However, the molecular identities of the components involved are less well-defined in non-nervous tissues (Burgoyne and Morgan, 2003).

1.1.4. Compensatory endocytosis and the exo-endocytic cycle

As a result of fusion, secretory organelle membrane becomes incorporated into the plasma membrane, creating an increase in surface area. Furthermore, if secreted cargo is ejected completely from the cell, there is a net decrease in cytoplasmic volume. Exocytosis may thus create a problem in the maintenance of cell surface area to volume ratio. Another potential problem of stimulated exocytosis from high activity secretory systems, such as the chemical synapse, is that vesicles will become depleted faster than biogenesis can form new vesicles *de novo*. One mechanism that prevents such problems is so called "kiss-and-run" exocytosis, a mechanism commonly employed by chemical synapses (An and Zenisek, 2004). In this mode of exocytosis, a transient fusion pore opens between the synaptic vesicle and the plasma membrane, and persists long enough to release neurotransmitter before re-sealing. The vesicle may then be refilled with neurotransmitter before undergoing another round of fusion. However, even at central synapses, full fusion of synaptic vesicles and incorporation of their membranes into the synaptic membrane is seen to predominate (Sankaranarayanan and Ryan, 2000).

In most secretory systems, catastrophic increases in surface area do not occur, since exocytosis is accompanied by compensatory endocytosis (Gundelfinger *et al.*, 2003). This again has been best characterized at the synapse, where compensatory endocytosis both removes excess plasma membrane, and leads to the recycling of synaptic vesicle components (De Camilli, 1995). Under physiological conditions, stimulated endocytosis is sufficiently fast to balance exocytosis from central synapses (Fernandez-Alfonso and Ryan, 2004). How exo-endocytosis is coupled is not yet clear, although endocytosis is stimulated by calcium and can still occur when exocytosis is blocked with botulinum toxin A (Neale *et al.*, 1999). Exocytosis can also be uncoupled from endocytosis: snake phospholipase A_2 neurotoxins (SPANS) cause depletion of synaptic vesicles by triggering their exocytosis, without compensatory endocytosis. This causes paralysis and massive increases in synaptic surface area (Rigoni *et al.*, 2004). Exocytosis may also regulate compensatory endocytosis indirectly via postsynaptic nitric oxide (NO) production, which diffuses back to the presynaptic terminal and accelerates recycling by a cGMP-dependent mechanism (Micheva *et al.*, 2001; Micheva *et al.*, 2003).

Although not well characterised at present, compensatory endocytosis also occurs after secretory granule exocytosis: there are specific reports of this in adrenal chromaffin cells (Smith and Neher, 1997; Engisch and Nowycky, 1998) and pancreatic beta cells (Takahashi *et al.*, 1997; Ohara-Imaizumi *et al.*, 2002).

1.1.5. Secretory vesicle priming

Before a recycled or *de novo* sythesised exocytic organelle may undergo exocytosis, it must be prepared for fusion. As observed in chromaffin cells, this involves trafficking along the microtubule and actin cytoskeletal elements (Neco *et al.*, 2002), followed by a process of docking at the plasma membrane (Parsons *et al.*, 1995). The latter is largely a morphological rather than functional term. The one exception is at the synapse, where synaptic vesicles dock in specific regions, know as active zones, containing a network of scaffolding proteins and calcium channels (Rosenmund *et al.*, 2003).

Early studies in chromaffin (Holz *et al.*, 1989) and PC12 cells (Hay and Martin, 1992) resolved functionally an ATP-dependent step that precedes fusion during exocytosis, know as vesicle priming. This has been extended to neurons, where pre-primed vesicles form a 'rapidly releasable pool', whilst other vesicles must undergo priming before fusion and form a 'slowly releasable pool' (Rettig and Neher, 2002). Although it has been assumed that morphological docking corresponds to primed vesicles, recent work has demonstrated that this is not the case at synapses (Rizzoli and Betz, 2004). The functional process of ATP-dependent priming is thus distinct from simple vesicle docking. Work by Eberhard and colleagues demonstrated that the requirement for ATP during priming was due, at least in part, to a process of lipid phosphorylation (Eberhard *et al.*, 1990). Before exploring this concept in further detail, it will be necessary to introduce the class of lipids into which ATP was incorporated: the phosphoinositides.

1.2. Phosphoinositides

The phosphoinositides (PIn) belong to a family of biological molecules that contain the hexameric cyclitol *myo*-inositol (figure 1.2). '*myo*' defines the isomer in which the hydroxyl groups at positions 1, 2, 3 and 5 lie above the plane of the carbon ring, whereas positions 4 and 6 lie beneath the plane (written (1,2,3,5/4,6-)-inositol); in its most relaxed chair conformation, all but the 2-hydroxl group are equatorial with respect to the carbon ring. Anti-clockwise numbering around the ring when viewed from above (with the axial hydroxyl projecting towards the viewer) distinguishes chiral derivatives such as D-*myo*-inositol-3-phosphate from its enantiomer, L-*myo*-inositol-1-phosphate; the D-enantiomer is referred to as standard in the biological literature (IUBMB, 1989). Since it is *myo*-inositol which predominates in cells, and which forms the inositol-containing molecules described below, inositol (or its abrevation Ins) will be used to refer specifically to this isomer from here on.

Ins is synthesised by cells from D-glucose-6-phosphate, which is converted enzymatically to D-*myo*-inositol-3-phosphate (Ins3*P*), or L-*myo*-inositol-1-phosphate (Maeda and Eisenberg, 1980). The human enzyme was recently cloned and named 1-*myo*-inositol-3-phosphate (MIP) synthase (Ju *et al.*, 2004). The

hydroxyl groups around the inositol ring may be reversibly phosphorylated, containing either mono or pyrophosphate moieties (Irvine and Schell, 2001). Collectively, these are referred to as inositol phosphates, or inositol polyphosphates (InsPP) where more than one phosphate group is present. There are at least 25 biologically relevant inositol phosphates and pyrophosphates.

Inositol can be esterified by a phosphodiester bond at the D-1 position to 1,2-diacyl-*sn*-glycerol (DAG). This occurs via the CDP-DAG pathway (Kent *et al.*, 1991) using the ER-associated phosphatidylinositol synthetase (Ghalayini and Eichberg, 1985). The resulting phospholipid, phosphatidylinositol (PtdIns), forms the parent compound for two families of biological molecules. In the first, glucosamine and three mannose residues are added in a step-wise fashion to the D-6 position, forming glycosylphosphatidylinositol (GPI); GPI can then be attached to the carboxyl terminus of a protein via a phosphoethanolamine moiety, forming GPI-anchored proteins (Eisenhaber *et al.*, 2003). These molecules are not examined in this thesis, so will not be described further.

The second family of PtdIns-derived compounds includes PtdIns itself, and are collectively known as the phosphoinositides (PIn), defined as lipids that contain D-*myo*-inositol as their headgroup. Like inositol phosphates, PtdIns can be reversibly phosphorylated on free hydroxyl groups around the inositol ring. However, to date, phosphorylation of only the D-3, -4 and -5 positions has been observed, and only monophosphate is added. This means that the resulting polyphosphoinositides (PPIn) contain only seven members: three *mono*phosphate and three *bis*phosphate isomers, as well as a single *tris*phosphate (figure 1.3). The metabolic pathways linking the PIn are described in the following two sections, with reference to mammalian cells unless otherwise stated.

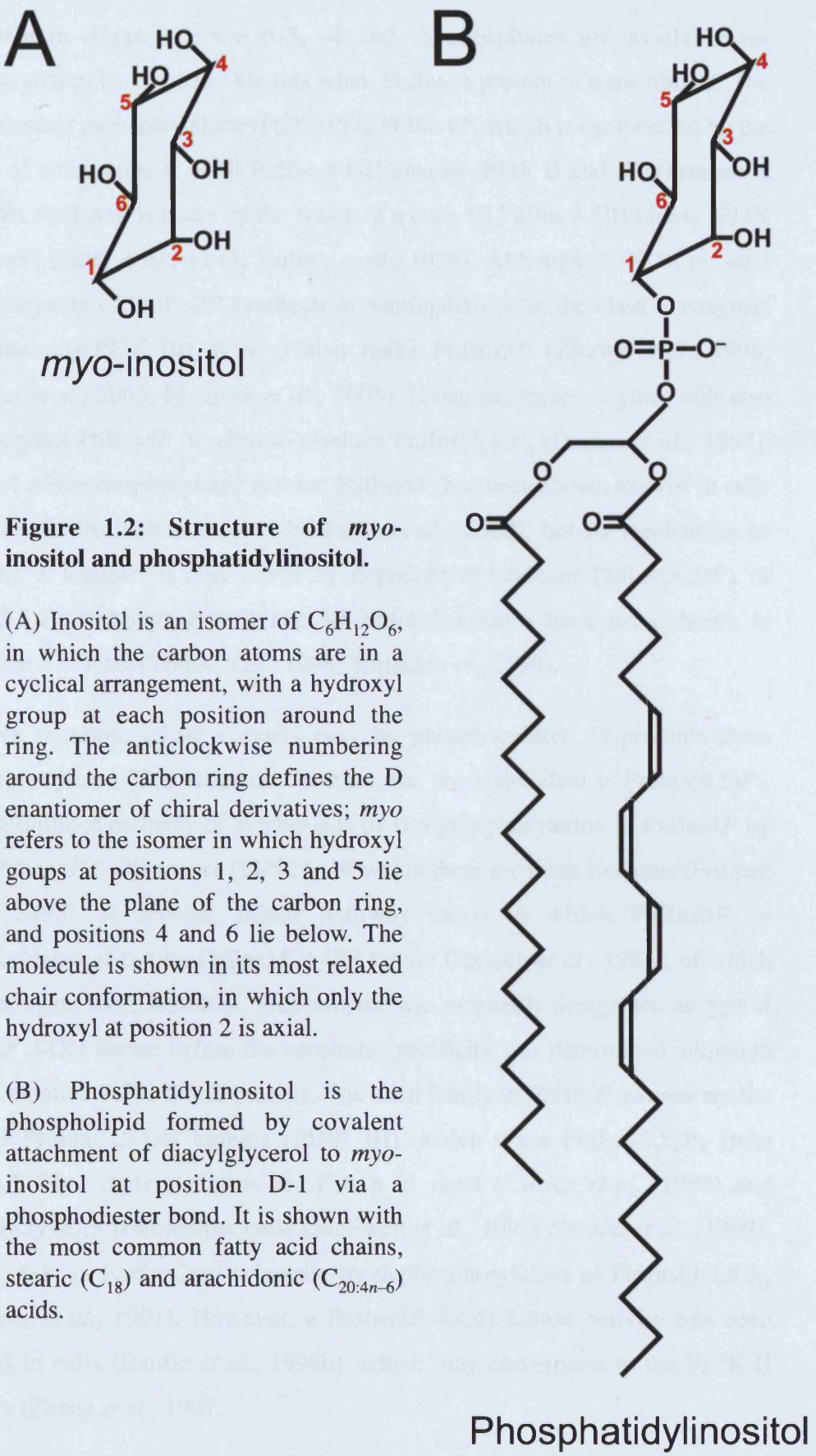

Figure 1.2: Structure of *myo*-inositol and phosphatidylinositol.

(A) Inositol is an isomer of $C_6H_{12}O_6$, in which the carbon atoms are in a cyclical arrangement, with a hydroxyl group at each position around the ring. The anticlockwise numbering around the carbon ring defines the D enantiomer of chiral derivatives; *myo* refers to the isomer in which hydroxyl goups at positions 1, 2, 3 and 5 lie above the plane of the carbon ring, and positions 4 and 6 lie below. The molecule is shown in its most relaxed chair conformation, in which only the hydroxyl at position 2 is axial.

(B) Phosphatidylinositol is the phospholipid formed by covalent attachment of diacylglycerol to *myo*-inositol at position D-1 via a phosphodiester bond. It is shown with the most common fatty acid chains, stearic (C_{18}) and arachidonic ($C_{20:4n-6}$) acids.

1.2.1. Synthesis of polyphosphoinositides

As shown in figure 1.3, the D-3, -4 and -5 phosphates are available for phosphorylation by cytosolic kinases when PtdIns is present in a membrane. The most abundant monophosphate (PtdIns*P*) is PtdIns4*P*, which is synthesized by the activity of either class II or III PtdIns 4-OH kinases (PI4K II and III) (Fruman *et al.*, 1998). PtdIns3*P* is made by the action of a class III PtdIns 3-OH kinase (PI3K III), Vps34 (Schu *et al.*, 1993; Volinia *et al.*, 1995). Although PI3K III account for the majority of PtdIns3*P* synthesis in mammalian cells, the class II enzymes (C2-containing PI3K IIα, β & γ) also make PtdIns3*P* (Zhang *et al.*, 1998; Maffucci *et al.*, 2005; Meunier *et al.*, 2005). However, these enzymes will also phosphorylate PtdIns4*P* *in vitro* to produce PtdIns(3,4)P_2 (Domin *et al.*, 1997). The final monophosphorylated isomer, PtdIns5*P*, has been shown to exist in cells at low levels (Rameh *et al.*, 1997; Morris *et al.*, 2000), but its mechanism of synthesis is unclear. It may occur by dephosphorylation of PtdIns(4,5)P_2 or PtdIns(3,5)P_2, although type I and III PtdIns*P*-kinases have been shown to synthesize it *in vitro* (Tolias *et al.*, 1998; Sbrissa *et al.*, 1999).

All three monophosphate isomers may be phosphorylated to produce three *bis*phosphorylated PPIn isomers. Of these, the most abundant is PtdIns(4,5)P_2. The predominant pathway of synthesis is by D-5 phosphorylation of PtdIns4*P* by type I PtdIns4*P* 5-OH kinase (PI*P*K I), of which there are three isoforms (Fruman *et al.*, 1998). A second, minor pathway exists in which PtdIns5*P* is phosphorylated at D-4 by PtdIns5*P* 4-OH kinase (Rameh *et al.*, 1997), of which there are again three isoforms. This enzyme was originally designated as type II PtdIns4*P* 5-OH kinase before the substrate specificity was determined, although the abbreviation PI*P*K II still persists. The third family of PtdIns*P* kinases are the type III PtdIns*P* 5-OH kinases (PI*P*K III), which make PtdIns(3,5)P_2 from PtdIns3*P*. The single isoforms are Fab1p in yeast (Cooke *et al.*, 1998) and p235/PIKfyve in mammalian cells (McEwen *et al.*, 1999; Sbrissa *et al.*, 1999). PtdIns(3,4)P_2 is synthesized primarily by dephosphorylation of PtdIns(3,4,5)P_3 (Stephens *et al.*, 1991). However, a PtdIns3*P* 4-OH kinase activity has been detected in cells (Banfic *et al.*, 1998b), which may correspond to the PI*P*K II enzymes (Zhang *et al.*, 1997).

PtdIns(3,4,5)P_3 is generally thought to be synthesized exclusively from PtdIns(4,5)P_2 via the action of class I PtdIns 3-OH kinases (PI3K I) (Auger *et al.*, 1989), which are stimulated in response to receptor activation (Vanhaesebroeck *et al.*, 2001). However, the fission yeast *Schizosaccharomyces pombe* does not contain PI3K I or II, yet synthesises PtdIns(3,4,5)P_3 (Mitra *et al.*, 2004). It is thought to do so via phosphorylation of PtdIns3*P* at the D-4 and -5 positions by the yeast PI*P*K, Mss4p. Interestingly, mammalian PI*P*K I can synthesize PtdIns(3,4,5)P_3 from PtdIns3*P* (Tolias *et al.*, 1998), and the same enzymes have been found to account for a PtdIns(3,4)P_2 5-OH kinase activity during oxidative stress (Halstead *et al.*, 2001). InsPP multi kinase (IPMK) has also recently been shown to posses PIn 3-OH kinase activity, and to utilise PtdIns(4,5)P_2 exclusively as a substrate (Resnick *et al.*, 2005).

Although not an enzyme that adds phosphate moieties to PtdIns, the PtdIns transfer protein (PITP) is thought to play an important role in the synthesis of PPIn. These proteins catalyse the transfer of PtdIns and phosphatidylcholine (PtdCho) between membranes *in vitro* (Thomas and Pinxteren, 2000), and were shown to maintain PtdIns(4,5)P_2 synthesis during receptor activation in permeabilised cells (Thomas *et al.*, 1993).

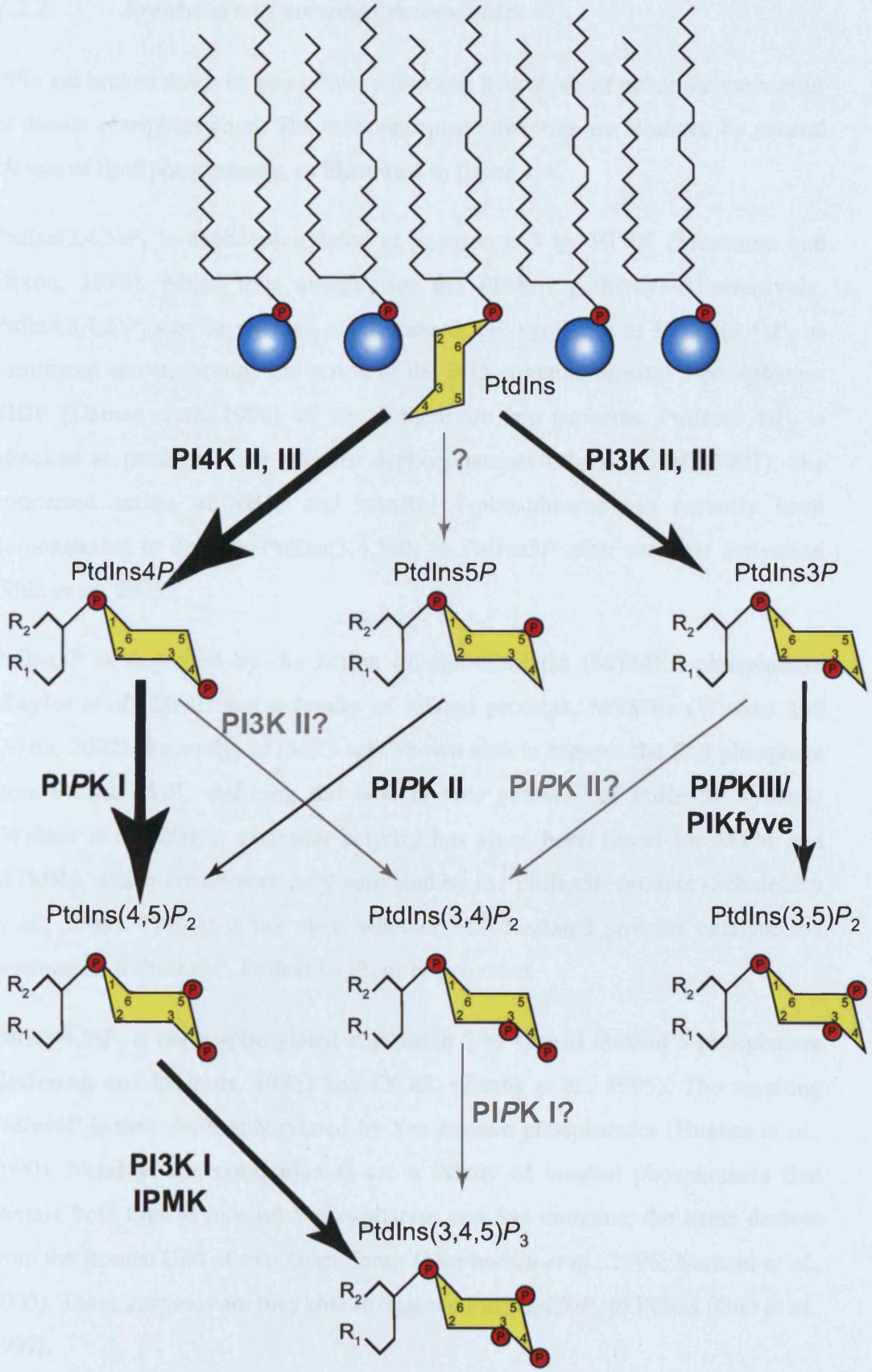

Figure 1.3: Anabolism of PPIn in mammalian cells. Thicker arrows indicate more prominent pathways; grey arrows indicate possible alternative pathways. R_1 and R_2 denote the fatty acid chains. See text for details.

1.2.2. Breakdown of polyphosphoinositides

PPIn are broken down by one of two pathways: hydrolysis of either the monoester or diester phosphate bond. The monophosphate moieties are removed by several classes of lipid phosphatases, as illustrated in figure 1.4.

PtdIns(3,4,5)P_3 is dephosphorylated at position D-3 by PTEN (Maehama and Dixon, 1998), which thus antagonises the PI3K I pathway. Alternatively, PtdIns(3,4,5)P_3 can be used as a substrate in the synthesis of PtdIns(3,4)P_2 as mentioned above, through the action of the SH2-containg inositol 5-phosphatase SHIP (Damen *et al.*, 1996), of which there are two isoforms. PtdIns(3,4)P_2 is attacked at position 4 by inositol 4-phosphatases (Norris *et al.*, 1997); the concerted action of SHIP and inositol 4-phosphatase has recently been demonstrated to degrade PtdIns(3,4,5)P_3 to PtdIns3*P* after receptor activation (Shin *et al.*, 2005).

PtdIns3*P* is degraded by the action of myotubularin (MTM) 3-phosphatase (Taylor *et al.*, 2000) and a family of related proteins, MTMRs (Wishart and Dixon, 2002). Recently, MTMR3 was shown also to remove the D-3 phosphate from PtdIns(3,5)P_2, defining the first *in vivo* pathway of PtdIns5*P* sythesis (Walker *et al.*, 2001); a similar activity has since been found for MTM and MTMR6, which are allosterically activated by the PtdIns5*P* product (Schaletzky *et al.*, 2003). Thus it is not clear whether MTM-related proteins catalyse the destruction of PtdIns3*P*, PtdIns(3,5)P_2 or both *in vivo*.

PtdIns(4,5)P_2 is dephosphorylated at position 5 by type-II inositol 5-phosphatase (Jefferson and Majerus, 1995) and OCRL (Zhang *et al.*, 1995). The resulting PtdIns4*P* is then dephosphorylated by Sac domain phosphatases (Hughes *et al.*, 2000). Notably, the synaptojanins are a family of inositol phosphatases that contain both type II inositol 5-phosphatase and Sac domains; the name derives from the Roman God of two faces, Janus (McPherson *et al.*, 1996; Nemoto *et al.*, 2000). These enzymes are thus able to degrade PtdIns(4,5)P_2 to PtdIns (Guo *et al.*, 1999).

As for its synthesis, little is known about the degradation of PtdIns5*P*, although a specific phosphatase, PLIP, has recently been identified that will remove the 5-phosphate *in vitro* (Pagliarini *et al.*, 2004). It is also possible that PtdIns5*P* is removed via hydrolysis of the phosphodiester bond (Roberts *et al.*, 2005).

The second pathway of degradation occurs by hydrolysis of the phosphodiester bond to produce DAG and the 1-phosphorylated inositol phosphate (figure 1.5). This reaction is catalysed by members of the phospholipase C family, of which there are 5 sub-families β-ζ (figure 1.6); α has since been shown to be a proteolytic fragment of δ (Rhee, 2001; Saunders *et al.*, 2002). The enzymes are calcium-dependent, and prefer PtdIns(4,5)P_2 as their substrate (Rhee *et al.*, 1989). The β-isoforms are regulated by G-protein coupled receptors, the γ by tyrosine kinases and the ε by Ras signalling. The other isoforms are less well characterized, but the δ isoforms seems to be regulated by calcium and lipids (Ochocka and Pawelczyk, 2003); the ζ isoform is delivered from the sperm upon fertilization to signal the activation of the oocyte, and thus constitutes the so-called "sperm factor" (Saunders *et al.*, 2002).

The importance of PIn in human health and disease is evident from the large number of genetic disorders caused by mutations in genes encoding the PIn phosphatases discussed above. These include *MTM*, which causes mytotubular myopathy, *MTMR2*, which causes Charcot Marie Tooth disease, *OCRL*, associated with occulocerebrorenal syndrome of Lowe, *SHIP2*'s implication in type 2 diabetes, and the frequent mutations in PTEN associated with a variety of cancers (Wishart and Dixon, 2002; Pendaries *et al.*, 2003; Lowe, 2005).

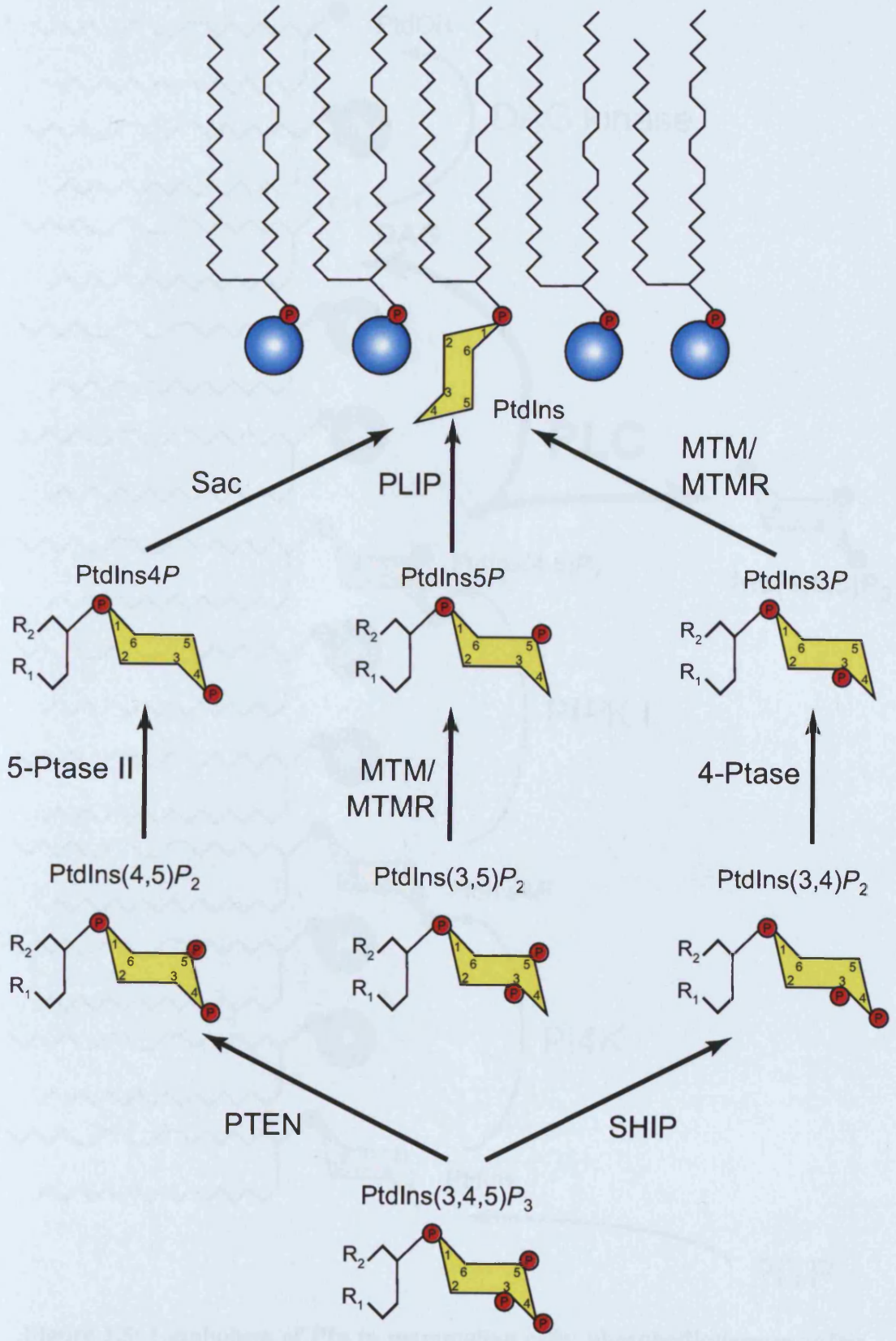

Figure 1.4: Catabolism of PIn in mammalian cells: phosphomonoesterases. See text for details. Ptase, phosphatase.

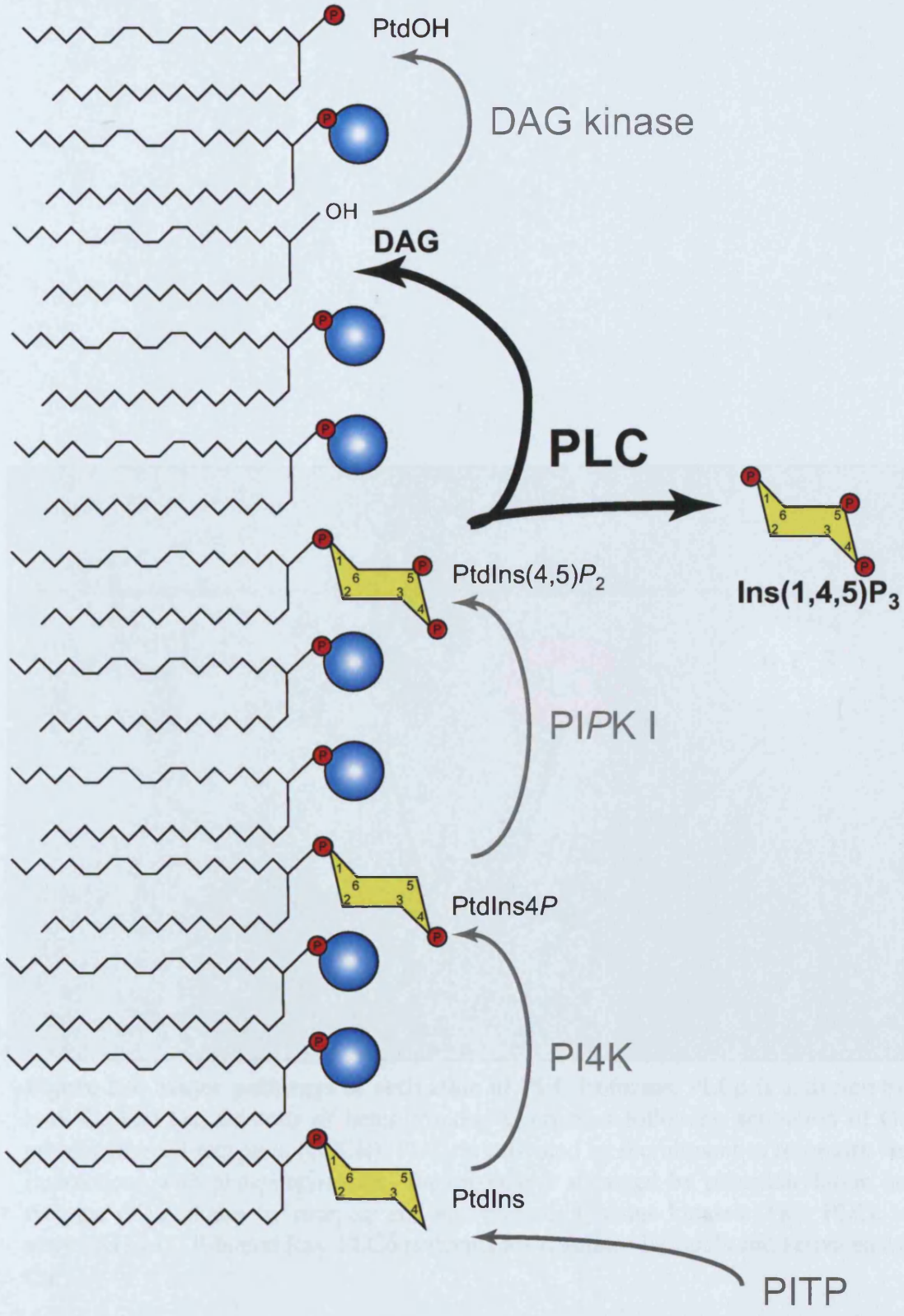

Figure 1.5: Catabolism of PIn in mammalian cells: phosphodiesterases. After synthesis of PtdIns(4,5)P_2, the 1-phosphate ester bond to diacylglycerol is cleaved by the phosphodiesterase, phospholipase C (PLC), generating DAG and Ins(1,4,5)P_3. DAG is rapidly phosphorylated to PtdOH; after dephosphorylation of Ins(1,4,5)P_3, PtdIns resynthesis can occur. PtdIns is transferred back to the plasma membrane by PITP. See text for details.

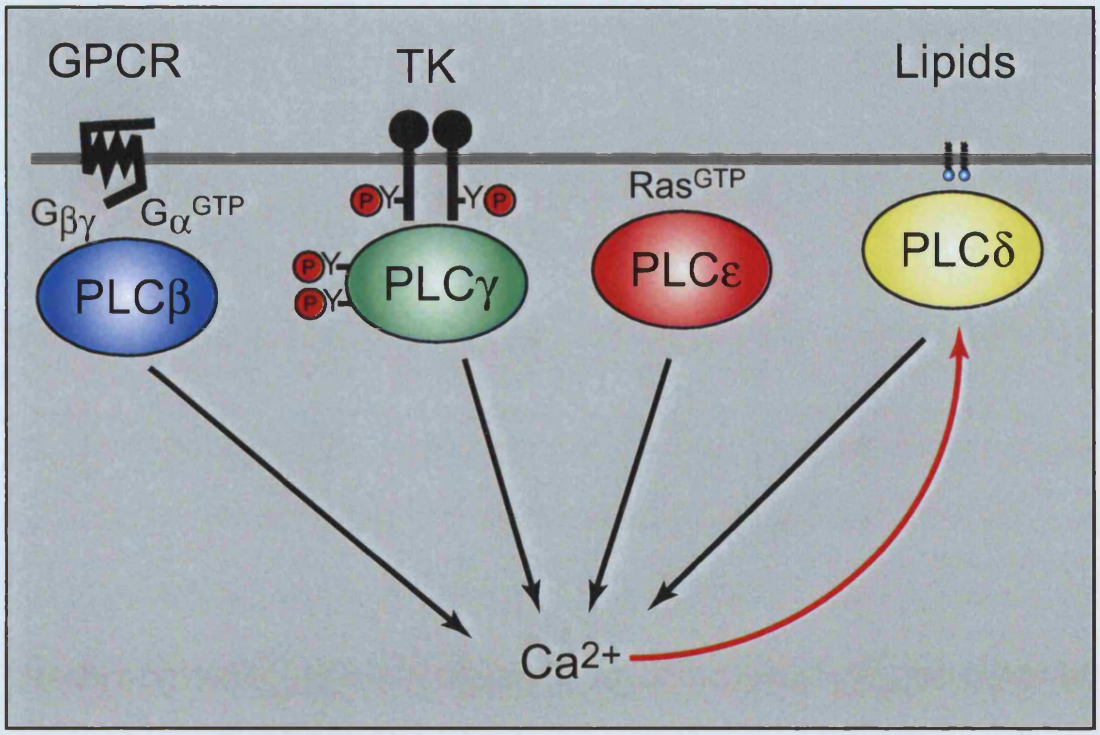

Figure 1.6: Major pathways of activation of PLC isoforms. PLCβ is activated by both G_{α} and $G_{\beta\gamma}$ subunits of heterotrimeric G-proteins following activation of G-protein coupled receptors (GPCR). PLCγ is activated by recruitment to receptors via interactions with phosphotyrosine; the enzyme is activated by phosphorylation on tyrosine (Y) residues by receptor and non-receptor tyrosine kinases (TK). PLCε is activated by GTP-bound Ras. PLCδ isoforms are regulated by lipids and activated by Ca^{2+}.

1.2.3. Regulatory interactions of the polyphosphoinositides

Aside from acting in the generation of the second messengers DAG and Ins(1,4,5)P_3, PPIn also act as regulatory molecules in their own right. Indeed, synthesis and degradation of PPIn is tightly regulated, spatially as well as temporally (Czech, 2003; Roth, 2004). Regulatory functions of the PPIn are mediated through their direct interaction with effector proteins, of which there are two classes. The first bind inositol lipids with high affinity and stereospecificity (Cullen *et al.*, 2001; Lemmon, 2003). As a rule, they contain a modular PIn-binding domain, and are found in several proteins from divergent families; a list of these is provided in table 1.1.

Domain	Common name	Lipid bound	References
Phox homology	PX	PtdIns3*P*, PtdIns(3,4)P_2	(Kanai *et al.*, 2001) (Xu *et al.*, 2001) (Yu and Lemmon, 2001)
Epsin/AP180 N-terminal homology	ENTH/ANTH	PtdIns(4,5)P_2, PtdIns(3,5)P_2	(Friant *et al.*, 2003) (Ford *et al.*, 2001) (Itoh *et al.*, 2001)
Protein 4.1, ezrin, radixin, moesin	FERM	PtdIns(4,5)P_2	(Hamada *et al.*, 2000)
Fab1p, YOTB, Vac1p, EEA1	FYVE	PtdIns3*P*	(Misra and Hurley, 1999) (Sankaran *et al.*, 2001)
Plant homeodomain	PHD	PtdIns5*P*	(Gozani *et al.*, 2003)
β-propeller	WD40	PtdIns(3,5)P_2	(Dove *et al.*, 2004)
Tubby	Tubby	PtdIns(4,5)P_2	(Santagata *et al.*, 2001)
Pleckstrin homology	PH	Various	(Harlan *et al.*, 1994) (Lemmon and Ferguson, 2000)

Table 1.1: PIn binding domains

The second class of PPIn effector protein do not display stereospecificty for the lipids, but may still bind with high affinity. Many of these proteins contain simple polybasic motifs; these bind to the acidic face of a membrane due to the presence of monobasic lipids such as PtdIns and phosphatidylserine (PtdSer), but once bound sequester PPIn in the plane of the membrane (McLaughlin *et al.*, 2002). Such motifs are commonly found in proteins associated with the actin cytoskeleton (Janmey and Lindberg, 2004). The pleckstrin homology domains

also appear for the most part to fall into this category; although some PH domains display specific, high-affinity interactions with one or two PPIn, many display low-affinity, promiscuous interactions (Kavran *et al.*, 1998). A recent screen of all thirty-three PH domains from *Saccharomyces cerevisiae* revealed that only a single example exhibited a specific, high-affinity interaction. The remaining domains bound PPIn promiscuously or not at all, and were targeted by other molecular interactions (Yu *et al.*, 2004). Lastly, C2 domains bind to acidic lipids via interactions with calcium, as the acidic residues in the lipids are thought to provide additional coordination for calcium (DiNitto *et al.*, 2003; Bai and Chapman, 2004). The C2B domain from synaptotagmin is also able to specifically bind PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 *in vitro* (Schiavo *et al.*, 1996; Mehrotra *et al.*, 2000; Bai *et al.*, 2004).

The biological function of the PPIn-protein interactions can be mediated in a number of ways. Firstly, and most simply, the PPIn may recruit a protein to a particular membrane, so restricting its sphere of influence or increasing the local concentration. This is exemplified by the recruitment of protein kinase B (PKB) to the plasma membrane by PtdIns(3,4,5)P_3, where it encounters its activator, phosphoinositide-dependent kinase (PDK) (Currie *et al.*, 1999). A second mode can be allosteric activation of a protein due to binding of its PPIn ligand. PKB once again provides a good example, as the binding of PtdIns(3,4,5)P_3 induces a conformational change that further advances activation (Calleja *et al.*, 2003). As mentioned above, polybasic motifs can bind to several PPIn molecules and sequester them in the plane of the membrane, as observed with the myristolated alanine-rich C-kinase substrate (MARCKS) effector domain (ED) (McLaughlin *et al.*, 2002; Gambhir *et al.*, 2004). Such local sequestration can also couple with allosteric activation of effector proteins, as demonstrated recently for the F-actin nucleating protein N-WASP (Papayannopoulos *et al.*, 2005). Finally, the interaction between PPIn and binding protein may lead to changes in membrane bilayer structure, as demonstrated for the interaction between epsin and PtdIns(4,5)P_2. This causes epsin to partially insert into the lipid bilayer, generating curvature that assists in the budding of a vesicle (Ford *et al.*, 2002).

Through these various modes of interaction, PPIn modulate an impressive diversity of biological functions. For ease of description, these have been separated into two groups: those that occur constitutively in the cell ("housekeeping"), and those that occur to transduce a signal within the cell, largely through bursts of PPIn synthesis. However, since all of these events are tightly regulated within the cell, and associated with turnover of PPIn (i.e. controlled synthesis and degradation), the distinction between the two may be somewhat arbitrary.

1.2.4. Housekeeping functions of PPIn

All of the PPIn are implicated in vesicular traffic within the cell, and are synthesised at restricted endomembrane compartments (Czech, 2003; Roth, 2004). Functions include control of early and late endocytic vesicular traffic by PtdIns3*P* and PtdIns(3,5)P_2, respectively (Gillooly *et al.*, 2000; Cooke, 2002; Jeffries *et al.*, 2004), Golgi trafficking by PtdIns4*P* (Godi *et al.*, 2004; Wang *et al.*, 2004; Balla *et al.*, 2005) and regulation of vesicle fusion and budding at the plasma membrane by PtdIns(4,5)P_2 (Martin, 2001). Furthermore, PtdIns(4,5)P_2 is implicated in the regulation of ion channel activity (Suh and Hille, 2005) and cytoskeletal dynamics (Takenawa and Itoh, 2001; Janmey and Lindberg, 2004) at the plasma membrane, as well as control of gene expression in the nucleus (Irvine, 2002; Hammond *et al.*, 2004).

Thus PPIn can perform multiple functions within specific cellular membranes. How can several functions be mediated by the same pool of lipid? A controversial but popular model is that there are spatially restricted pools of PPIn within cellular membranes. This has been demonstrated for PtdIns3*P* at the endosomal membrane, where it mediates sorting between the recycling and degradative pathways (Gillooly *et al.*, 2003). Early reports using the PtdIns(4,5)P_2-specific PH domain from PLCδ1 fused to green fluorescent protein (GFP-PH-PLCδ1) showed that this lipid was concentrated in F-actin-rich ruffles (Honda *et al.*, 1999; Botelho *et al.*, 2000). A subsequent study showed that this was in fact an artefact caused by increased membrane density at the ruffle (van Rheenen *et al.*, 2005). However, ultrastructural studies have demonstrated enrichment of PtdIns(4,5)P_2 in lamellipodia of cultured cells (Watt *et al.*, 2002). Plasma membrane patches of

PtdIns(4,5)P_2 were also reported by Laux *et al.*; however, these patches were shown to be induced by formaldehyde fixation, and were not observed after glutaraldehyde treatment (Laux *et al.*, 2000). Taken together, these studies indicate that there may indeed by spatially restricted pools of PPIn within individual membranes, but spatial resolution of these pools is unclear. An alternative mechanism involves local sequestration and release of PtdIns(4,5)P_2 at the membrane, which may create the sub-pools required to mediate the plethora of functions (McLaughlin *et al.*, 2002).

1.2.5. Signalling by PPIn

The first function ascribed to PPIn was PLC-catalysed generation of the second messengers Ins(1,4,5)P_3 and DAG, which cause the release of calcium from intracellular stores and activation of novel and classical protein kinase C (PKC) isoforms, respectively (Berridge, 1993; Parker and Murray-Rust, 2004). A second important PPIn signalling pathway is agonist-induced generation of PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, which controls many cellular functions such as growth, apoptosis and migration (Vanhaesebroeck *et al.*, 2001). Signalling is initiated by receptor mediated recruitment and activation of PI3K, specifically the class IA for tyrosine kinase signalling and class IB for G-protein coupled receptors (GPCRs). As discussed above, these enzymes phosphorylate the D-3 position of PtdIns(4,5)P_2; the resulting PtdIns(3,4,5)P_3 then transduces singals by recruiting and in some cases activating downstream signalling enzymes. These include PKB (Gray *et al.*, 1999), p-REX1 (Welch *et al.*, 2002), Bruton's tyrosine kinase (Kojima *et al.*, 1997; Bolland *et al.*, 1998) and PLCγ (Falasca *et al.*, 1998). However, PI3K is not always required to recruit and activate PLCγ (Matsuda *et al.*, 2001).

As discussed above, class I PI3K are antagonised by conversion of PtdIns(3,4,5)P_3 back to PtdIns(4,5)P_2 by PTEN. This forms one manner in which to terminate signalling, although a second pathway exists whereby SHIP phosphatases remove the 5-phosphate, generating PtdIns(3,4)P_2. This lipid will still recruit proteins such as PKB, although others such as General Receptor for PIn-1 (GRP1) are not recruited (Gray *et al.*, 1999). In this way, certain PtdIns(3,4,5)P_3-dependent signals will be terminated, whilst others, such as those

transduced by PKB, will persist. Interestingly, it appears that PKB is responsible for the most prominent aspects of PtdIns(3,4,5)P_3-mediated signalling, since mutations in its PH domain that reduce PtdIns(3,4,5)P_3 binding will rescue the phenotype observed in PTEN null *Drosophila* (Stocker *et al.*, 2002). PtdIns(3,4)P_2 also appears to have signalling functions that are independent of PtdIns(3,4,5)P_3, through its interaction with the monogamous PtdIns(3,4)P_2-binding proteins TAPP1 and 2 (Marshall *et al.*, 2002). It appears that PtdIns(3,4)P_2 signalling is terminated by a 4-phosphatase, generating PtdIns3P at early endosomes as the activated receptor complex enters the endocytic pathway (Shin *et al.*, 2005).

Cross-talk between pools of PPIn that mediate signalling reactions and housekeeping functions may also occur. For example, PtdIns(4,5)P_2 regulates many housekeeping functions at the plasma membrane as described above. This explains why in certain cells there is a burst of PtdIns(4,5)P_2 synthesis in response to receptor activation. This provides a pool of PtdIns(4,5)P_2 for hydrolysis by PLC; there is thus a comparatively small decrease in the resting PtdIns(4,5)P_2 level after PLC activation (Xu *et al.*, 2003). Furthermore, the yeast *Saccharomyces cerevisiae* does not contain Ins(1,4,5)P_3-receptors, so does not release calcium in response to this molecule. Despite this, yeast contain a PLC isoform whose function appears to be in a pathway of rapid Ins(1,2,3,4,5,6)P_6 (InsP_6) synthesis, which regulates the constitutive process of mRNA export (York *et al.*, 1999). Higher inositol phosphates such as InsP_6 also perform a plethora of functions in mammalian cells (Irvine and Schell, 2001). Another consequence of PLC activation is signalling by phosphatidic acid (PtdOH), which is generated rapidly after PLC activation through the catalytic activity of DAG-kinase (Luo *et al.*, 2004). Furthermore, despite its housekeeping function in the endosomal network, PtdIns3P was shown recently to be synthesised on the plasma membrane in response to agonists, where it functions in Glut4 exocytosis and cell migration (Maffucci *et al.*, 2003; Maffucci *et al.*, 2005).

1.3. Phosphoinositides in regulated exocytosis

As noted above, PIn regulate many stages of membrane traffic, including constitutive exocytosis. However, PIn have additional roles in the control of regulated exocytosis, which are outlined in detail in this section. The importance

of PIn in regulated exocytosis can be underscored by the fact that PIn metabolism was first observed in the context of regulated exocytosis (Hokin and Hokin, 1953; Hokin and Hokin, 1955, 1958). Figure 1.7 outlines the various points of regulation of the SV cycle by PtdIns(4,5)P_2. Specific interactions at individual stages are outlined below.

1.3.1. PtdIns(4,5)P_2, vesicle priming and fusion

Despite many studies demonstrating that PIn metabolism was associated with exocytosis (e.g. (Harwood and Hawthorne, 1969; Schacht and Agranoff, 1972; Pickard and Hawthorne, 1978; Whitaker, 1985)), it was not until 1990 that the first direct link between the PIn and exocytosis was published. In this seminal study, Eberhard and colleagues showed that depletion of PIn using a bacterial PtdIns-specific PLC could abolish ATP-dependent chromaffin cell exocytosis, i.e. prevent vesicle priming (Eberhard *et al.*, 1990). Similar effects were achieved after removal of ATP; neomycin, which prevented depletion of PPIn after ATP removal, permitted the maintenance of a primed state. Together, these observations highlighted the importance of PPIn in the priming reaction. Subsequently, work by Martin and co-workers in PC12 cells found that priming could be reconstituted by soluble cytosolic factors in broken cells (Hay and Martin, 1992); two of these factors were later identified as PITP (Hay and Martin, 1993) and PIPK (Hay *et al.*, 1995). Recently, it has been established that the isolated kinase domains from PIPK Iα, β and γ are sufficient for priming activity, with the efficiencies of the three isoforms correlating with their *in vitro* kinase activities (Wang *et al.*, 2005). Soon after discovery of the priming activity of PIPK came the observation that a PI4K activity is required for chromaffin cell and synaptic vesicle exocytosis (Wiedemann *et al.*, 1996; Wiedemann *et al.*, 1998), which was later identified as type IIα at the synapse (Guo *et al.*, 2003). Therefore, enzymes involved in the synthesis of PtdIns(4,5)P_2 were demonstrated to be important for exocytosis in neuroendocrine cells.

Similar evidence exists in endocrine cells. Maintenance (via PI4K activity) or addition of PtdIns4*P* and PtdIns(4,5)P_2 to pancreatic beta cells was shown to maintain a primed state, whereas decreases in free PtdIns4*P* or PtdIns(4,5)P_2 levels decreased priming (Olsen *et al.*, 2003). Furthermore, neuronal calcium

sensor 1 (NCS1) was shown to act through PI4K IIIβ to maintain a primed state in these cells via increases in PtdIns4*P* and PtdIns(4,5)P_2 levels (Gromada *et al.*, 2005). Thus although specific isoforms differ, a similar requirement for PPIn sythesis for priming of exocytosis exists in the endocrine system.

Notably, inhibitors of PI3K were shown to have no effect on priming in PC12 cells (Martin, 1997). PtdIns(4,5)P_2 represents the metabolic "end-point" of PPIn synthesis in the absence of PI3K activity, so it is this lipid that is believed to function during priming. On which membranes does PtdIns(4,5)P_2 perform its priming function? PI*P*K was identified as a soluble factor, so could mediate its effect at the plasma or vesicular membranes. However, the PI4K activity necessary for exocytosis was isolated on the vesicular membranes (Wiedemann *et al.*, 1996; Wiedemann *et al.*, 1998). It therefore came as somewhat of a surprise when Holz and co-workers identified a plasma membrane pool of PtdIns(4,5)P_2 as important for chromaffin cell exocytosis, using the GFP-PH-PLCδ1 probe, and confirmed with fluorescent neomycin. Similar observations have been made in intact PC12 cells, where PI*P*K Iγ was also recruited to the plasma membrane (Aikawa and Martin, 2003). This was confirmed in mechanically permeabilised cells using anti-PtdIns(4,5)P_2 antibodies (Grishanin *et al.*, 2004). Finally, a plasma membrane pool of PtdIns(4,5)P_2 also regulates priming in pancreatic beta cells (Lawrence and Birnbaum, 2003).

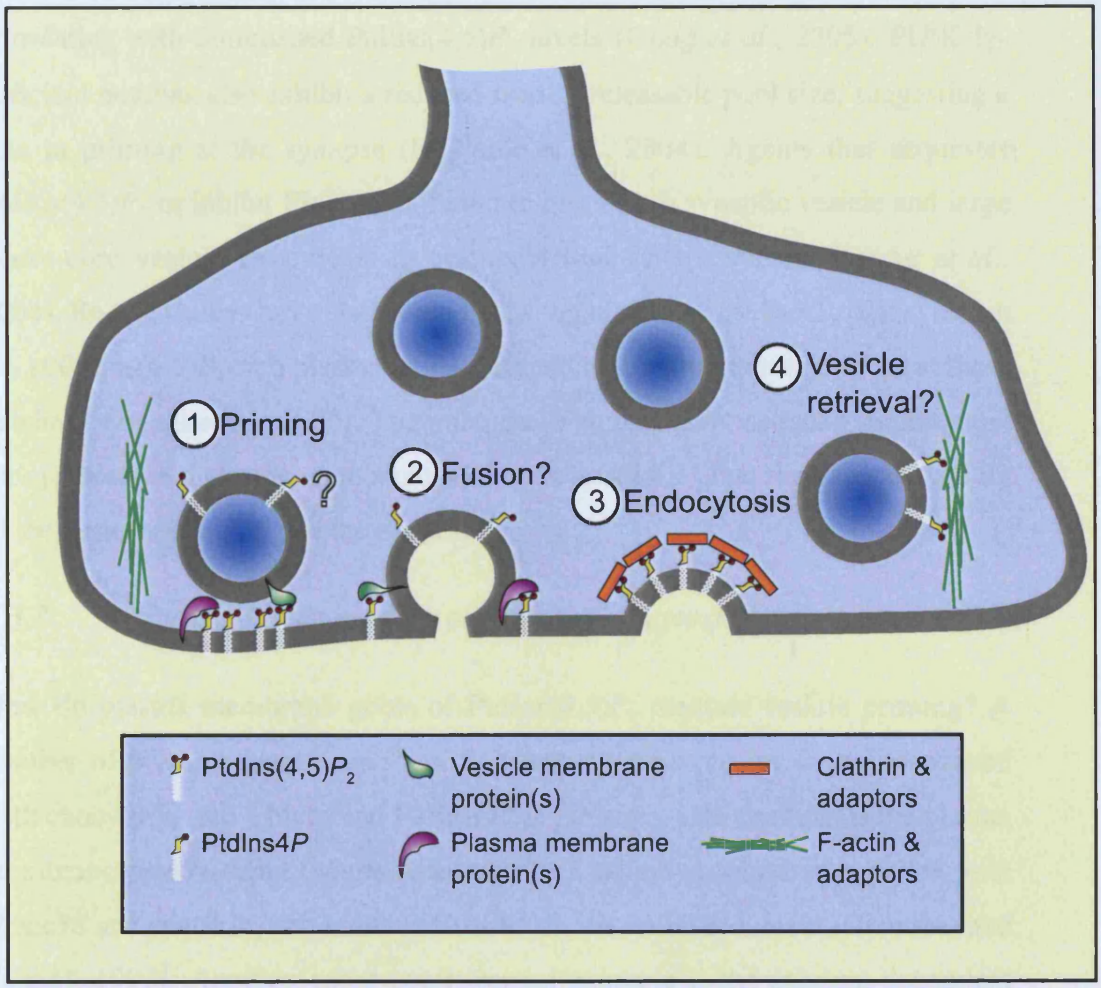

Figure 1.7: PtdIns(4,5)P_2 and the SV cycle. ATP-dependent priming produces interactions between vesicle and/or plasma membrane associated protein(s) with PtdIns(4,5)P_2 that renders the vesicle competent to undergo fusion in response to calcium influx. The question mark beside PtdIns4P denotes the undefined role for this lipid on SV and granules. At the moment of fusion, PtdIns(4,5)P_2 may expedite the fusion reaction via interactions with plasma membrane and/or vesicle proteins. Next, local PtdIns(4,5)P_2 accumulation recruits the clathrin-associated machinery that mediates vesicle budding and fission. Finally, interactions between PtdIns(4,5)P_2 and the actin cytoskeleton may assist in vesicle retrieval and recycling. Question marks indicate those stages where PtdIns(4,5)P_2 function is inferred, rather than being supported by direct experimental evidence. See text for details of the specific candidate proteins.

Recent work confirms the requirements for PtdIns(4,5)P_2 in intact cells. Artificial manipulation of PtdIns(4,5)P_2 levels produced corresponding changes in the number of primed vesicles in chromaffin cells (Milosevic *et al.*, 2005), and priming is severely reduced when these cells are devoid of PIPK Iγ, again correlating with diminished PtdIns(4,5)P_2 levels (Gong *et al.*, 2005). PIPK Iγ-deficient neurons also exhibit a reduced readily-releasable pool size, suggesting a role in priming at the synapse (Di Paolo *et al.*, 2004). Agents that sequester PtdIns(4,5)P_2 or inhibit PI4K were found to block both synaptic vesicle and large dense-core vesicle exocytosis in permeabilised synaptosomes (Zheng *et al.*, 2004). Recent studies have also noted the accumulation of the R-SNARE syntaxin 1A at PtdIns(4,5)P_2-rich plasma membrane patches, with granules docked at these patches (Aoyagi *et al.*, 2005). Together, these studies demonstrate a requirement for synthesis of a plasma membrane pool of PtdIns(4,5)P_2 that regulates the ability of the vesicles to undergo exocytosis.

1.3.2. PtdIns(4,5)P_2 binding proteins in exocytosis

How do plasma membrane pools of PtdIns(4,5)P_2 mediate vesicle priming? A number of proteins have been identified that are required for or are associated with exocytosis, and which bind PtdIns(4,5)P_2 *in vitro*. One example is the plasma membrane protein Mint (Munc18-interacting), which is found in complex with Munc18 and syntaxin, and binds PtdIns(4,5)P_2 via its PTB domain (Okamoto and Sudhof, 1997). Another, more controversial example is the calcium-dependent activator protein for secretion (CAPS), which is a cytosolic factor that restores secretory competence in broken PC12 cells (Walent *et al.*, 1992). CAPS undergoes a conformational change upon binding PtdIns(4,5)P_2 (Loyet *et al.*, 1998), and the lipid is required to target the protein to the plasma membrane in permeabilised cells (Grishanin *et al.*, 2004). However, PtdIns(4,5)P_2 binding is redundant for CAPS function when it is overexpressed (Grishanin *et al.*, 2002), and the major defect in CAPS1-deficient (knock-out, KO) chromaffin cells is at the stage of vesicle loading, rather than exocytosis (Speidel *et al.*, 2005). Thus in embryonic chromaffin cells from homozygous KO mice, vesicles fuse as normal, but do not release catecholamines since these are not taken up by the vesicle. However, it is possible that CAPS2 is able to compensate for an exocytic function

in the absence of CAPS1 at this developmental stage. In CAPS1 KO heterozygous chromaffin cells from one-month old mice, CAPS2 is no longer expressed and CAPS1 is expressed at reduced levels. In this situation, there is indeed a deficit in vesicle priming; homozygous mice were not viable after birth, so it was not possible to asses the effect of a complete absence of CAPS (Speidel *et al.*, 2005).

Two vesicle-associated proteins have been shown to interact with PtdIns(4,5)P_2 *in vitro*. One is the Rab3A interacting protein rabphillin, which binds PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 via its C2 domain. A second example is the proposed calcium sensor for exocytosis, synaptotagmin. Synaptotagmins I and II bind to PtdIns(4,5)P_2 via their C2B domains in a calcium-dependent manner (Schiavo *et al.*, 1996; Mehrotra *et al.*, 2000). Recently, this binding was shown *in vitro* to cause insertion of the domain into a lipid bilayer; this suggests a model where the calcium/PtdIns(4,5)P_2 interaction can stimulate a change in bilayer structure that may assist in fusion and thus triggering of exocytosis (Bai *et al.*, 2004).

Together, these studies suggest that binding of proteins to PtdIns(4,5)P_2 may localise the fusion machinery present on both the plasma and vesicular membranes, thus leaving factors in place to expedite fusion on arrival of the stimulus. In this way, PtdIns(4,5)P_2 would mediate priming by acting simply as a locally-produced factor that defined the site of fusion. However, the calcium-dependent interaction with synaptotagmin suggests that there may be a role for PtdIns(4,5)P_2 in the fusion process itself. However, given that depletion of PPIn does not inhibit calcium-dependent, ATP-independent fusion (Eberhard *et al.*, 1990), this seems unlikely to occur *in vivo*.

1.3.3. Regulation of compensatory endocytosis

Plasma membrane pools of PtdIns(4,5)P_2 are required for SV cycling (Micheva *et al.*, 2001). In synapses from PIPK Iγ KO cells, the major PIPK isoform at the synapse (Wenk *et al.*, 2001), the major defect is in compensatory membrane retrieval after exocytosis (Di Paolo *et al.*, 2004). Combined with a second study (Kim *et al.*, 2002), these data points to a cycle of PtdIns(4,5)P_2-synthesis and degradation as being important for efficient synaptic vesicle endocytosis: synapses devoid of synaptojanin fail to efficiently uncoat clathrin-coated vesicles

after endocytosis, impairing vesicle recycling. Furthermore, plasma membrane pools of PtdIns(4,5)P_2 were shown to regulate synaptic vesicle recycling in hippocampal neurons (Micheva *et al.*, 2001). The rate of recycling was subsequently shown to be modulated by postsynaptic NO signals, which increased PtdIns(4,5)P_2 levels in a cGMP-dependent manner (Micheva *et al.*, 2003).

PtdIns(4,5)P_2 is also known to regulate clathrin mediated endocytosis in a variety of other cell types (Itoh *et al.*, 2001). Although not well characterized in endocrine cells, it is likely that clathrin mediated membrane retrieval, and by extension PtdIns(4,5)P_2, is necessary for endocytosis in these cells, too. Furthermore, given the role of PtdIns(4,5)P_2 in actin dynamics, the lipid may also impinge on stages of vesicle transport. Interestingly, Micheva *et al* noted an effect of PtdIns(4,5)P_2 downstream of vesicle endocytosis, but could not resolve any effect of PtdIns(4,5)P_2 on synaptic vesicle exocytosis (Micheva *et al.*, 2003). Therefore, despite an impressive weight of evidence implicating PtdIns(4,5)P_2 during priming of exocytosis, it may be difficult to define precisely at which stage(s) of the exo-endocytic cycle the lipid acts.

1.4. Mast Cells

1.4.1. Biology and pathology of the mast cell

Mast cells are haemopoietic cells originating from bone marrow precursors. These precursors express the c-kit receptor, and proliferate in response to the c-kit ligand, otherwise known as stem cell factor (SCF). The cells differentiate in the tissues in response to local factors, such as interleukins. Broadly speaking, there are two classes of mast cells: the connective tissue or serosal mast cells (CTMCs), and the mucosal mast cells; the latter contain fewer, smaller cytosplasmic granules and have a histamine content ~10% that of the CTMCs (Metcalfe *et al.*, 1997). Typical experimental models for CTMCs are rat peritoneal mast cells (RPMCs); common models for mucosal mast cells include cultured bone-marrow derived mast cells (BMMCs) and the rat basophilic leukaemia cell line (RBL).

The physiological functions of mast cells include mediating inflammatory and allergic responses, clearing of microbial infections, and co-ordinating the adaptive

immune response in the peripheral tissues (Abraham and Malaviya, 1997; Metcalfe *et al.*, 1997; Theoharides and Cochrane, 2004). This occurs in two stages: firstly, pre-formed mediators such as histamine and proteases are released from the cytoplasmic granules via regulated exocytosis. Secondly, mediators such as arachidonic acid metabolites and interleukins are synthesised *de novo* and secreted from the cells. The first step is initiated very rapidly (in seconds or minutes), whereas the second produces a long-term response (tens of minutes to hours)(Metcalfe *et al.*, 1997).

Due to their ability to initiate inflammatory and allergic reactions, mast cell dysfunction is associated with a large number of human diseases. Among these is systemic mastocytosis, associated with over-proliferation of mast cells in specific tissues, with a spectrum of symptoms depending on the affected tissue and the degree of proliferation (Akin and Metcalfe, 2004). Other prominent examples include inflammatory diseases such as arthritis and migraine (Theoharides and Cochrane, 2004) and complex roles in asthma (Marone *et al.*, 2005).

1.1.2. Activation of mast cells

Mast cells become activated through two pathways. The archetypal pathway involves cross-linking of antigen receptors of the FCεR1 family. These receptors interact with IgE antibodies via their α subunits, which are cross-linked by binding of multivalent antigen. This in turn leads to phosphorylation of β and γ receptor subunits on their immune receptor tyrosine-based activation motifs (ITAM) by the Lyn tyrosine kinase. In turn, activation of the downstream tyrosine kinases Fyn and Syk occurs, adaptor proteins such as Vav, SLP-76, LAT and Gab2 are recruited, and activation of the signalling enzymes PLCγ and PI3K IA takes place (Kinet, 1999; Siraganian, 2003; Blank and Rivera, 2004). Studies with BMMCs from genetically manipulated mice have underscored the importance of PLCγ2 (Wen *et al.*, 2002) and PI3K Iδ (Ali *et al.*, 2004). However, studies with human umbilical cord mast cells have given rise to doubts as to how general the requirement for PLCγ is, since these cells instead rely on sphingosine kinase to mediate calcium signalling (Melendez and Khaw, 2002).

The second pathway involves activation by polycationic agonists, such as the synthetic agonist compound 48/80, the wasp venom mastoparan, and neuropeptides such as substance P (SP) and neurotensins (NT) (Ferry *et al.*, 2002). Antimicrobial peptides secreted from neutrophils, the defensins, have also been shown to activate mast cells (Befus *et al.*, 1999), as have the aminoglycoside antibiotics, which include neomycin (Raab, 1968; Aridor and Sagi-Eisenberg, 1990). Activation occurs through the heterotrimeric G-proteins G_{i2} and G_{i3}, as formally demonstrated for 48/80, spermine and mastoparan (Aridor *et al.*, 1993; Ferry *et al.*, 2001), with the signal most likely transduced by the $G_{\beta\gamma}$ subunits (Pinxteren *et al.*, 1998; Ferry *et al.*, 2001). Polycations are thought to activate the G-proteins directly after penetration of the plasma membrane by an ill-defined mechanism (Ferry *et al.*, 2002). The resulting exocytosis occurs via activation of PLCβ, but is independent of PI3K (Aridor *et al.*, 1993; Shefler *et al.*, 1998). Mast cells are often observed in the vicinity of neurons in the peripheral tissues, where they can be directly activated by exocytosis of SP and NT from synaptic granules (Furuno and Nakanishi, 2005). Therefore, this second activation pathway could reflect a neuroparacrine role for mast cells.

There is also evidence of autocrine signalling during mast cell exocytosis after activation of the antigen receptor pathway. This came from studies on BMMCs from mice devoid of the GPCR-coupled PI3K Iγ (Laffargue *et al.*, 2002). These cells exhibit severely impaired exocytosis, because they are unable to mediate stimulatory signalling by PI3K Iγ coupled to A3 receptors, which are activated by adenosine. The adenosine is secreted from mast cell granules during exocytosis, activating PI3K Iγ via A3 receptors, which leads to further degranulation. Thus adenosine mediates an autocrine-like, self-amplifying response. Similar observations were made with RPMCs, which activate calcium signalling in response to ATP secreted by neighbouring cells (Osipchuk and Cahalan, 1992).

Therefore activation of exocytosis by either pathway is believed to require G-proteins. In support for this, RPMC exocytosis is stimulated by the non-hydrolysable GTP analogue GTPγS when introduced via a patch pipette, by microinjection or through cell permeabilisation (Gomperts, 1983; Fernandez *et al.*, 1984; Howell *et al.*, 1987; Tatham and Gomperts, 1991). In addition to the

heterotrimeric G_i proteins discussed above, GTPγS is thought to act through small monomeric GTPases. Indeed, the small GTPases RhoA, Rac2 and Cdc42 activate exocytosis when introduced into permeabilised cells, whereas antagonists of these proteins inhibit exocytosis (Price *et al.*, 1995; Brown *et al.*, 1998). Furthermore, a cytosolic complex that supports exocytosis from permeabilised RPMCs was identified as Rac2 and Rho guanine nucleotide dissociation inhibitor (RhoGDI). The Rac2:RhoGDI complex supports exocytosis, whereas RhoGDI alone inhibits (O'Sullivan *et al.*, 1996). As is the case for neuronal cells, the Rab GTPases have also been implicated in mast cell exocytosis. Expressly, these include Rab27 (Goishi *et al.*, 2004) and the mast-cell specific Rab37 (Masuda *et al.*, 2000).

Thus in contrast to neuronal and endocrine systems where calcium alone provides the trigger for exocytosis, mast cells rely on the dual action of GTP- and Ca^{2+}-binding proteins (Howell *et al.*, 1987). Under certain conditions, either effector alone is sufficient to trigger exocytosis in the presence of MgATP (Churcher and Gomperts, 1990; Koffer and Churcher, 1993). However, exocytosis triggered in response to guanine nucleotide is rather less dependent on MgATP (Koffer and Churcher, 1993); the dependence of calcium-induced secretion on ATP can be ascribed, in part, to transphosphorylation of GDP from ATP by an endogenous guanine nucleoside diphosphate kinase (Lillie and Gomperts, 1992; Koffer, 1993). Thus GTP is believed to be essential for exocytosis, whereas calcium only acts in a modulatory role.

1.4.3. Cell biology of exocytosis

The morphology of CTMCs is exemplified by the RPMC, as shown in figure 1.8A. The cytosol is packed with around a thousand dense-core granules. These are of lysosomal origin (Blott and Griffiths, 2002), and form by homotypic fusion of smaller granules as the cell matures over a period of weeks (Alvarez de Toledo and Fernandez, 1990b). The surface morphology of the cells is characterized by a taut plasma membrane overlying cortical granules, and is decorated with numerous thread-like membrane folds (Kessler and Kuhn, 1975; Burwen and Satir, 1977).

Complete exocytosis of all the granules occurs from RPMC within approximately 30 s upon stimulation with antigen (Lawson *et al.*, 1975; Lawson *et al.*, 1977) or polycations (Kagayama and Douglas, 1974). During exocytosis, compensatory endocytic events are comparably rare (Fernandez *et al.*, 1984). However, catastrophic increases in surface area:volume ratio are prevented by the exploitation of a specialised mode of exocytosis, called compound exocytosis. This occurs by virtue of the fact that only the cortical granules fuse at the plasma membrane; these granules become targets for sequential fusion of internal granules (Rohlich *et al.*, 1971). In this manner, heterotypic fusion between granule and plasma membranes is accompanied by homotypic fusion between granules, forming large vacuole-like structures that communicate with the extracellular space (figure 1.8B). A model of this process is presented in figure 1.9A.

Studies on increases in membrane capacitance during RPMC exocytosis noted step-like increases in capacitance representing fusion of single granules. However, larger step sizes were also observed which could represent fusion of a large, or compound granule formed by homotypic fusion of the smaller granules (Fernandez *et al.*, 1984). This suggests a modified model for exocytosis (figure 1.9B) whereby homotypic fusion between granules is not always sequential, but can occur before fusion at the plasma membrane. A third model was proposed by Alvarez de Toledo and Fernandez, after further examination of capacitance (figure 1.9C): as well as larger increases in capacitance, large decreases in capacitance could be observed. Therefore, sequential exocytosis of a cortical granule with the plasma membrane followed by fusion of internal granules could form an internal, compound granule after fission of the cortical granule from the plasma membrane. When this granule re-fuses at the plasma membrane, a large compound fusion event is thus observed (Alvarez de Toledo and Fernandez, 1990a). This model suggests that some factor is transferred from the plasma membrane to confer fusion competence on the granules.

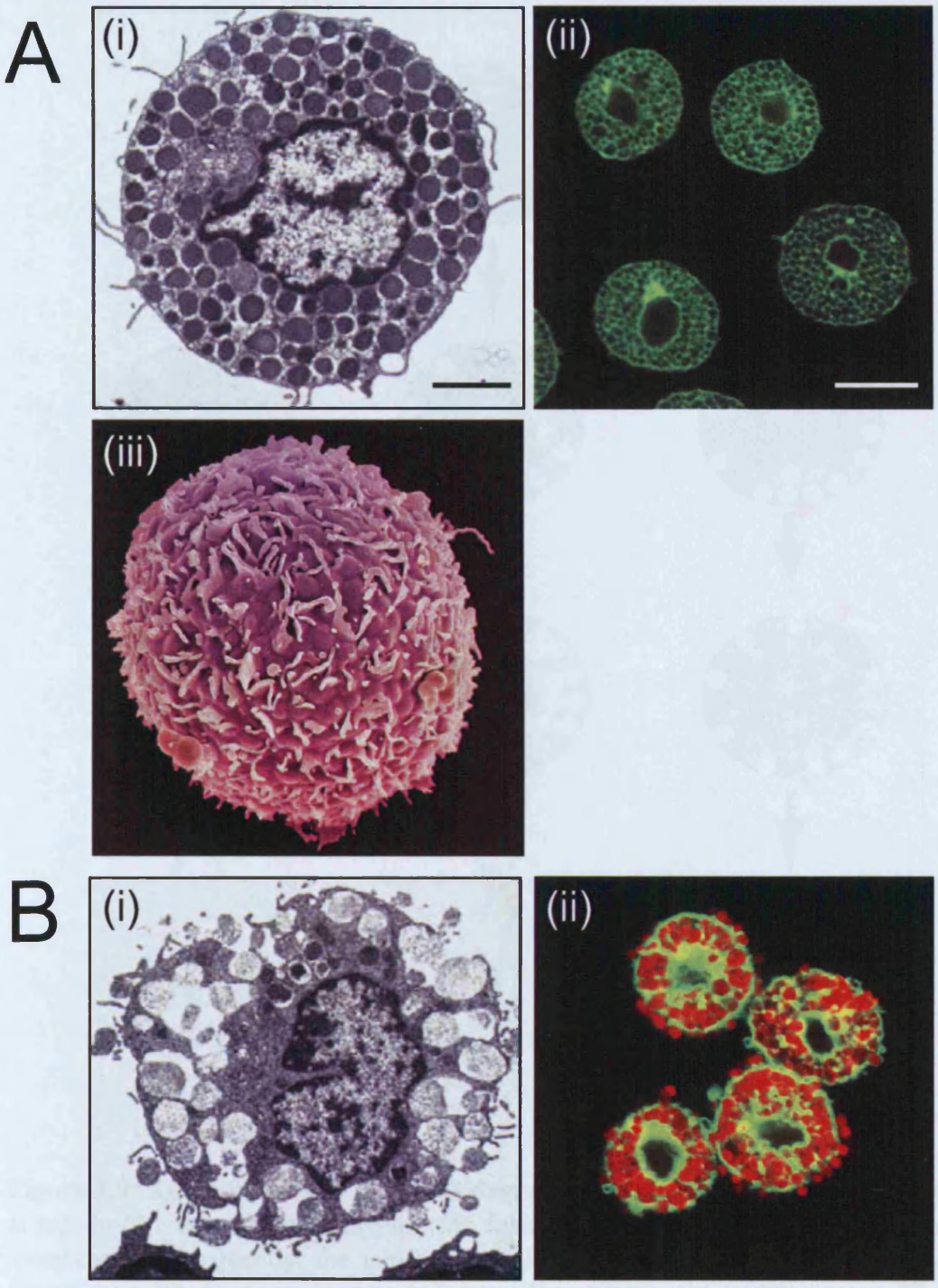

Figure 1.8: Resting and stimulated rat peritoneal mast cells (RPMCs). (A) Resting RPMCs viewed by transmission electron microscopy (TEM) (i), confocal microscopy after staining with BODIPY-ceramide for membranes (ii) or by field-emission electron microscopy (FESEM) with false-colour (iii). Note the electron-dense granule cores. (B) RPMCs after exocytosis (degranulation), viewed by TEM (i) or confocal (ii) microscopy. For the latter, cell membranes were stained with BODIPY-ceramide (green) and granule cores with Alexa647-concanavalin A (red). Bars = 5 μm (TEM micrographs) or 10 μm (confocal micrographs). FESEM micrograph was kindly provided by Stephen Gschmeissner (CR-UK electron microscopy unit). TEM micrographs are reproduced from Alberts *et al* "Molecular Biology of the Cell" 4th Ed. Garlund Publishing 2004.

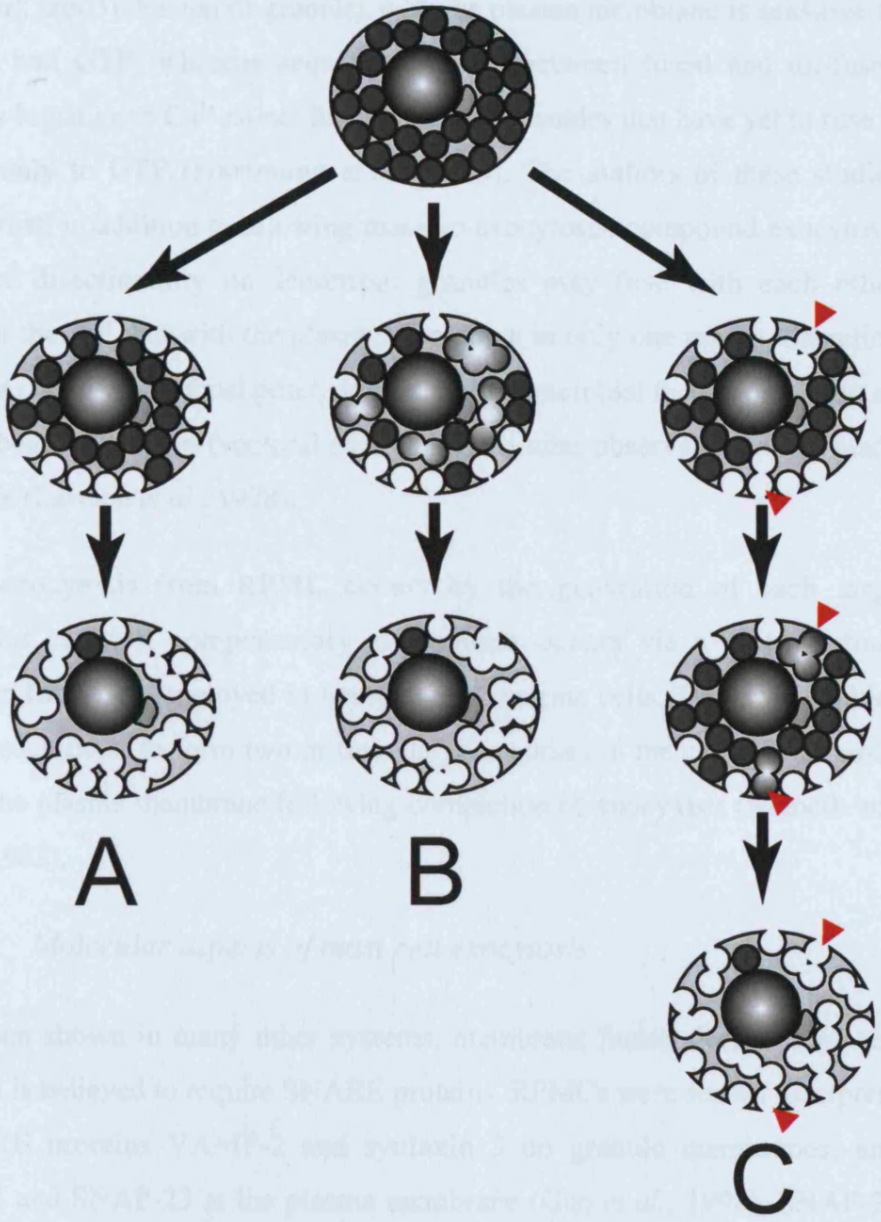

Figure 1.9: Three models of mast cell degranulation. A resting mast cell is shown at top. In (A), degranulation begins with fusion of cortical granules with the plasma membrane. Subsequently, the internal granules fuse with these plasma membrane-fused granules to form compound granules, which are continuous with the extracellular milieu. In a second model of degranulation (B), fusion of cortical granules with the plasma membrane is accompanied by homotypic fusion between granules, forming compound granules that are yet to fuse with the plasma membrane (lighter shading). Degranulation is completed after fusion of single and compound granules with the plasma membrane or plasma membrane-fused granules. In the third (C), granules fuse with the plasma membrane and each other in a sequential manner as in (A). However, certain granules that have fused with the plasma membrane then re-seal (red arrow heads); these re-fuse with the plasma membrane later on as a multi-granule compound. The end result of all three models is thus the same, with compound granules continuous with the extracellular milieu.

All three models of compound exocytosis were shown to occur in the eosinophil (Hafez *et al.*, 2003). Fusion of granules with the plasma membrane is sensitive to both Ca^{2+} and GTP, whereas sequential fusion between fused and un-fused granules is sensitive to Ca^{2+} alone; fusion between granules that have yet to fuse is sensitive only to GTP (Hartmann *et al.*, 2003). The authors of these studies proposed that, in addition to allowing massive exocytosis, compound exocytosis can confer directionality on secretion: granules may fuse with each other throughout the cell, but with the plasma membrane in only one region. Secretion would thus occur from a focal point, delivering anti-microbial factors only onto an adjacent, bound pathogen (vectoral exocytosis). Similar observations were made for RPMCs (Lawson *et al.*, 1978).

Because exocytosis from RPMC occurs by the generation of such large intracellular cavities, compensatory endocytosis occurs via a quite distinct mechanism from that employed in neural and endocrine cells. The large cavities fuse with each other to form two or three large vacuoles in the cell, which pinch off from the plasma membrane following completion of exocytosis (Nemeth and Rohlich, 1982).

1.4.4. Molecular aspects of mast cell exocytosis

As has been shown in many other systems, membrane fusion during mast cell exocytosis is believed to require SNARE proteins. RPMCs were shown to express the SNARE proteins VAMP-2 and syntaxin 3 on granule membranes, and syntaxin-4 and SNAP-23 at the plasma membrane (Guo *et al.*, 1998). SNAP-23 was found associated with the actin cytoskeleton in the surface membrane folds, but relocated to granule membranes to expedite compound exocytosis (Guo *et al.*, 1998). Therefore, SNAP-23 may constitute a plasma membrane factor conferred to the granules to enable their fusion during sequential exocytosis. However, SNAP-25 has recently been demonstrated on RPMC granules (Salinas *et al.*, 2004). Several studies have also been performed on the mucosal RBL mast cell model (Blank *et al.*, 2002). These have detected expression of syntaxins 2-4, SNAP-23 and VAMP 2, 3, 7 and 8; overexpressed syntaxin 3 and VAMP7 were found on granule membranes, with syntaxin 3 also present at the plasma membrane (Hibi *et al.*, 2000). Endogenous syntaxin 4 and SNAP-23 could be

detected in complex with all four Vamp proteins but not syntaxin 3 from RBL cell lysates (Paumet *et al.*, 2000). Furthermore, overexpression of syntaxin 4 lead to an inhibition of exocytosis, leading the authors to propose syntaxin 4 as the relevant isoform in mast cell exocytosis (Paumet *et al.*, 2000).

Several regulatory proteins have also been discovered in mast cells. RBL cells express Munc18-2 and Munc18-3 (Martin-Verdeaux *et al.*, 2003). Munc18-3 is found at the plasma membrane in complex with syntaxin 4, whereas Munc18-2 is present at granule membranes in complex with syntaxin 3; overexpression of Munc18-2 but not Munc18-3 led to inhibition of exocytosis (Martin-Verdeaux *et al.*, 2003). The authors therefore proposed that the Munc18-2:syntaxin 3 complex was required for RBL cell exocytosis, in contrast with their earlier proposal (Paumet *et al.*, 2000). Munc13-4 has also been detected in RBL cells as an effector of Rab27 (Goishi *et al.*, 2004; Neeft *et al.*, 2005), and overexpression of Munc13-4 potentiates exocytosis (Neeft *et al.*, 2005).

Synaptotagmin I has been demonstrated to enhance RBL exocytosis when ectopically expressed (Baram *et al.*, 1998). However, subsequent studies showed that this isoform is not endogenously expressed, and the predominant isoform, synaptotagmin II, was a negative regulator of exocytosis (Baram *et al.*, 1999), since a reduction in expression levels potentiates exocytosis. Recently, complexin II has also been identified in RBL cells, where it is required for efficient coupling of calcium transients to exocytosis (Tadokoro *et al.*, 2005).

Several novel regulators of exocytosis have been identified in RPMCs. A cytosolic factor from bovine brain, secernin, is able to enhance the calcium sensitivity and extent of exocytosis from RPMCs (Way *et al.*, 2002). Sequestration of endogenous secernin with antibodies was also shown to inhibit exocytosis. Secretory carrier associated membrane proteins (SCAMPs) have also been demonstrated to be important regulators of exocytosis. Peritoneal mast cells from SCAMP-1 deficient mice display a reduced extent of exocytosis and impaired fusion pore kinetics (Fernandez-Chacon *et al.*, 1999). SCAMP-2 was

also demonstrated to be required at a late stage of RPMC exocytosis (Guo *et al.*, 2002), as was the case in PC12 cells (Liu *et al.*, 2002).

Together, these studies demonstrate that the molecular machinery controlling SNARE-dependent membrane fusion is present in mast cells. However, it is still far from clear which isoforms are required, and when they interact during the fusion process.

Mast cell exocytosis is also accompanied by dynamic changes in the cytoskeleton. Resting mast cells contain an F-actin cortex, which is partially disassembled in response to stimulation in RPMCs (Norman *et al.*, 1994) and BMMCs (Nishida *et al.*, 2005). Subsequently, polymerisation of actin occurs to produce centripetal filaments in RPMCs (Norman *et al.*, 1994). This is regulated by the same Rho-family GTPases as exocytosis, but by a parallel pathway, since actin dynamics can be blocked without effect on exocytosis (Norman *et al.*, 1996; Sullivan *et al.*, 1999). However, partial disassembly of the F-actin cortex with latrunculin B or gelsolin potentiates exocytosis in RPMCs, BMMCs or RBL cells (Borovikov *et al.*, 1995; Martin-Verdeaux *et al.*, 2003; Sasaki *et al.*, 2005), whereas complete disassembly prevents exocytosis in RPMCs (Pendleton and Koffer, 2001). Therefore, actin appears to play a complex and poorly-defined role in mast cell exocytosis. On the other hand, there is clear evidence for a role of microtubule formation in RBL and BMMC exocytosis (Martin-Verdeaux *et al.*, 2003; Nishida *et al.*, 2005). Polymerisation of microtubules in the interior of BMMCs was proposed to facilitate translocation of granules to the plasma membrane, where they fuse (Nishida *et al.*, 2005).

1.4.5. Priming and the role of phosphoinositides in mast cell exocytosis

Evidence for priming of exocytosis in mast cells comes from experiments conducted on streptolysin-O (SL-O) permeabilised RPMCs. SL-O binds to membrane cholesterol in a temperature-independent manner, before oligomerising to form ~30 nm circular and arc-shaped pores in a cholesterol-independent, temperature-dependent fashion (Bhakdi *et al.*, 1985; Sekiya *et al.*, 1996; Palmer *et al.*, 1998). RPMCs permeabilised in this way leak soluble proteins and lose

responsiveness to stimulation by Ca^{2+} and GTPγS (Koffer and Gomperts, 1989; Pinxteren *et al.*, 2000). This system also permits the effect of exogenously applied proteins in maintaining secretory competence to be tested (Gomperts and Tatham, 1992).

No ATP is required if stimulation (Ca^{2+} and GTPγS) is provided at the moment of permeabilisation with SL-O (Howell *et al.*, 1987). However, if the stimulus is delayed, RPMCs lose responsiveness within 5 minutes; the presence of ATP prolongs the period of responsiveness for approximately half an hour (Howell *et al.*, 1989). Notably, these requirements persist if endogenous ATP is depleted by metabolic inhibition. From these experiments, it was concluded that a phosphorylation state is required for exocytosis, and that ATP preserves responsiveness to stimulation by maintaining this phosphorylation state (Pinxteren *et al.*, 2000). By analogy with the terminology of vesicle priming applied to neuroendocrine cells, (Holz *et al.*, 1989; Hay and Martin, 1992), it may be concluded that the mast cell granules exist in a primed state, but that priming is lost after cell permeabilisation. Rundown of cell responsiveness thus occurs due to the loss of soluble factors required for both priming and the coupling of the stimulus to the exocytic response.

As for neural and endocrine cells, several studies have furnished evidence that PPIn are required for priming RPMC exocytosis. A PH domain that sequesters endogenous PPIn was shown to inhibit exocytosis from permeabilised RPMCs (Pinxteren *et al.*, 1998; Pinxteren *et al.*, 2001). Furthermore, neomycin, which tightly binds PPIn (Schacht, 1978), was found to mimic the effect of ATP depletion on the loss of secretory reponsiveness from RPMCs; on the other hand, responsiveness could be prolonged using PITP (Pinxteren *et al.*, 2001). The basic effector peptide from SCAMP2 that antagonizes exocytosis in RPMC (Guo *et al.*, 2002) was recently show to sequester PtdIns(4,5)P_2 (Ellena *et al.*, 2004). Maintenance of secretory competence in SL-O permeabilised RBL cells was also shown to require phospholipase D (PLD) and Arf1-driven synthesis of PtdIns(4,5)P_2 (Way *et al.*, 2000).

As described above, activation of PI3K is important for stimulation of mast cell exocytosis in response to antigen. Inhibitors of PI3K were found to block

exocytosis from BMMCs evoked using calcium ionophores (Marquardt *et al.*, 1996), although they had no such effect on SL-O permeabilised RPMCs activated with Ca^{2+} and GTPγS (Pinxteren *et al.*, 1998). PI4K IIIβ was shown to be necessary for exocytosis from antigen-stimulated RBL cells, though not for cells stimulated with calcium ionophore (Kapp-Barnea *et al.*, 2003). Furthermore, stimulation of RBL cells with antigen also activates a type II PI4K activity (Naveen *et al.*, 2005), apparently required for exocytosis. Finally, accelerated PPIn metabolism is observed in RPMCs activated with either antigen or polycations (Cockcroft and Gomperts, 1979).

1.5. Aims

As discussed above, it is clear that the PPIn, especially PtdIns4*P* and PtdIns(4,5)P_2, are required for exocytosis. Their synthesis seems to be a requisite step in the acquisition of fusion competence for a regulated secretory organelle. Mast cell exocytosis is certainly associated with PPIn metabolism, and this may even be required for the maintenance of a primed state. However, it is not clear which PPIn are required. Furthermore, precisely at which stage and within which membranes the lipids act is unresolved, as are the molecular interactions that mediate their function during exocytosis. RPMCs possess certain advantages as a model system in which to resolve these issues. Firstly, as in neuroendocrine cells, the ATP-dependent priming step can be easily dissected out from the ATP-independent triggering step. Secondly, exocytosis occurs in a single, rapid burst and can therefore be resolved from other stages of the exocytic/endocytic cycle. Finally, the PPIn-dependent membrane recycling steps do not accompany mast cell exocytosis.

Therefore, the express aims of the experiments described in this thesis were to discover (i) which PPIn are required for mast cell exocytosis, (ii) in which membranes they reside and (iii) what the mechanisms of action of these lipids are.

In chapter 3, results are presented from studies in permeabilised cells that attempted to resolve which PPIn need to be synthesised to maintain secretory competence, i.e. which lipids are involved in priming. In the following chapter, conditions for immunofluorescent detection of PPIn are established. Using this

technique, it is established that PtdIns(4,5)P_2 resides at the plasma membrane, but becomes transiently depleted during RPMC exocytosis. Chapter 5 aims to resolve which enzyme activity is responsible for this decrease, and whether it is mandatory for exocytosis to occur. Finally, these results are discussed in the context of a model for PPIn function at multiple stages of the exo-endocytic cycle.

Chapter 2: Materials and Methods

2.1. Materials

2.1.1. Antibodies

The following monoclonal antibodies were from CR-UK monoclonal antibody services: anti-PI4K II 4C5G (Endemann *et al.*, 1991), anti c-erbB3 2E11(Rajkumar *et al.*, 1993), anti-PtdIns(4,5)P_2 2C11 and 10F8 (Thomas *et al.*, 1999). All were purified, except 10F8, which was culture supernatant from clone 10F8. Monoclonal anti-PtdIns(4,5)P_2 kt3g (Matuoka *et al.*, 1988) was from Assay Designs. Monoclonal anti PtdIns(3,4)P_2 antibody PO34 was a kind gift of Echelon. Polyclonal anti-GST was from Chemicon; monoclonal anti-GST was from Sigma. Alexa fluorophore conjugated goat secondary antibodies were from Molecular probes. Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse antibodies were from Dako.

2.1.2. Chemicals

Synthetic PPIn and InsPP were from Cell Signals. Lipids were dissolved in $CHCl_3$:MeOH (1:1), whereas InsPP were dissolved in water. Gro*P*Ins(4,5)P_2 was from Sigma and dissolved in EtOH; 1-oleoyl 2-palmitoyl PtdCho was from Avanti and dissolved in $CHCl_3$:MeOH (1:1). All were stored in sealed glass vials (except InsPP, which were in plastic vials) at –20°C.

Neomycin and amikacin were from Alexis and Sigma, respectively. 100 mM stocks were dissolved in water and stored in small single-use aliquots at –20°C. U73122, U73343 and Et-18-OMe were from Calbiochem. U73122 and U73343, dissolved in $CHCl_3$, were split into aliquots, dried under nitrogen and stored at –20°C. Aliquots were warmed to room temperature, dissolved in anhydrous DMSO to 2 mM and used within a day; they were diluted immediately prior to use. PAO (Sigma) was dissolved in $CHCl_3$; aliquots were dried and stored under nitrogen at room temperature. Immediately prior to use, aliquots were dissolved in anhydrous DMSO to 20 mM and diluted. Et-18-OMe was dissolved to 9 mM in EtOH and stored at –20°C. Aliquots were warmed and diluted immediately before use. LY294002 and LY303511 (Sigma) were prepared as 5 μl single-use aliquots in anhydrous DMSO at 65 mM; they were stored at –20°C,

thawed and diluted immediately before use. Wortmannin was from Sigma; a 20 mM stock solution in anhydrous DMSO was prepared in the dark, split into aliquots and stored at –20°C. Aliquots were thawed and diluted immediately prior to use. β-glycerophosphate (Sigma) was dissolved in water to 1 M and stored at –20°C. DAG analogues phorbol 12-myristate 13-acetate (PMA), 4-α-PMA, phorbol 12,13-dibutyrate (PDBu) and 1-oleoyl 2-acetyl-*sn*-glycerol (OAG) were from Sigma. Stock solutions were frozen as 20 mM aliquots in DMSO at –20°C. 100 mM solutions of MgATP were prepared by dissolving Na_2ATP (Roche) in 100 mM $MgCl_2$, 200 mM Tris-Cl, pH 6.8. 100 μl single-use aliquots were stored at –20°C. GTPγS (Li salt solution; Roche) was stored at –20°C. *Myo*-[2-^{3}H]-inositol, *myo*-[2-^{3}H]-PtdIns, [^{14}C]-PtdCho and [γ-^{32}P]-ATP were from Amersham. *Myo*-[2-^{3}H]-Ins(1,4,5)P_3 was from Perkin Elmer.

2.1.3. Constructs

The following constructs were obtained as detailed:

Construct	Vector	Source	Reference
GST-PH-βARK^{WAA}	pGEX-2T	J. Pinxteren (Univeristy of Gent)	(Touhara *et al.*, 1995)
GST-2xFYVE^{Hrs}	pGEX-5X-3	H. Stenmark (The Norwegian Radium Hospital)	(Gillooly *et al.*, 2000)
GST-PH-FAPP1	pGEX-4T-1	O. Gozani (Stanford University)	(Dowler *et al.*, 2000)
GST-PH-TAPP1	pGEX-4T-1	D. Alessi (University of Dundee)	(Dowler *et al.*, 2000)
GST-PH-DAPP1	pGEX-4T-1	D. Alessi (University of Dundee)	(Dowler *et al.*, 2000)
GST-PH-PLCδ1	pGEX-2T	M. Katan (ICR, London)	(Lemmon *et al.*, 1995)
GFP- PH-PLCδ1	pEGFP-N1	M. Katan (ICR, London)	(Varnai and Balla, 1998)
His_6-SigD	pET28a	B. Finlay (University of British Columbia)	(Marcus *et al.*, 2001)
His_6-SigD^{C462S}	pET28a	B. Finlay (University of British Columbia)	(Marcus *et al.*, 2001)

Table 2.1: Constructs used in this thesis and their sources

PH-GRP1 (2-G splice variant), defined according to (Klarlund *et al.*, 2000), was cloned from a pQE30-GRP1 construct (a kind gift of G. Thomas, University College London) by polymerase chain reaction using the following primers:

5': GAGCTGCTGAGGAATTCGTATGAGAGCATTAAGAACGAGCC

3': GTCTTTTAGCCTGTCGACCTATTTTTTATTGGCAATCCTTCG

The coding sequence of PH-GRP1 is boxed and the stop codon is shaded. Restriction sites are underlined. The resulting oligomeric nucleotide was digested with EcoR1 (5') and Sal1 (3'), and ligated into the same sites of a pGEX-4T3-Cys vector (Lalli *et al.*, 2003). This vector contains an alanine-rich helical linker between the GST tag and cloned protein, which contains 4 cysteines so may be covalently bound to maleimide conjugated reporters.

2.1.4. Proteins

PtdIns-specific PLC from *Bacillus cereus* was purchased from Sigma. Aliquots were dissolved in PIPES buffered salts solution (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.05% NaN_3, 20 mM PIPES-NaOH, pH 6.8) at 10 U/ml, flash frozen in liquid nitrogen and stored at –80°C. $MARCKS_{151-175}$ was produced by the peptide synthesis laboratory, CR-UK; it was dissolved to 10 mM in PIPES buffered salts solution and split into small single use aliquots; these were flash-frozen under liquid nitrogen and stored at –80°C.

DNA was freshly transformed into the indicated strain of *E. coli*, then grown in 2 l cultures (with ampicillin or kanamycin, as appropriate) until OD_{600} = 0.8-1.4. They were induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) and left to express as indicated in Table 2.2.

Protein	Strain of *E. coli*	IPTG (μM)	Induction time (h)	Induction temperature (°C)
GST-PH-βARK^{WAA}	TG1	400	8	25
GST-2xFYVE^{Hrs}	BL21 (DE3)	500	3	37
GST-PH-FAPP1	BL21 (DE3)	400	3	30
GST-PH-TAPP1	BL21 (DE3)	400	3	30
GST-PH-DAPP1	BL21 (DE3)	400	3	30
GST-PH-PLCδ1	TG1	400	3	30
GST-PH-GRP1	BL21 (DE3)	400	3	30
His_6-SigD	BL21 (DE3)	100	18	16
His_6-SigD^{C462S}	BL21 (DE3)	100	18	16

Table 2.2. Conditions for induction of recombinant protein expression

After induction, bacteria expressing GST-fusion proteins were harvested, washed twice in phosphate-buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.2) containing 0.05% Tween-20 and lysed by two passages in a French Press maxi cell (Aminco) at 1300 p.s.i. in breaking buffer (PBS with EDTA-free protease inhibitor tablets [Roche], 2 mM EDTA, 4 μg/ml pepstatin, 0.1% β-mercaptoethanol and 0.05% Tween-20). Lysates were then

cleared of insoluble material by centrifugation at 27,000 g_{AV} for 15 min to produce a bacterial suspension, followed by 160,000 g_{AV} for 20 min to produce a bacterial extract. Supernatants were then bound to glutathione-agarose (Sigma) for 1-2 hours at 4°C with rotation. Unbound proteins were removed by extensive washing with PBS-0.05% Tween-20; bound proteins were eluted twice with 2.5 mM reduced glutathione (Roche), 100 mM NaCl (in 50 mM Tris-Cl, pH 8.0), and once with 2.5 mM reduced glutathione, 500 mM NaCl (in 50 mM Tris-Cl, pH 8.0) for 5 minutes at room temperature. Proteins were concentrated against either 10-40% PEG (40,000, MWCO = 14,000) or by centrifugation through Amicon Ultra centrifuge filter devices (Millipore) (MWCO = 15,000) until > 1 mg/ml. Concentrations were determined by the Bradford method. Finally, proteins were dialyzed into PIPES buffered salts solution, flash-frozen in liquid nitrogen and stored at –80°C.

PH-βARK^{WAA} required further purification by gel filtration on a Superdex High resolution preparatory grade 16/60 column (Amersham) at a flow rate of 0.1 ml/minute with PIPES buffered salts solution. The columns were connected to an ÄKTA™ fast protein liquid chromatography (FPLC) system running Unicorn™3.10 software (Amersham Pharmacia). 0.5 ml fractions were analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining; purified fractions were pooled, concentrated and frozen as above.

For His_6-tagged proteins, a similar procedure was used with modifications. Firstly, breaking buffer consisted of PBS with EDTA-free protease inhibitor tablets, 2 mM EDTA, 4 μg/ml pepstatin, 0.1% β-mercaptoethanol, 10 mM imidazole and 0.2% Triton X-100. Secondly, proteins were bound on Ni-agarose (Qiagen), and unbound proteins removed with PBS-0.2% Triton X-100. Finally, recombinant protein was eluted from the Ni-agarose resin using a solution consisting of 250 mM imidazole, 150 mM NaCl and 50 mM Tris-Cl, pH 8.0.

Purity of all proteins was assessed by SDS-PAGE (figure 2.1), and was ≥ 80% of the total protein content, with the exception of SigD and SigD^{C462S}, which were ~50%.

2.1.5. Streptolysin-O

Steptolysin-O (SL-O) was purchased from three separate suppliers during the course of the experiments described in this thesis, as it became unavailable from various sources. SL-O obtained from Corgenix or Sigma-RBI (reduced and lyophilised with phosphate-buffered saline) was dissolved in water to 20 IU/ml. 4.8 IU aliquots were frozen in liquid nitrogen and lyophilised, before storing at –80 °C for up to 6 months. SL-O from iTEST came as a purified, lyophilised powder in 22 IU aliquots. This was reduced for 1h at 37 °C with 20 mg sodium dithionite in PBS (supplemented with 1 mg/ml BSA) at a concentration of 20 IU/ml. 4.8 IU aliquots were flash frozen, stored at –80°C and used within 1 month. SL-O from different sources showed comparable activities on the loss of secretory competence of mast cells after permeabilisation (figure 2.2).

2.2. Calcium buffers

An approximately 284 mM (prepared in error; the solution would ideally have been 300 mM) solution of EGTA (Sigma, ≥ 97% purity) was prepared with 60 mM PIPES-NaOH, pH 6.8. 25 ml of this solution was end-point titrated against a volumetric solution of 0.4 M $CaCl_2$, using 5 ml 10 M NaOH and 2.5 ml potassium oxalate as an indicator (end-point was reached when persistent cloudiness of the solution formed). From this titration, it was apparent that the EGTA solution was 96.4% pure, i.e. at a concentration of 0.2736 M. To 100 ml of this solution, an equimolar quantity of $CaCl_2$ was added. Both the equimolar CaEGTA and the EGTA solutions were adjusted to pH 6.8, and made up to 200 ml with Millipore water, giving a final stock solution of EGTA or CaEGTA of 136.8 mM. These solutions were mixed in appropriate ratios to form a buffered solution with $[Ca^{2+}]_{free}$ between 10^{-8} and 10^{-5} M at 30 °C, pH 6.8. This was achieved using LigandY software as described previously (Gomperts and Tatham, 1992).

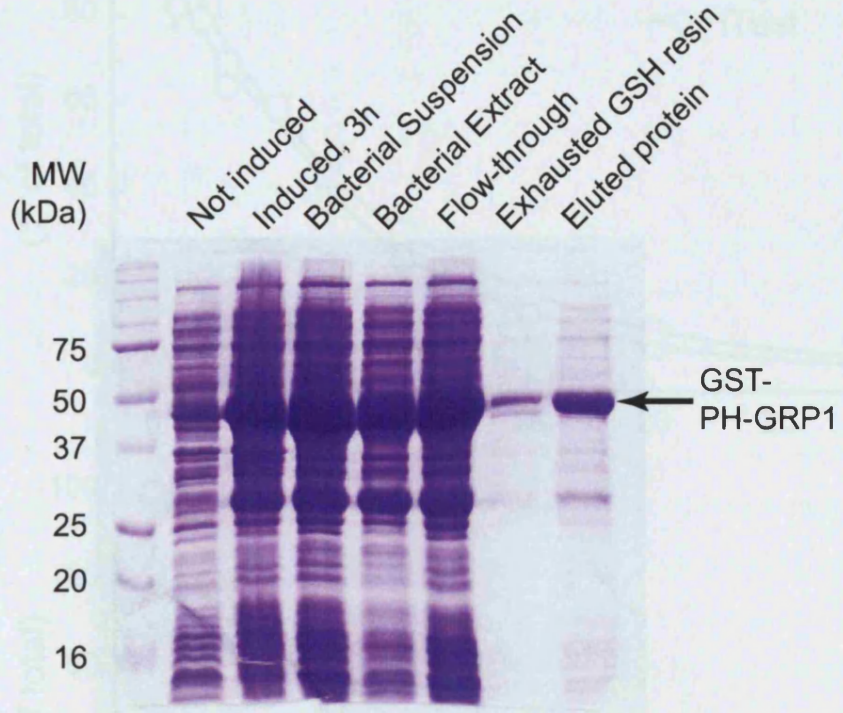

Figure 2.1: Purification of GST-PH-GRP1. (A) Coomassie stained polyacrylamide gel (10%); lanes show different stages of purification as indicated in the text. "Not induced" was sampled just prior to addition of IPTG (400 μM). "Flow-through" is the material that did not bind to the reduced glutathione-agarose resin; "exhausted resin" is the protein remaining bound after elution with free reduced glutathione. The arrow points to the GST-PH-GRP1 band with expected molecular mass of 46 kDa.

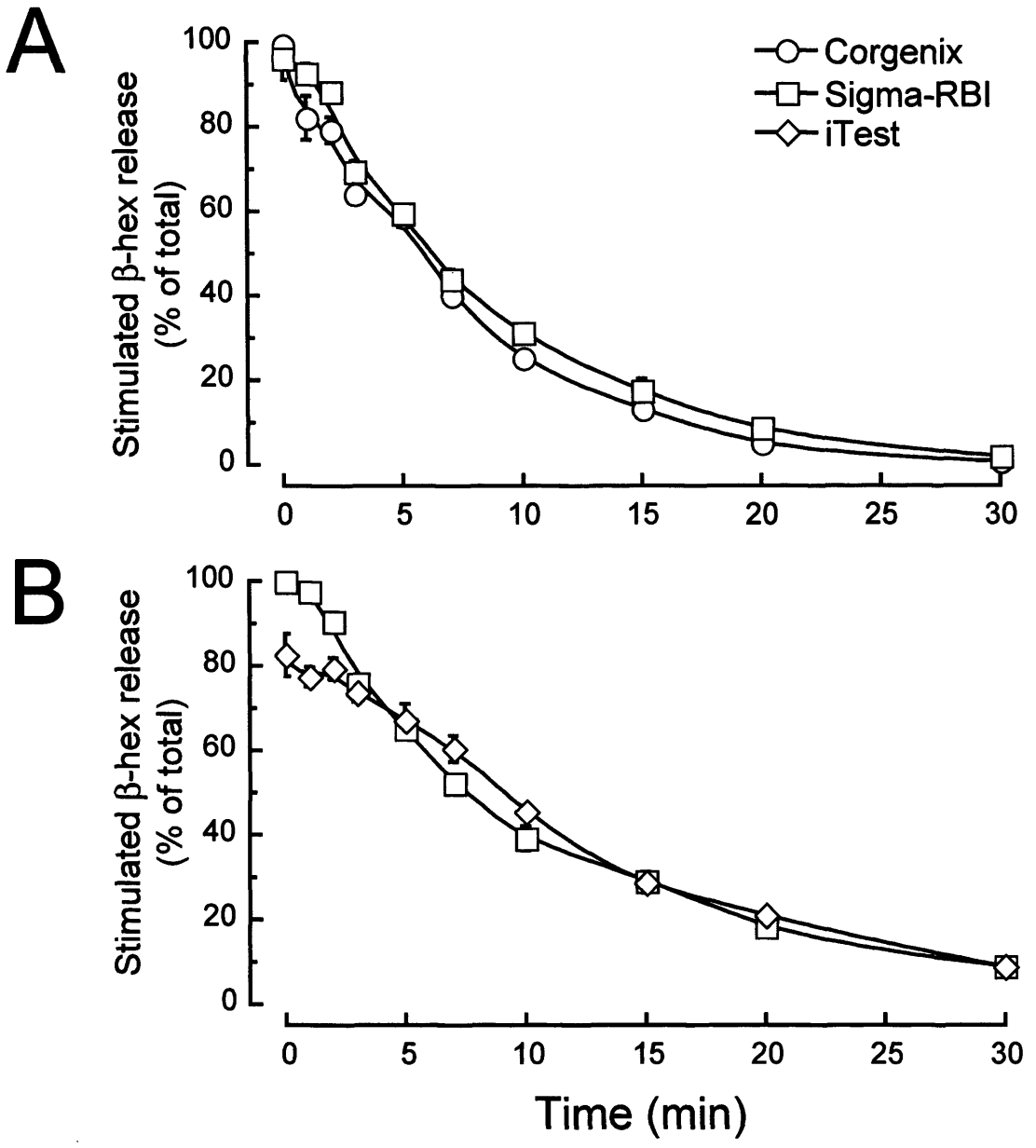

Figure 2.2: Rundown after permeabilisation with SL-O from different sources. (A) RPMCs pre-incubated with 1.2 IU/ml SL-O from the indicated supplier were permeabilised by warming to 30°C at time = 0 in 0.3 mM Ca:EGTA at pCa 8, 100 μM MgATP. Thereafter, they were stimulated at the indicated time with 3 mM Ca:EGTA (at pCa 5) and 100 μM GTPγS in the continuing presence of 100 μM MgATP. Secretion was allowed to proceed for 10 minutes, before cells were quenched on ice with 5 mM EGTA. Data are means ± S.E.M. of triplicate determinations. (A) compares SL-O from Corgenix and Sigma-RBI; (B) compares SL-O from Sigma-RBI with iTest.

2.3. Cells

2.3.1. Rat peritoneal mast cells

Rat peritoneal mast cells (RPMCs) were purified from the peritoneal cavities of Sprague Dawley rats (Charles River). For most experiments, male retired-breeder rats were used. Rats were killed in a rising concentration of CO_2 followed by cervical dislocation. Peritoneal cells were isolated by peritoneal lavage using two approximately 50 ml washings of 0.9% NaCl, 0.1% BSA. Washings were centrifuged at 300 g_{AV}, resuspended in a buffered salts solution and pooled to a volume of ~8 ml. The buffered salts solution was either Intracellular Buffer (IB: 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 20 mM PIPES-NaOH, pH 6.8, 1 mg/ml BSA) for cells that would be kept in suspension, or Extracellular Buffer (EB: 137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM D-glucose, 20 mM HEPES-NaOH, pH 7.2, 1 mg/ml BSA) for cells that would be used adherent (see below). The suspension was filtered through a nylon mesh (made of 'net curtain' material, John Lewis), and layered over ~ 2 ml of a 1.114 g/ml Percoll cushion (Amersham) in a 'v'-bottom, 15 ml polystyrene centrifuge tube. The Percoll cushion consisted of (for 20 ml) 15.91 ml Percoll stock at 1.13 g/ml, 2 ml 10xPBS at 1.1056 g/ml (Gibco) and 2.09 ml sterile Millipore water (density taken as 1.000 g/ml).

Mast cells were purified by centrifugation over this cushion for 5 minutes at 300 g_{AV}; the dense mast cells pass through the Percoll and form a pellet at the base of the tube, whereas contaminating neutrophils and macrophages form an interface between the buffered salts and Percoll. After aspirating away the buffered salts, interface and Percoll, the mast cell pellet was resuspended in 200 μl of buffered salts. This suspension was transferred to a fresh 15 ml tube, and the cells were washed once by dilution with buffered salts and centrifugation at 300 g_{AV}. Finally, cells were resuspended in a convenient volume depending on the experiment (between 1-5 ml). Yield was typically 10^6 cells/rat with a purity > 98%.

For adherent cells, 40 μl of the above suspension was pipetted into wells of 8-well multitest slides (MP Biomedicals) or flat-bottom 96-well plates (Costar). Cells were left to adhere for 30 minutes at room temperature.

2.3.2. NIH-3T3 and HEK-293 cells

NIH-3T3 and HEK-293 cells were maintained in complete medium consisting of Dulbecco's minimal essential medium (DMEM; CR-UK Cell Services) supplemented with 2 mM L-glutamine (CR-UK Cell Services) and 10% foetal calf serum (FCS; Sigma) in a humidified atmosphere at 10% CO_2, 37°C in T80 tissue culture flasks fitted with ventilated filter caps (Nunc). They were passaged every 4-5 days by dissociation with a trypsin/versene solution (CR-UK Cell Services), dilution and re-plating. For imaging, cells were seeded overnight at approximately 20% confluence on 8-well, multitest test slides in the above media. For HEK-293 cells, the wells were first coated with a 1 mg/ml solution of poly-L-lysine (Sigma) to assist with adhesion of the cells.

2.3.3. Transfection

25 μl of pEGFP-N1-PH-PLCδ1 at 1.6 ng/μl was mixed with 25 μl of 0.1% lipofectamine (LFA; invitrogen) in DMEM. After 20 minutes' incubation at room temperature to allow DNA:LFA complexes to form, the mixture was diluted with 50 μl DMEM, and mixed by pipetting. 40 μl of this solution was added per well of HEK-293 cells on 8-well multitest slides, i.e. 16 ng DNA/well. After 4 hours at 10% CO_2, 37°C in a humidified atmosphere, the media was removed and replaced with complete media. Cells were fixed after 24 hours; transfection efficiency was ~25%.

2.4. SDS-PAGE and Coomassie staining

Protein samples to be analysed by SDS-PAGE were mixed with an equal volume of Laemmli sample buffer (20% glycerol, 5% β-mercaptoethanol, 1-5 mg bromophenol blue, 4% SDS and 120 mM Tris-Cl, pH 6.8) and boiled for 3 minutes. After spinning for a brief period to collect liquid in the bottom of a 0.5 ml or 1.5 ml tube, samples were loaded on a polyacrylamide protean III™ minigel (BioRad). These gels were freshly prepared, and consisted of a separating gel (8-12% acrylamide [ratio of acrylamide:bis-acrylamide 37.5:1), 0.1% SDS, 375 mM Tris-Cl, pH 8.8), overlaid with a stacking gel (4.5% acrylamide, 0.1% SDS, 125 mM Tris-Cl, pH 6.8) into which crenellation had been moulded for sample

loading. Gels were polymerized with 3 mM ammonium persulphate (AMPS) and 0.25% Temed. Samples were run at 20 mA/minigel in running buffer (190 mM glycine, 0.1% SDS, 25 mM Tris-Cl, pH 8.5). When the sample front had reached the end of the gel, proteins were fixed and stained with Coomassie staining solution (50% MeOH, 10% acetic acid, 0.1% Coomassie stain) for 15 minutes at room temperature; bands were revealed by de-staining the gel using 10% acetic acid in 10% isopropanol.

2.5. Activation of mast cell exocytosis

2.5.1. Stimulation of intact cells

Adherent RPMC in 40 μl of EB in 96-well plates were treated with 20 μl of the indicated inhibitor (e.g. U73122) at 3x final concentration for the indicated time at room temperature. Subsequently, exocytosis was activated with 20 μl EB in the presence or absence of 40 μg/ml 48/80 (to give $[48/80]_{final}$ = 10 μg/ml). After 10 minutes, secretion was stopped on ice with 80 μl of 5 mM EGTA in IB. The plate was spun for 5 minutes at 4°C, 300 g_{AV} to pellet detached cells. 50 μl aliquots were then withdrawn for assay of secreted β-hexosaminidase (see below)

For cells adhered on 8-well multitest slides, EB was aspirated and the cells treated with 20 μl of the stated compounds for time periods as indicated for individual experiments. Subsequently, cells were stimulated by the addition of 20 μl 48/80 at 20 μg/ml (giving $[48/80]_{final}$ = 10 μg/ml) in EB. When no pre-incubation was required, cells were directly activated with 40 μl of EB with 10 μg/ml 48/80. Approximately 5 s before the indicated time period, 48/80 was aspirated and the secretion was stopped on ice at the appropriate time by addition of ice-cold 3% glutaraldehyde in PBS.

2.5.2. Permeabilisation of intact cells

RPMC on 8-well, multitest slides in EB were placed on an ice-cold metal plate and rinsed twice with 5 mM EGTA in IB to remove Ca^{2+}. They were then incubated with ice-cold SL-O at 1.6 U/ml in IB for 8 minutes. Streptolysin-O binding to membranes is temperature-independent, whereas pore formation only occurs above 12°C (Sekiya *et al.*, 1996); therefore, unbound SL-O can be

removed from the cells before permeabilisation. Unbound SL-O was removed by rinsing once with ice-cold IB, and ice-cold IB containing Ca:EGTA ($[Ca^{2+}]_{free}$ as indicated), nucleotides, proteins and compounds as described for individual experiments was added. Permeabilisation was then initiated by transferring the slide to a heated metal plate at 30°C. If stimulation was immediate, the cells were permeabilised in 40 μl of buffer. If the stimulus was to be delayed, permeabilisation was in 20 μl; stimulation was initiated by addition of 20 μl stimulation buffer at 2x final concentration of effectors. Buffer was removed ~5 s before the end of the incubation; cells were then quenched at the indicated times on ice with ice-cold 3% glutaraldehyde.

2.5.3. Permeabilisation in suspension and the rundown assay

Immediately after purification and washing, RPMCs were resuspended in 1 ml of IB. Cellular metabolism was then blocked by addition of 0.6 mM 2-deoxyglucose and 10 μM antimycin-A for 5 minutes at 30°C; ~90% depletion of ATP occurs under these conditions (Koffer and Churcher, 1993). RPMCs were next chilled on ice for ~30 s, then transferred to 3 ml ice-cold SL-O in IB to give a final [SL-O] of 1.2 IU/ml. After 5 minutes' incubation on ice, cells were washed free of unbound SL-O by dilution with ice-cold IB, centrifugation for 5 minutes at 4°C, 300 g_{AV}, and aspiration of the supernatant. The RPMC-pellet was immediately re-suspended in ice-cold IB (~2.5 ml/96-well plate) and transferred to a plastic trough in an ice/water bath. Permeabilisation was initiated by transferring 20 μl aliquots of the cell suspension to 96-well 'v'-bottom plates (Greiner) in a water bath at 30°C, as rapidly as possible using a multi-channel pipette. When exocytosis was evoked at the time of permeabilisation, the cells were transferred to wells containing a cocktail of the compound or protein to be tested (in 40 μl at twice the final concentration) and 20 μl of the stimulus (at four-times the final concentration, i.e. 12 mM Ca:EGTA at pCa 7 or 5 and 400 μM GTPγS as indicated).

For the rundown assay, where the stimulus is withheld for a designated time after permeabilisation, cells were transferred to wells containing a cocktail of the compound to be tested (in 20 μl at three-times the final concentration) and rundown buffer (in 20 μl at three-times the final concentration, i.e. 0.9 mM

Ca:EGTA at pCa 8 and 300 μM MgATP). After the indicated rundown times, cells were stimulated by the addition of 20 μl of the pre-warmed stimulus at four-times the final concentration (i.e. 12 mM Ca:EGTA at pCa 7 or 5, 100 μM MgATP to maintain $[MgATP]_{final}$ = 100 μM, and where indicated 400 μM GTPγS).

For both assays, stimulation was allowed to proceed for 10 minutes, before the 96-well plates were transferred to ice and quenched with an equal volume (i.e. 80 μl) of ice-cold 5 mM EGTA in IB. Cells were pelleted by centrifugation at 4°C, 300 g_{AV} for 5 minutes, and 50 μl aliquots of supernatant were sampled for secreted β-hexosaminidase activity (see below).

2.6. Assay for secreted β-hexosaminidase

One of the secretory products of RPMCs is *N*-acetyl-β-D-glucosaminidase (β-hexosaminidase; β-hex). Therefore, measurement of secreted hexosaminidase activity in the supernatant provides a quantitation of the extent of exocytosis. This is achieved through the provision of the substrate 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide, which is cleaved by β-hexosaminidase, liberating fluorescent 4-methylumbeliferone (Gomperts and Tatham, 1992).

The 50 μl samples of supernatant from secretion assays (see preceding section) were transferred to black flat-bottom 96-well plates (Labsystems) containing 50 μl 1 mM 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide (Sigma) in 0.2 M Na citrate, pH 4.5. After sealing the plate to prevent evaporation, the reaction was allowed to proceed at 37 °C typically for 1 hour. The reaction was quenched with 150 μl Tris base, and fluorescence measured with a fluorescence plate reader (PerSeptive Biosystems) (excitation = 360 nm, emission = 450 nm).

Exocytosis was calibrated for a given sample x in terms of β-hex release according to one of two formulae:

$$\text{(i) } \beta\text{-hex release (\% of total)} = 100 \times \frac{(x - \text{Blank})}{(\text{Total} - \text{Blank})}$$

$$\text{(ii) } \beta\text{ - hex release (\% of control)} = 100 \times \frac{(x - \text{Blank})}{(\text{Control} - \text{Blank})}$$

"Blanks" are the fluorescence quantified in the absence of cells, "Total" is given by cells lysed with 0.2% Triton X-100 and "Control" is a sample of stimulated cells to which the other samples are normalised. Data are expressed as single values, means ± range of duplicates or mean ± S.E.M. of triplicates and quadruplicates. For some experiments, secretion was expressed as "Stimulated β-hex release". This was calculated as above, followed by subtraction of unstimulated (at pCa 7) from stimulated (at pCa 5 with GTPγS) values.

2.7. *Myo*-[2-^{3}H] inositol labelling and HPLC

RPMC were prepared from four Sprague Dawley rats (≥ 500 g) as described above, except that after the Percoll step cells were suspended and washed in Medium 199 (Gibco RBL) supplemented with 1 mg/ml BSA, 60 μg/ml penicillin and 100 μg/ml streptomycin. Cells were seeded in four 35 mm dishes (2 ml/dish) in the presence of 25 μCi/ml *myo*-[2-^{3}H] inositol (Amersham Biosciences). After 19 hours at 37°C, 10% CO_2, cells were rinsed five times with EB, and stimulated with 48/80. Reactions were stopped by removing 48/80 and killing cells at the indicated times with 0.5 ml 1 M HCl, supplemented with 5 mM tetrabutyl ammonium hydrogen sulphate (TBAHS, Fisher). Lipids were then extracted following a previously described protocol (Jackson *et al.*, 1992). The cells in HCl were scraped and transferred to a glass bottle (Chromacol). The empty well and rubber policeman were washed with 0.667 ml MeOH, which was then pooled with the acid-killed cells in the glass bottle. 1.333 ml of $CHCl_3$ was added to this mixture, giving a ratio of $CHCl_3$:MeOH:acid of 8:4:3 (Folch *et al.*, 1957). The combination was mixed by vortex and resolved into organic and aqueous phases by centrifugation at 300 g_{AV}. The lower, organic phase (~ 1.5 ml) was isolated and transferred to a fresh glass bottle The aqueous phase (~1 ml) was re-extracted with 1.5 ml synthetic organic phase (prepared from $CHCl_3$:MeOH:1 M HCl with 5 mM TBAHS and 5 mM EDTA in the ratio 8:4:3). The bottle was then mixed, centrifuged and the synthetic organic layer isolated as before. Both organic phases

were washed sequentially with 1 ml synthetic aqueous phase (prepared as above), before pooling the two phases and drying under nitrogen.

The extracts were deacylated with monomethylamine reagent (a kind gift of Dr. F.T. Cooke, University College London) for 35 minutes at 53°C. Samples were then extracted with petroleum ether and analysed by high performance liquid chromatography (HPLC) by Dr. Stephen Dove, University of Birmingham as previously described (Dove *et al.*, 1997).

2.8. Extraction of Ins(1,4,5)P_3

These experiments were used to test the efficiency of Ins(1,4,5)P_3 extraction in the presence of neomycin, and were performed exactly as described in (Cockcroft *et al.*, 1987). 1 μM Ins(1,4,5)P_3 in a buffer consisting of 137 mM NaCl, 2.7 mM KCl, 10 mM LiCl, 5.6 mM D-glucose, 20 mM PIPES-NaOH, pH 6.8 and 1 mg/ml BSA was spiked with 3 nCi [2-^{3}H]-Ins(1,4,5)P_3 in glass scintillation vials with or without 0.3 mM neomycin. To 0.4 ml of this solution, 0.5 ml $CHCl_3$ and 1 ml MeOH were added, and the mixture vortexed. 0.5 ml each of $CHCl_3$ and either water or 0.2 M HCl were then added, giving a ratio of $CHCl_3$:MeOH:water of 1:1:0.9 to achieve a two-phase system (Bligh and Dyer, 1959). The phases were resolved by centrifugation at 300 g_{AV}. 1 ml of the aqueous phase and 0.5 ml of the organic phases were then transferred to fresh scintillation vials. The remaining liquid was designated as 'interface'; all three samples were evaporated under a stream of nitrogen to remove $CHCl_3$ and MeOH, which acts as potent quenching agents for scintillant; in contrast, almost no quenching with HCl (at ~ 0.1M) was observed. 5 ml of scintillation fluid (Ultima Gold; Perkin Elmer) was then added to each sample, and radioactivity was quantified by scintillation counting on a Beckman LS6500.

2.9. *In vitro* enzyme assays

2.9.1. PtdIns-PLC assay

0.3 U/ml PtdIns-PLC was assayed in IB (without BSA) with or without PLC inhibitors in a volume of 20 μl. Heat-inactivated PtdIns-PLC was treated at 110°C for 15 min. To start the reaction, 20 μl of 1 μM PtdIns spiked with 10 nCi [2-^{3}H]-

PtdIns in 0.16% octylglucoside was added, and the reaction allowed to proceed for 10 min at 30°C. The reaction was stopped with 50 μl 1 M HCl; 200 μl of $CHCl_3$:MeOH (1:1) was then added, the mixture vortexed and the aqueous phase separated from the organic by brief centrifugation. 50 μl of aqueous phase was then assayed for released [2-^{3}H]-Ins*P* by scintillation counting as described above.

2.9.2. PI 4-kinase assay

300 μl SL-O permeabilised cells were quenched with 300 μl ice-cold 5 mM EGTA in IB, and pelleted at ~400 g_{AV} for 3 min. After removing the supernatant, PI4K assays were performed essentially as described (Meyers and Cantley, 1998). Briefly, cells were lysed using 25 μl PtdIns lysis buffer, which consisted of 40 mM HEPES-NaOH, pH 7.4, 0.3% Triton X-100, 0.4 mg/ml PtdIns. DiC_{16}-PtdIns in $CHCl_3$ was dried under a stream of nitrogen gas and dispersed into PtdIns lysis buffer by sonication for 5 min at 80% output, 50% duty cycle on a Branson Sonifier 250 with a bath sonicator attachment.

Reactions were started by addition of 25 μl 20 mM $MgCl_2$, 100 μM MgATP, 5 μCi [γ-^{32}P]-ATP, and allowed to proceed for 30 minutes at room temperature. Reactions were stopped with 50 μl 2 M HCl and lipids were extracted by addition of 200 μl $CHCl_3$:MeOH (1:1) spiked with 25 nCi [^{14}C]-PtdCho as a loading control; the lower organic phase was removed and washed with 80 μl 1 M HCl:MeOH (1:1). Samples were spotted onto 1.2% potassium oxalate-impregnated TLC plates (Whatman), dried and run with $CHCl_3$:MeOH:H_2O:NH_4OH (90:90:20:7), before drying and developing on phosphorimager plates. Spots were identified by co-migration with 10 μg diC_{16}-PtdIns4*P* (revealed with copper molybdate staining) and analysed by densitometry using NIH Image.

2.10. Protein-lipid overlay assay

These assays were performed essentially as described previously (Dowler *et al.*, 2002). 1:2 serial dilutions of diC_{16}-PPIn were prepared in a 2:1:0.8 mixture of MeOH, $CHCl_3$ and water at concentrations from 100 μM down to 0.78 μM. 1 μl of these dilutions (working up the dilutions for each PPIn) was spotted onto a grid

(0.5 mm intervals) on H-bond+ membranes (Amersham), so that spots contained 0.78-100 pmol of the selected lipid. Membranes were left to dry for 1 hour at room temperature and then blocked for 1 hour at room temperature with antibody solution, consisting of 3% fatty-acid free BSA (prepared by cold EtOH precipitation, Sigma) in TBST (150 mM NaCl, 50 mM Tris-Cl pH 7.5, 0.1% Tween-20). Indicated concentrations of GST-tagged proteins were then incubated with the membranes for 1 hour at room temperature or overnight at 4°C, with gentle rocking. The blots were then washed six times over 30 minutes with TBST, and incubated with monoclonal anti-GST (1:1000) in antibody solution either for 1 hour at room temperature or overnight at 4°C, with gentle rocking. After washing as before, membranes were incubated with HRP-conjugated rabbit anti-mouse secondary antibodies for 30-60 minutes at room temperature, and washed 12 times over 1 hour with TBST. Bound proteins were revealed with ECL (Amersham). Exposure time was between 10 and 30 s.

2.11. Immunofluorescence

2.11.1. Immunostaining before fixation

Adherent RPMCs on 8-well multitest slides were SL-O permeabilised as described above, except IB was replaced with potassium glutamate buffer (KGB: 137 mM potassium glutamate, 2 mM $MgCl_2$, 20 mM PIPES-KOH, 1 mg/ml BSA). After removing unbound SL-O, cells were permeabilised in 40 μl KGB with 3 mM EGTA (KGB-E) and the indicated concentration of MgATP by warming the slides to 37°C. After 10 minutes, this buffer was removed and replaced with 40 μl KGB-E blocking solution, which contained 32% normal goat serum (NGS, Gibco). After RPMCs were blocked for 30 minutes at room temperature, blocking solution was replaced with antibody solution (KGB-E with 16% NGS) containing 10 μg/ml 2C11. RPMCs were stained for 1 hour at room temperature, washed twice with KGB-E and incubated with 6.7 μg/ml Alexa488 anti-mouse IgG secondary in antibody solution for 30 minutes. Finally, cells were washed 4 times in KGB-E, fixed for 20 minutes in 4% paraformaldehyde (PFA) in PBS, rinsed thrice in PBS, once in water and mounted in Mowiol4-88 (Calbiochem).

2.11.2. *Immunostaining post-fixation at room temperature*

Cells were fixed with 4% PFA (unless otherwise stated) for 20 minutes, and rinsed thrice with PBS. A blocking reaction was next performed for 30-60 minutes in sodium glutamate buffer (NaGB: 137 mM sodium glutamate, 2 mM $MgCl_2$, 20 mM PIPES-NaOH, 1 mg/ml BSA) containing 5-10% NGS, 50 mM NH_4Cl and 0.5% saponin. Subsequently, this solution was removed and replaced with antibody solution (NaGB with 5% NGS) containing 16 μg/ml 2C11 or 5 μg/ml PO34. After 1 hour, cells were washed twice for 5 minutes with NaGB and stained for 30 minutes with 6.7 μg/ml Alexa488 anti-mouse IgG, Alexa488 anti-mouse IgG_3 or 10 μg/ml Alexa555 anti-mouse IgM. Finally, cells were washed four times for 5 minutes each with NaGB and mounted with Mowiol4-88.

2.11.3. *Immunostaining post-fixation ≤ 4°C*

For this protocol, cells were fixed with ice-cold aldehydes. All handling of the samples was on an ice-cold metal plate in an ice-water bath. All solutions were ice-cold before use. For incubations, slides were transferred rapidly to a fridge so that at no point did the slides warm > 4°C.

Cells were fixed in 3% glutaraldehyde (GA) or a mixture of PFA and GA as indicated (such as 0.2% GA in 4% PFA) for 3 hours at 4°C. They were next rinsed thrice with PBS with 50 mM NH_4Cl. At this point, RPMCs were stained with 200 μg/ml Alexa647 concanavalin-A (Molecular Probes) for 5 min in PBS, and rinsed twice in PBS. Cells were then blocked for 4 hours with 5% NGS, 50 mM NH_4Cl and 0.5% saponin in NaGB. When cells were incubated with GST-tagged recombinant proteins, these were included at this stage, and cells were subsequently washed twice with NaGB. Blocking solution was removed and replaced with antibody solution (NaGB with 5% NGS and 0.1% saponin) containing the following antibodies as indicated: 2C11 (16 μg/ml), 10F8 (1:10 dilution), kt3g (10 μg/ml), PO34 (5 μg/ml) or polyclonal anti-GST. Staining was typically 13-14 hours at 4°C. Cells were then washed twice for 10 minutes each with NaGB, and stained for 4 hours with antibody solution containing the following additions: 6.7 μg/ml Alexa488 anti-rabbit IgG, 10 μg/ml Alexa555 anti-mouse IgM, 10 μg/ml Alexa555 anti-mouse IgG, 5 μM BODIPY-ceramide

(Molecular Probes) or 5 U/ml Alexa488 phalloidin (Molecular Probes). They were then washed 4 times for 10 minutes with NaGB; when used, DAPI (Roche) or Draq5 (Alexis) were included at 1:2000 in the second wash. Finally, cells were fixed as before for 10 minutes on ice and 5 minutes at room temperature, in order to immobilise antibodies. They were rinsed four times in PBS with 50 mM NH_4Cl, once in water, and mounted with Mowiol4-88.

2.11.4. Staining for nuclear PtdIns(4,5)P_2

In order to stain for nuclear PtdIns(4,5)P_2, RPMCs were seeded in EB on 13 mm glass coverslips placed in four-well tissue culture dishes (Costar) for 30 min at room temperature. This was because detergent-containing solutions would run off the glass wells of multiwell test slides onto the Teflon-coated surrounding. Steps were performed at ≤ 4°C as in the previous section, except where indicated. Cells were fixed for 3 hours with 4% PFA, rinsed thrice with PBS containing 50 mM NH_4Cl, and blocked for 4 hours at room temperature with TX solution (5% NGS and 0.2% Triton X-100 in PBS). This solution was replaced on ice with TX solution containing 16 μg/ml 2C11, and cells were stained overnight. Subsequently, coverslips were washed twice for 10 minutes with PBS, and stained for 4 hours with 10 μg/ml Alexa555 anti-mouse IgM and 5 U/ml Alexa488 phalloidin in TX solution. They were next washed four times for 10 minutes each with PBS, including 1:2000 Draq5 in the second wash. Finally, cells were fixed with 3% GA in PBS for 10 minutes on ice and 5 minutes at room temerature, before rinsing 4 times with 50 mM NH_4Cl in PBS, once in water, and mounting in Mowiol4-88.

2.12. Confocal microscopy and image analysis

Images were acquired on a Zeiss 510 LSM confocal microscope equipped with 405, 488, 543 and 633 nm laser excitation lines, using a 63x 1.4 NA PlanApochromat oil-immersion lens. Image intensity profiles were created with the Zeiss LSM 3.2 software.

For quantitative image analysis, image stacks comprising four (nuclei) or six (whole cell) 4 μm sections were acquired using a 40x 1.3 NA PlanApochromat

oil-immersion lens and 4x averaging. Image stacks were saved in the original Zeiss format, with 2C11 (Alexa555, 543 nm excitation) in the red channel. Laser power and detector gain/offset were set such that 2C11 signal was never saturated, and background fluorescence from secondary antibody just detectable. BODIPY-ceramide (488 nm excitation) was in the green channel and nuclei (DAPI, 405 nm excitation or Draq5, 633 nm excitation) in the blue; the detector gain was set such that both signals just saturated the detector.

Image stacks were then analysed in MetaMorph 6.3 image analysis software (Molecular Devices), using a journal written by Drs Alastair Nicol and Daniel Zicha (CR-UK Light Microscopy Laboratory). The journal performed the following operations: Image stacks were separated into red, green and blue channels and the fluorescence intensities for each image channel in the stacks summed. Nuclei were detected in the blue channel using the "Count Nuclei" application module and a binary mask of the nuclei generated. The detected nuclei were used as markers for watershed segmentation of the green channel (cell location) image. This generated boundaries between cells and between touching cells. Visual inspection of such images revealed accurate separation of touching cells in the vast majority of cases. To analyse 2C11 fluorescence in individual cells, a cell body mask obtained from the segmented green image was used to extract the total red (2C11 labelling) intensities of the individual cells from the red channel image. To quantify nuclear PtdIns(4,5)P_2, a "Count Nuclei" derived nuclear mask was used to extract the total intensities of the individual nuclei from the red channel image. Data were analysed in Excel spreadsheet software (Microsoft).

2.13. Measurement of intracellular calcium

Cells were seeded in the presence of 2 μM Fluo3/AM (Molecular Probes) for 30 min, before incubating in the presence of the stated compounds. Cells were then stimulated with 48/80 whilst images were acquired at approximately 3 frames/s with a 100x 1.25 NA PlanApochromat oil-immersion lens (Nikon) mounted on a Nikon Diaphot 200 inverted microscope, using a standard Nikon FITC B-2A filter. Exposure time was 111 ms. Fluorescence changes were measured within defined regions of interest encompassing whole cells using Tracker software

(Kinetic Imaging), and fluorescence intensity at a given frame (F_t) normalised to the initial fluorescence intensity (F_0).

2.14. Preparation of liposomes

Sufficient 1-oleoyl 2-palmitoyl-PtdCho and diC_{16}-PtdIns(3,4)P_2 or diC_{16}-PtdIns(3,4,5)P_3 were dispensed into a glass vial (Chromacol) such that $[lipid]_{final}$ = 1.3 mM with 5 mole % PIn. Lipids were dried under nitrogen, resuspended in 200 μl ethanol and dried again. Finally, lipids were washed with 200 μl diethyl ether, and dried completely under nitrogen followed by vacuum for 30 minutes. Finally, liposomes were formed by bath sonication in an ice/water bath for 15 minutes at 80% output, 60% duty cycle using a Branson Sonifier 250. Liposomes were mixed with an equal volume of 10 μg/ml PO34; this gave final concentrations of 5 μg/ml (33 nM) PO34, 650 μM total lipid and 33 μM PIn (i.e. 1000-fold molar excess over antibody, or 500-fold if 50% of the PIn is on the inner leaflet of the vesicle).

Chapter 3: Phosphoinositide regulation of exocytosis

3.1. Introduction

Experiments that established the role of heterotrimeric G-protein βγ-subunits during exocytosis from permeabilized RPMCs first indicated the requirements for PPIn (Pinxteren *et al.*, 1998). This study relied in part on the use of recombinant PH domains to sequester endogenous βγ-subunits and thus prevent their facilitation of exocytosis. One such PH domain from β-adrenergic receptor kinase (PH-βARK) binds to both G-protein βγ-subunits and PtdIns(4,5)P_2 (Touhara *et al.*, 1995). To clarify which interaction impaired exocytosis, specific mutants were employed which retained βγ-subunit binding at the expense of PtdIns(4,5)P_2 binding, and *vice versa*; both types of mutant were in fact shown to inhibit exocytosis (Pinxteren *et al.*, 1998). Further studies showed that ATP acted to maintain secretory competence in permeabilised RPMCs, at least in part, by phosphorylation of PtdIns: sequestration of endogenous PPIn with neomycin mimicked the effect of ATP-depletion on the rundown of RPMC exocytosis (Pinxteren *et al.*, 2001). Furthermore, PtdIns transfer proteins were found to maintain secretory competence in a manner that correlated with their ability to maintain PIn metabolism (Pinxteren *et al.*, 2001).

Neomycin was demonstrated to bind tightly to *bis*-phosphorylated, and with lower affinity to mono-phosphorylated PIn and other acidic phospholipids (Schacht, 1978). Furthermore, the PH domain from βARK was shown to bind promiscuously to PPIn which included PtdIns3*P*, PtdIns4*P*, PtdIns(3,4)P_2, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (Kavran *et al.*, 1998). Therefore, although a role for PPIn during RPMC exocytosis has been established, the identity of the specific PIn isomer(s) involved is yet to be determined. In this chapter, the results from experiments that were designed to clarify this issue are presented. Three broad experimental strategies were employed in permeabilised cells. First, specific interactions between neomycin or PH-βARK^{WAA} (an alanine insertion mutant that has lost its βγ-subunit but not PIn-binding properties) (Touhara *et al.*, 1995) with endogenous lipid were tested by competition with exogenous short-chain lipid analogues. Second, reagents that would interfere with specific aspects of PIn metabolism were tested for their effects on the maintenance of secretory competence. Finally, proteins with defined PIn-binding properties were

introduced into the cells in order to establish if sequestration of the respective lipid would inhibit exocytosis.

3.2. Results

The inhibition of RPMC exocytosis by neomycin was shown to be reversed so long as soluble di-octanoyl (diC_8) PtdIns(4,5)P_2 was present in excess or at equimolar concentrations (Pinxteren *et al.*, 2001). This was interpreted as displacement of neomycin from endogenous PtdIns(4,5)P_2 by the soluble analogue. However, given the interactions between neomycin and other PPIn (Schacht, 1978), it could equally be possible that exogenous PtdIns(4,5)P_2 displaces neomycin from other PIn. To test this hypothesis, neomycin (100 μM) was pre-absorbed with a two-fold molar excess of diC_8 analogues of all seven PPIn. This led to substantial increases in the level of β-hex secretion from permeabilized cells for several of the PPIn in the presence of neomycin (figure 3.1A). However, in the case of PtdIns(4,5)P_2, PtdIns5P and PtdIns(3,4,5)P_3 the release occurred independently of exocytosis since it was observed at resting calcium levels in the absence of GTPγS. The most likely explanation for this phenomenon was that neomycin preferentially forms complexes with these lipids, which have detergent-like properties and lead to lytic release of β-hex from the granules. Only PtdIns4P produced a substantial reduction in the level of inhibition when stimulated exocytosis was considered (i.e. the release of β-hex in response to Ca^{2+} and GTPγS, calculated by subtraction of the non-stimulated release), although this was not reproducible in all experiments (figure 3.1B).

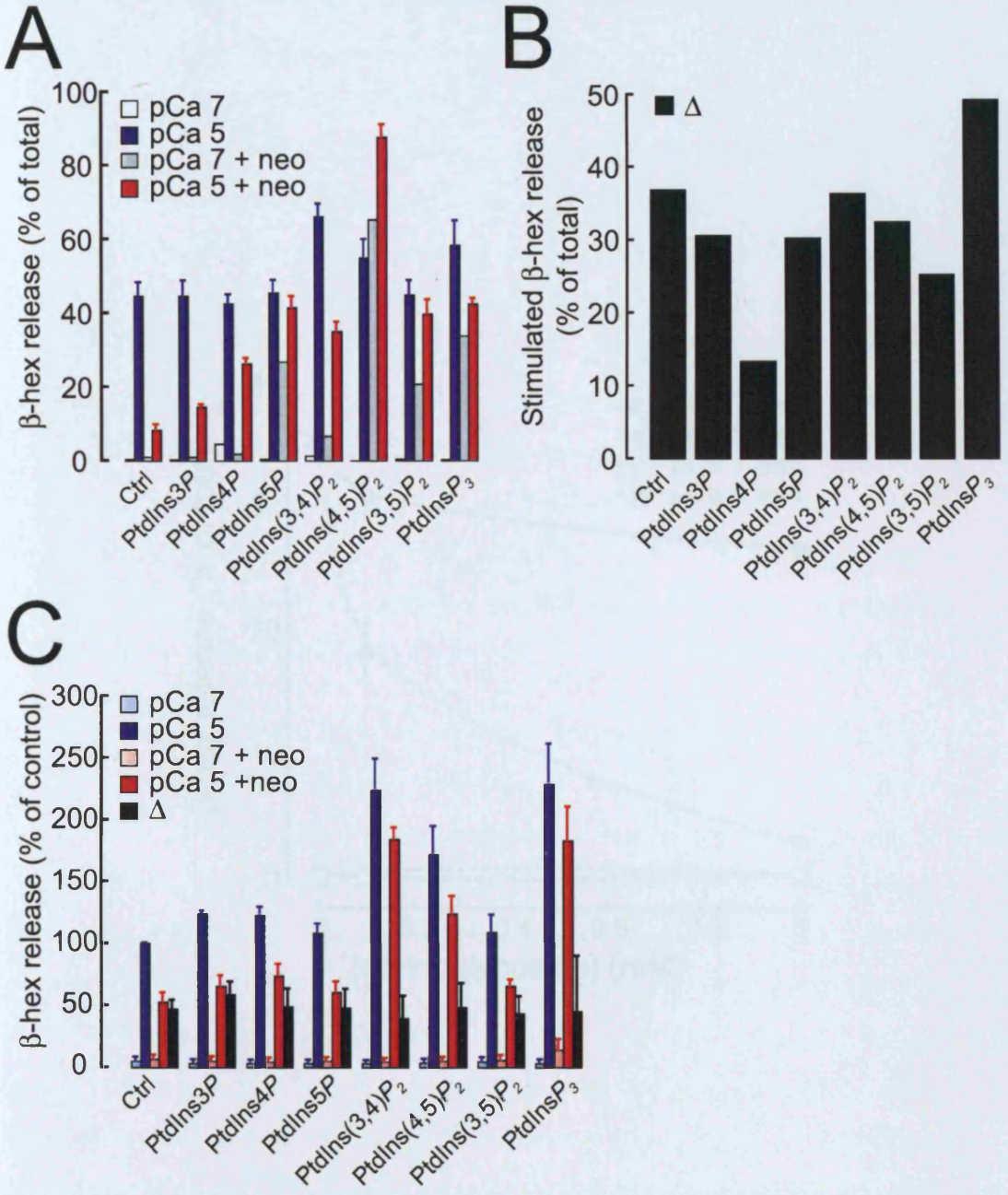

Figure 3.1: Effect of neomycin at 10 minutes rundown. RPMC were permeabilised with SL-O and left to run down in a solution containing 100 μM MgATP and 300 μM Ca:EGTA (pCa 8) with the indicated reagents at 30°C. After 10 min, they were stimulated by addition of buffer containing 3 mM Ca:EGTA (pCa 7) or 100 μM GTPγS + 3 mM Ca:EGTA (pCa 5), as well as maintaining 100 μM MgATP; β-hex release was measured as described in Materials and Methods. (A & B). Cells were run down in the presence of 100 μM neomycin ± 200 μM of the indicated diC_8-PIn. Δ refers to the change in β-hex release due to the presence of neomycin, calculated by subtracting release in its presence from that in its absence. Data are from a single, representative experiment and are means ± S.E.M. (n = 3). (C) as A & B, but with 30 μM neomycin and 90 μM diC_8-PIn. Data are the mean of 4 experiments carried out in triplicate ± S.E.M. Note neomycin:diC_8-PIn complexes were left to form for 30-60 minutes at room temperature before applying to cells.

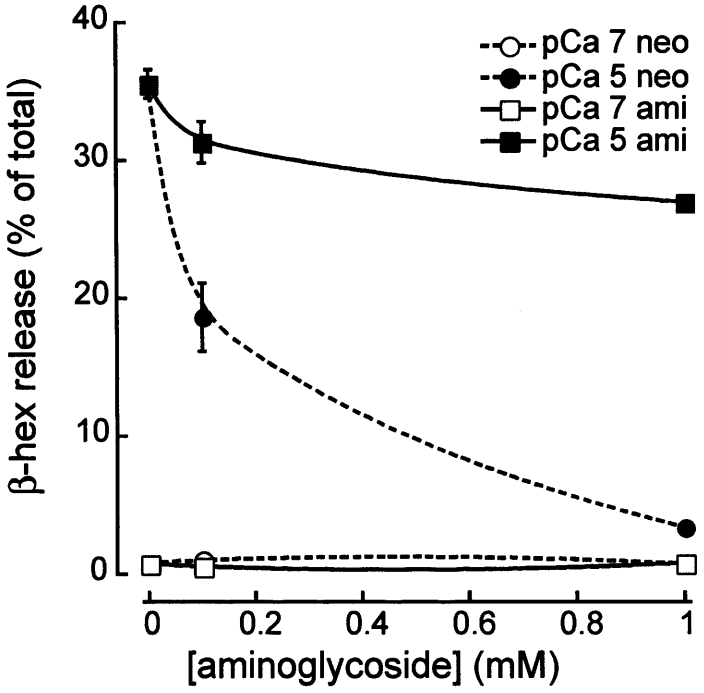

Figure 3.2: Effect of amikacin at 10 minutes rundown. RPMC were permeabilised with SL-O and left to run down in a solution containing 100 μM MgATP and 300 μM Ca:EGTA (pCa 8) with the indicated concentrations of aminoglycosides at 30°C. After 10 min, they were stimulated by addition of buffer containing 3 mM Ca:EGTA (pCa 7) or 100 μM GTPγS + 3 mM Ca:EGTA (pCa 5), as well as maintaining 100 μM MgATP. Data are from a single, representative expriment, and are means ± S.D. (n = 3).

To circumvent this problem, it was established that pre-absorption of 30 μM neomycin with a three-fold molar excess of diC_8-PPIn would not lead to such lytic behaviour, whilst neomycin still produced an ~50% inhibition of exocytosis (figure 3.1C). However, in this case no reversal of the inhibitory effect of neomycin on exocytosis was observed with any of the PPIn. Therefore, it could not be confirmed that neomycin caused inhibition of exocytosis through sequestering an endogenous PIn. To rule out an alternative effect of this aminoglycoside antibiotic on exocytosis, the effect of amikacin, another aminoglycoside with greatly reduced affinity for PIn (Marche *et al.*, 1983; Marche *et al.*, 1987), was tested. Indeed, whereas neomycin at 100 μM and 1 mM substantially reduced the levels of exocytosis from mast cells permeabilised for 10 minutes before stimulation, amikacin had only a minor effect (figure 3.2). Therefore, although neomycin appears to act through binding to an endogenous phospholipid, it was not possible to identify the molecules with this approach.

A similar strategy was employed to define the endogenous lipids bound by PH-βARK^{WAA}. Pre-incubation of 5 μM PH-βARK^{WAA} with a 10-fold molar excess of any of the diC_8-PPIn caused an almost complete ablation of the inhibitory effects of this protein (figure 3.3A). The complexes formed between the exogenous proteins and PtdIns(4,5)P_2 did not cause non-stimulated release of β-hex from the granules (figure 3.3B). Note that in the latter experiment, a different batch of PH-βARK^{WAA} was used with a higher specific activity than that of figure 3.3A. Hence a lower concentration (2.5 μM) was used to produce a similar inhibition, and a larger molar excess (40-fold) of lipid was required for effective competition. Given that any of the PPIn could alleviate the inhibition of exocytosis observed with PH-βARK^{WAA} (figure 3.3A), the protein may be interacting with any of these lipids within the cell via a non-specific electrostatic interaction. To test that the protein did not cause inhibition simply by interaction with acidic phospholipids, PH-βARK was preincubated with the same molar excess of phosphatidic acid (PtdOH, figure 3.3B). Under these conditions, PtdOH produced only a small effect on the resulting inhibition. Thus although the inhibitory effect of PH-βARK^{WAA} may be ascribed to interaction with a PPIn, the identity of the isomer(s) involved was not apparent from these experiments.

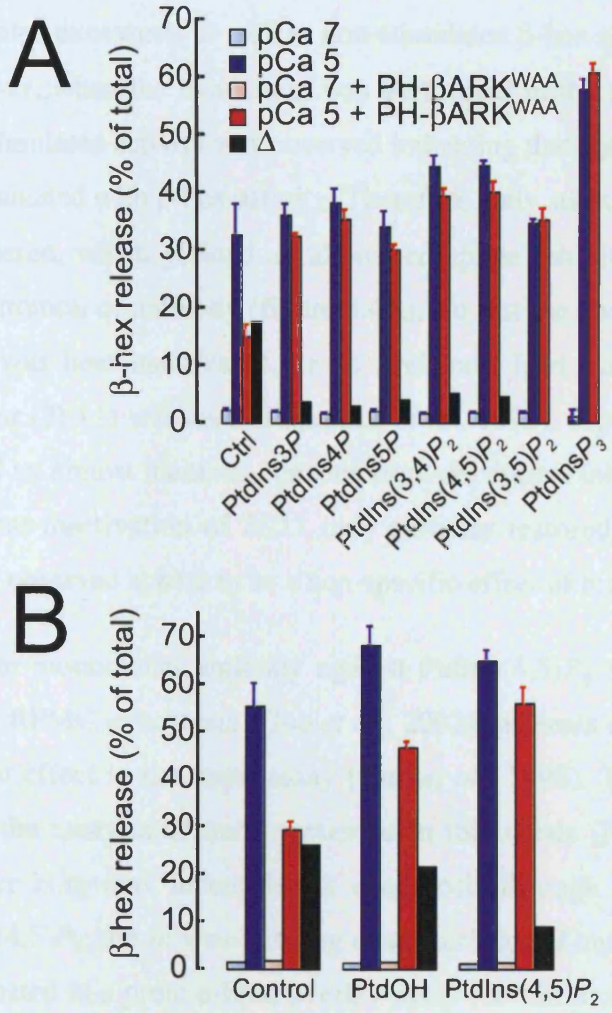

Figure 3.3: Effect of PH-βARK^{WAA} at 10 minutes rundown. RPMC were permeabilised for 10 minutes before stimulation as described in figure 3.1, in the presence of 5 (A) and 2.5 (B) μM PH-βARK^{WAA}. Where indicated, PH-βARK^{WAA} was incubated for 1 hour with 50 (A) or 100 (B) μM of the indicated DiC_8 lipid before applying to cells. Single representative experiments are shown; data are means ± S.E.M. (n = 3) for stimulated cells (pCa 5). For non-stimulated cells (pCa 7), n = 2 (A, mean ± range) and n = 1 (B).

Given that both neomycin and PH-βARK^{WAA} may inhibit exocytosis by interaction with any of the seven PPIn, the data would be compatible with a role for PtdIns(4,5)P_2. To test if this were true, the effect of sequestering this PPIn with a specific monoclonal IgM antibody 2C11 (Thomas *et al.*, 1999) raised against this lipid was tested. 2C11 caused a concentration-dependent inhibition of stimulated exocytosis as well as non-stimulated β-hex activity (figure 3.4A, inset). However, when the incubation was performed in the absence of cells, the same non-stimulated activity was observed indicating that the antibody preparation was contaminated with β-hex activity. Therefore, only stimulated release of β-hex was considered, which yielded an almost complete inhibition of exocytosis at high concentration of antibody (figure 3.4A). To test the specificity of this interaction 2C11 was heat inactivated, or an irrelevant IgM raised against the c-erbB-3 receptor (2E11) was used (Rajkumar *et al.*, 1993). Figure 3.4B shows that 2E11 caused an almost identical concentration-dependent inhibition of exocytosis, and that heat inactivation of 2C11 only partially restored secretion. Therefore, the effects observed appear to be a non-specific effect of high concentrations of IgM.

Another monoclonal antibody against PtdIns(4,5)P_2 was previously shown to inhibit RPMC exocytosis (Guo *et al.*, 2002), whereas other IgG antibodies were without effect in the same assay (Guo *et al.*, 1998). This result was confirmed under the assay conditions presented in this thesis (figure 3.5A). To establish whether kt3g may indeed block exocytosis through sequestering endogenous PtdIns(4,5)P_2, the *in vitro* binding characteristics of anti-PtdIns(4,5)P_2 antibodies were tested in a protein-lipid overlay assay (or "fat blot") (Dowler *et al.*, 2002)). Both 2C11 and kt3g interacted strongly with PtdIns(4,5)P_2 in this assay; however, kt3g displayed a more potent interaction with PtdIns(3,4)P_2 (figure 3.5B), indicating this that lipid is a potential endogenous target of kt3g in RPMCs. Further evidence on the specificities of kt3g and 2C11 are presented in the next chapter.

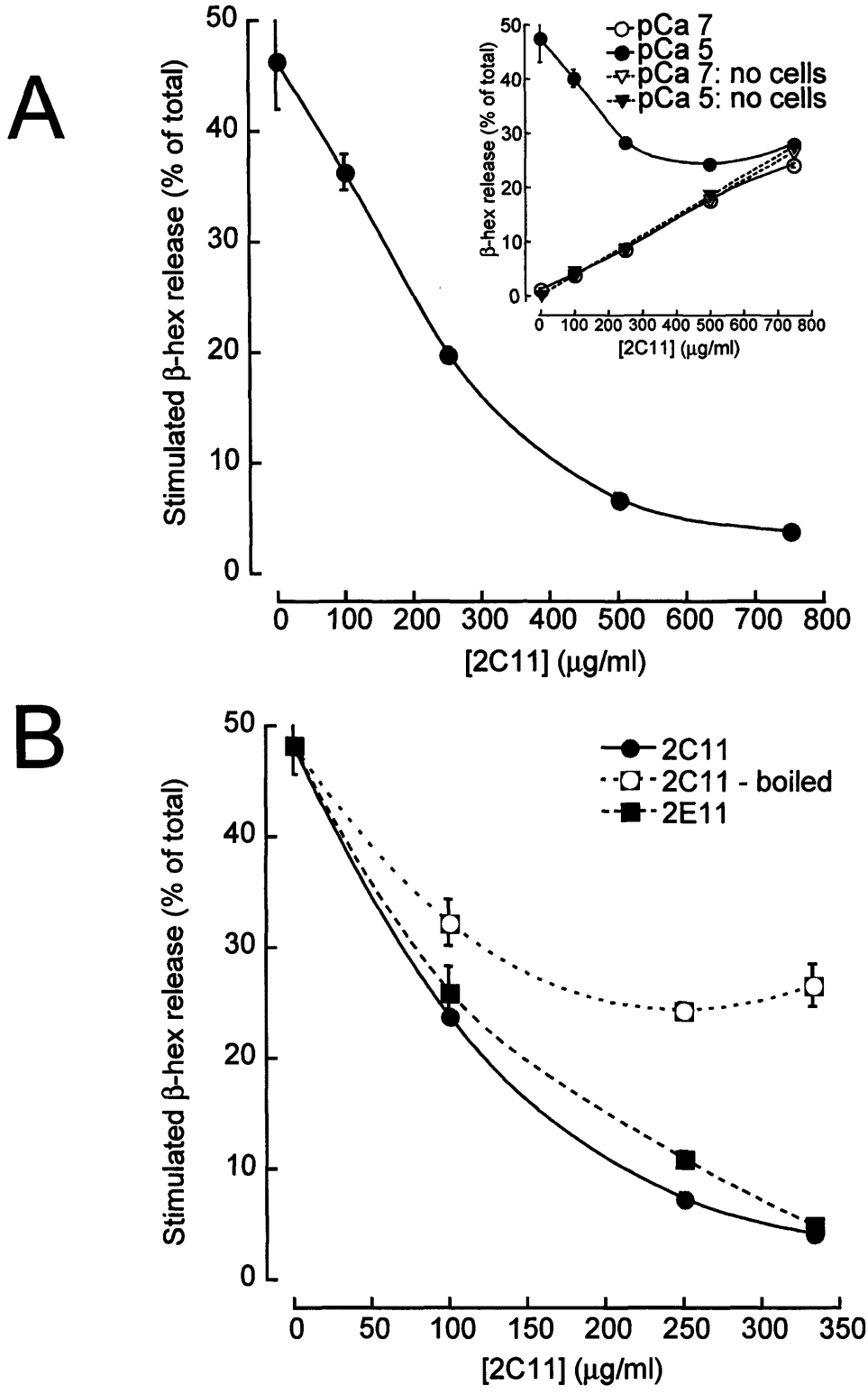

Figure 3.4: Effect of anti-PtdIns(4,5)P_2 antibody 2C11 at 10 minutes rundown. RPMC were permeabilised in the presence of the indicated concentration of anti-PtdIns(4,5)P_2 antibody 2C11 (A), or another IgM, 2E11 (anti-cerbB-3), and stimulated as described in figure 3.1. "Boiled" refers to 2C11 that was heat-inactivated for 3 minutes at 110°C. The inset of A shows total β-hex release. In the main panels, secretion is expressed as "stimulated" (= pCa 5 – pCa 7). Data are from representative experiments, and are means ± S.E.M. (n = 3).

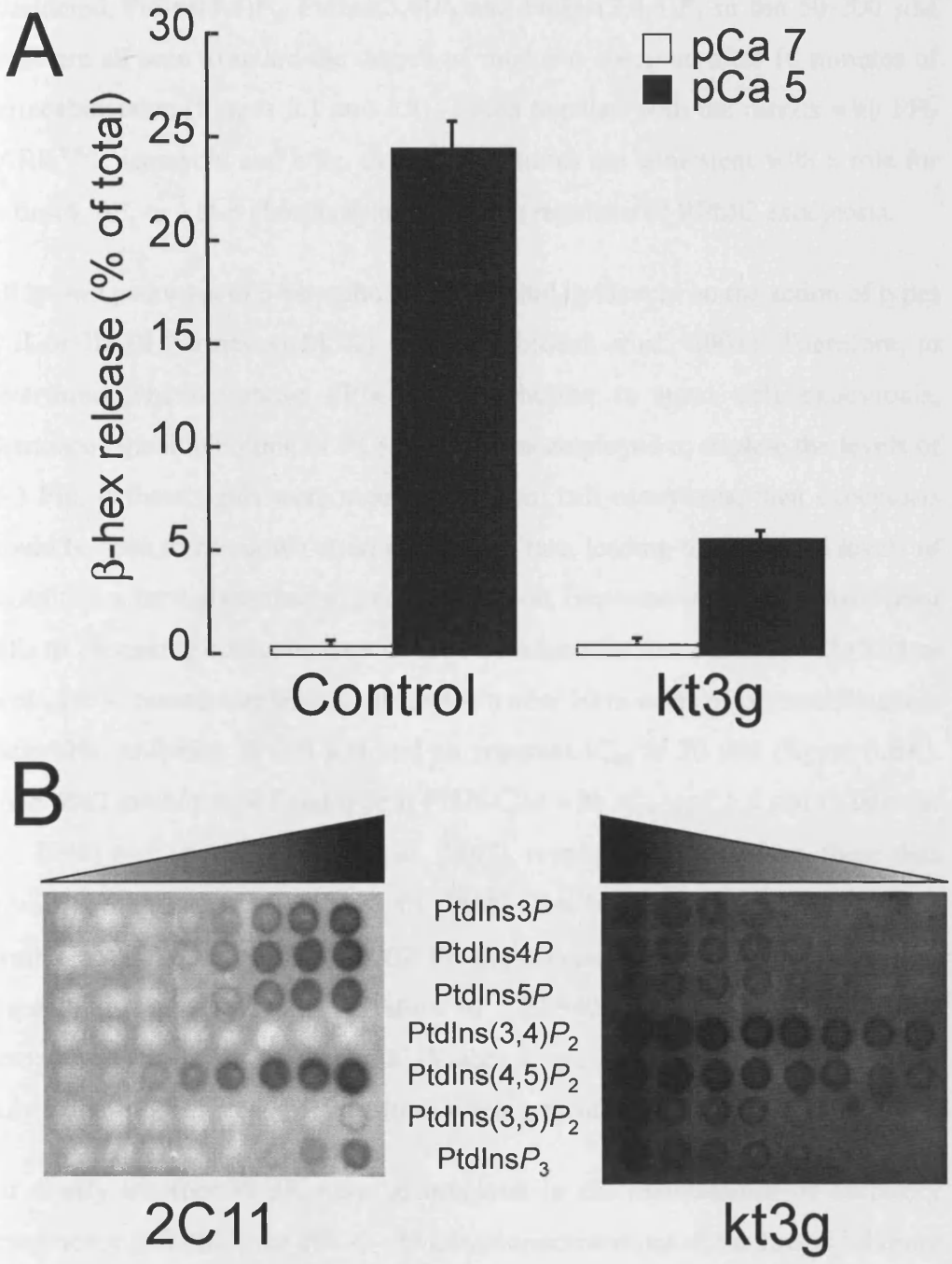

Figure 3.5: Specificity of anti-PtdIns(4,5)P_2 antibodies 2C11 & kt3g. (A) RPMC were permeabilised in the presence of 100 µg/ml kt3g for 10 min prior to stimulation as in figure 3.1. (B) Specificity of anti-PtdIns(4,5)P_2 antibodies in a protein-lipid overlay assay (Material and Methods). 1:2 serial dilutions of the indicated diC_{16}-PIn isomers from 100 pmol to 0.8 pmol were spotted onto H-bond+ membranes, incubated with 1.6 µg/ml 2C11 or 1 µg/ml kt3g, and revealed with HRP-conjugated secondary antibodies and ECL.

The effects of diC_8-PPIn furnished further clues as to the nature of the PPIn regulating mast cell exocytosis. When the effects of the diC_8-lipids alone are considered, PtdIns(4,5)P_2, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in the 50-200 μM range are all seen to retard the degree of rundown observed after 10 minutes of permeabilisation (figures 3.1 and 3.3). Taken together with the results with PH-βARK^{WAA}, neomycin and kt3g, these observations are consistent with a role for PtdIns(4,5)P_2 or a D-3 phosphorylated PIn as a regulator of RPMC exocytosis.

All known pathways of 3-phosphorylated inositol lipids rely on the action of types I, II or III PI 3-kinases (PI3K) (Vanhaesebroeck *et al.*, 2001). Therefore, to determine whether these PPIn could function in mast cell exocytosis, pharmacological inhibition of PI 3-kinases was employed to deplete the levels of D-3 PIn. If these lipids were required for mast cell exocytosis, then exocytosis would be seen to run down at an accelerated rate, leading to a reduced levels of exocytosis after a given time of permeabilisation. Exposure of permeabilised mast cells to increasing concentrations of the PI 3-kinase inhibitor LY294002 (Vlahos *et al.*, 1994) caused acceleration of rundown after 10 minutes of permeabilisation, with 40% inhibition at 100 μM and an apparent IC_{50} of 30 μM (figure 3.6A). LY294002 inhibits type I and type II PI3K-C2α with IC_{50}'s of 1.4 μM (Vlahos *et al.*, 1994) and 19 μM (Domin *et al.*, 1997), respectively. Therefore, these data would be consistent with a role for PI3K-C2α in this process. No data are available on the IC_{50} of LY294002 for the mammalian type III PI3K Vps34. Notably, a single atom substitution of LY294002 produces LY303511, a compound with no effect on PI-3K (Vlahos *et al.*, 1994; Ding *et al.*, 1995) and only a minor effect on RPMC rundown when present at 50 μM (figure 3.6A).

To clarify whether PI-3K may be involved in the maintenance of secretory competence in RPMC, the effects of various concentrations of the fungal inhibitor wortmannin (Arcaro and Wymann, 1993) were tested (figure 3.6B). This compound had no effect on exocytosis up to 1 μM; beyond this concentration, results were variable with a maximal inhibition of 50%, and an IC_{50} of 3 μM. Wortmannin inhibits classical, type II C2α and Vps34 PI-3Ks with IC_{50}'s of 1-5 nM (Vlahos *et al.*, 1994), 420 nM (Domin *et al.*, 1997) and 2.5 nM (Volinia *et al.*,

1995), respectively. Thus when taken together with the LY294002 results, effects of classical, type II PI3K-C2α and Vps34 PI3Ks can be ruled out.

Another hypothesis could be that PI-3K inhibitors were without effect because the pool of 3-phosphorylated lipids required turned over too slowly to be depleted by inhibition of their synthesis for 10 minutes. Therefore, other high-affinity D-3 PIn binding domains were tested. Introduction of a high affinity PtdIns3*P* binding protein, a tandem fusion of the FYVE domain from Hrs (Gillooly *et al.*, 2000), had no effect on RPMC rundown (figure 3.6C). Neither the PH domains from GRP1 or DAPP1 affected exocytosis, despite their respective abilities to bind PtdIns(3,4,5)P_3 (Klarlund *et al.*, 2000) or both PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Dowler *et al.*, 1999) *in vitro* (figure 3.6C). It was not possible to produce sufficient quantities of recombinant Svp1, which binds with great specificity and affinity to PtdIns(3,5)P_2 (Dove *et al.*, 2004), to directly test a role for this last 3-phosphorylated PIn in mast cell exocytosis (not shown). Despite this shortcoming, it seems unlikely that synthesis of this lipid is required to maintain secretory competence, as sequestration of its precursor lipid, PtdIns3*P* (Cooke, 2002), was without effect.

Thus no direct evidence could be discerned that 3-phosphorylated PPIn were required for exocytosis from RPMC. This leaves possible roles for PtdIns4*P*, PtdIns(4,5)P_2 and PtdIns5*P*. PH-βARK was demonstrated to bind the headgroup of PtdIns(4,5)P_2 with a K_d of ~200 μM (Fushman *et al.*, 1998). If this PH domain inhibited exocytosis by sequestering endogenous PtdIns(4,5)P_2, then domains with a higher affinity for this PIn would also impair secretion. The PH domain from PLCδ1 binds to the headgroup of PtdIns(4,5)P_2 with a K_d of 200 nM (Lemmon *et al.*, 1995), yet fails to produce significant effects on secretion at concentrations sufficient for PH-βARK to inhibit > 50% of exocytosis (figure 3.7A). When the lipid binding properties of these domains were tested in a protein-lipid overlay assay (figure 3.7B), PLCδ1 indeed yielded a higher affinity for PtdIns(4,5)P_2. However, PH-βARK interacts with mono-phosphorylated PIn with greater affinity than PH- PLCδ1. Since the FYVE domain has been shown to bind to PtdIns3*P* with a K_d of 2.5 μM (Sankaran *et al.*, 2001), it seems unlikely that this interaction with PH-βARK is relevant, given that a tandem fusion of the

former protein failed to affect exocytosis (figure 3.6C). This leaves PtdIns4*P* and PtdIns5*P*, both of which may function independently of their role as substrates for PI*P*-kinases (Fruman *et al.*, 1998).

To test for a role of PtdIns4*P*, the PH domain from FAPP1 was used, which interacts with PtdIns4*P* (Godi *et al.*, 2004; Roy and Levine, 2004). The loss of secretory competence was retarded in RPMC when 10 μM of this PH domain was present (figure 3.8A). This probe may retard rundown of secretion by protecting endogenous PtdIns4*P* from degradation, whilst still permitting access of a higher affinity effector protein. PtdIns4*P* is synthesised by type II and III PI4K activities (Fruman *et al.*, 1998). A role for the type III activity in mast cells may be ruled out for several reasons. Firstly, these enzymes have a K_m of ~400 μM for ATP (Downing *et al.*, 1996), whereas the effect of ATP in maintaining secretory competence in mast cells is saturating at 50 μM (Pinxteren *et al.*, 2001). Secondly, LY294002 and wortmannin inhibit type III PI4K with IC_{50}'s of 100 μM and 50 nM, respectively. These concentrations are incompatible with the observed IC_{50}'s for RPMC exocytosis (figure 3.6A & B). On the other hand, type II PI4Ks are insensitive to LY294002 and wortmannin, and possess a K_m of ~100 μM with respect to ATP. Conditions are therefore amenable for the action of these enzymes in maintaining secretory competence in RPMCs.

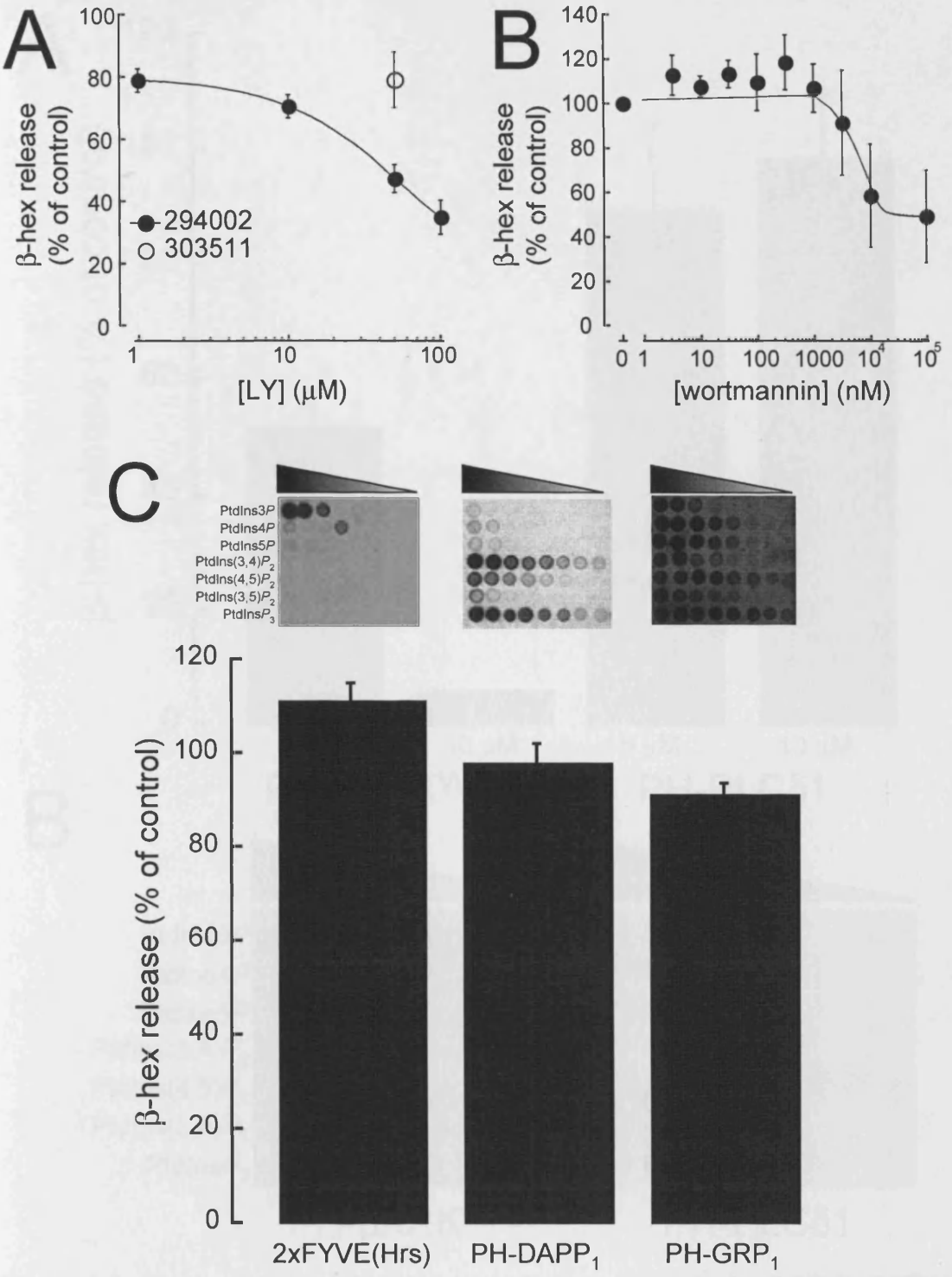

Figure 3.6: Effects of anatagonists of D-3 PIn function at 10 minutes rundown. RPMC were permeabilised for 10 minutes prior to stimulation in the presence of the indicated concentrations of LY294002 & LY303511 (A), wortmannin (B) or 10 μM recombinant GST-tagged protein (C), as described in figure 3.1. The panels at the top of C are protein-lipid overlay assays with 1 μg/ml 2xFYVE(Hrs), 0.2 μg/ml PH-DAPP1 and 0.2 μg/ml PH-GRP1, revealed with anti-GST and HRP secondaries as described in figure 3.4B. Again, blots were 1:2 serial dilutions from 100 to 0.8 pmol. Data are means ± S.E.M. from 3 or more experiments, with the exception of 2xFYVE from 2 experiments (mean ± range).

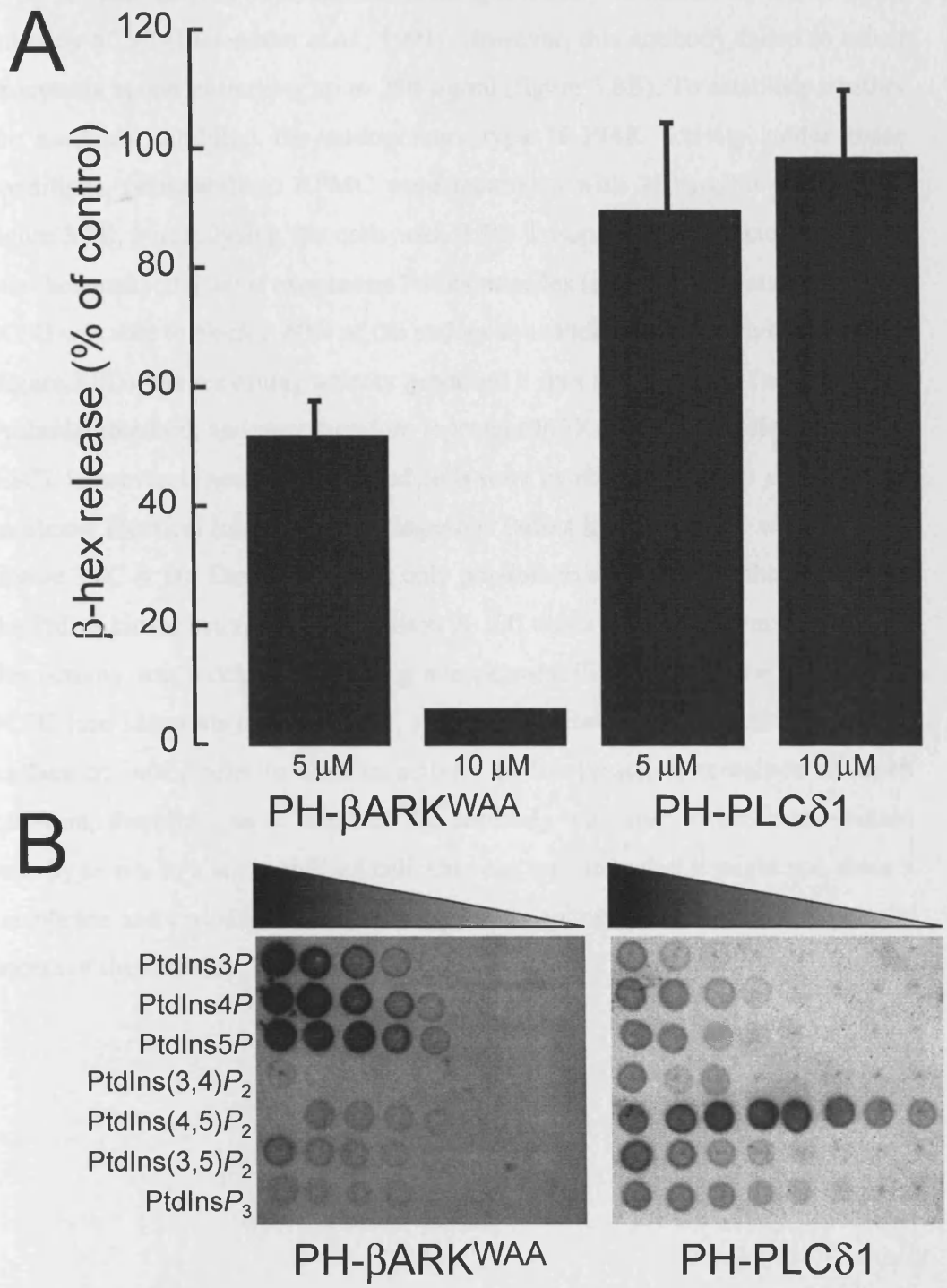

Figure 3.7: Contrasting lipid binding and effects on exocytosis of PH-βARK^{WAA} (WAA) and PH-PLCδ1. (A) RPMC were permeabilised in the presence of the indicated concentration of GST-tagged PH domain for 10 min prior to stimulation as in figure 3.1. Data are means ± S.E.M. of 16 experiments (5 μM PH-βARK^{WAA}), 5 experiments (10 μM PH-PLCδ1), mean ± range of 2 experiments (5 μM PH-PLCδ1) and from a single experiment (10 μM PH-βARK^{WAA}). (B) Specificity of GST-tagged PH domains in a protein-lipid overlay assay (Material and Methods). 1:2 serial dilutions of lipid from 100 pmol to 0.8 pmol were spotted onto H-bond+ membranes, incubated with 0.2 μg/ml PH-βARK^{WAA} or 0.2 μg/ml PH-PLCδ1, and revealed as described in figure 3.5.

Type II PI4K activity was shown to be specifically inhibited by monoclonal antibody 4C5G (Endemann *et al.*, 1991). However, this antibody failed to affect exocytosis at concentrations up to 250 μg/ml (figure 3.8B). To establish whether the antibody inhibited the endogenous type II PI4K activity under these conditions, permeabilised RPMC were incubated with 250 μg/ml 4C5G as in figure 3.7B, before lysing the cells with 0.2% Triton X-100. PIn-kinase activity was then assayed against exogenous PtdIns micelles (see Materials and Methods). 4C5G was able to block > 60% of the endogenous PtdIns kinase activity in RPMC (figure 3.8D); the remaining activity produced a spot that migrated faster than the PtdIns4*P* standard, and may therefore represent PI3K-generated PtdIns3*P* (figure 3.8C). However, if non-permeabilised cells were incubated with 250 μg/ml 4C5G, an almost identical inhibition of endogenous PtdIns kinase activity was observed (figure 3.8C & D). Therefore, it was only possible to conclude that the majority of the PtdIns kinase activity in 0.2% Triton X-100 was a type II PI4K activity. Since this activity was blocked after lysing non-permeabilised cells in the presence of 4C5G (see Materials and Methods), sufficient antibody was associated with the surface of intact cells to abolish activity in the lysate. It remained an open question, therefore, as to whether the antibody was able to block the kinase activity *in situ* in a permeabilised cell. One can speculate that it might not, since a membrane associated PI4K may not expose its epitope in a manner that permits access of the antibody.

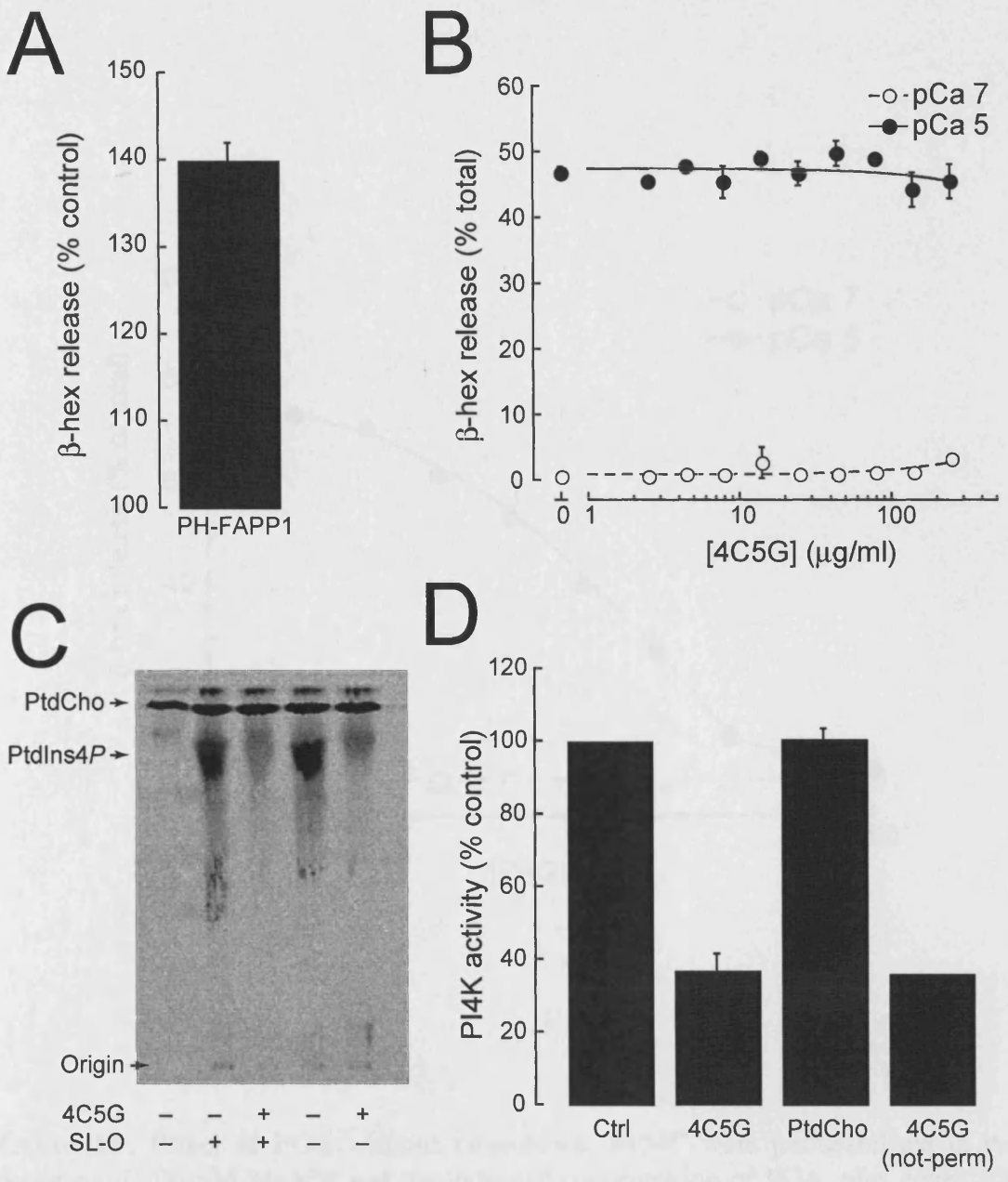

Figure 3.8: Activity and effect of type II PI4K. RPMC were permeabilised with SL-O in the presence of 10 μM PH-FAPP1 (A) or the indicated concentration of monoclonal anti-PI4K II antibody 4C5G (B) for 10 minutes before stimulation as described in figure 3.1. Data are means ± range of two experiments (A), or are results from a representative experiment (B) and are means ± S.E.M. (n = 3). (C & D) RPMC were treated with 250 μg/ml 4C5G as in B, except after 10 minutes incubation, the cells were lysed in 0.2% Triton X-100 and assayed for PI 4-kinase activity (Materials and Methods). [^{14}C]-PtdCho was included as a loading control, and was not saturated in the original exposure. (C) shows a representative radiograph from a single experiment (PtdIns4*P* was identified by co-migration with an unlabelled standard), whereas (D) shows normalised results after densitometric analysis of 4 experiments (Ctrl and 4C5G, means ± S.E.M.), 2 experiments ([^{14}C]-PtdCho, mean ± range) or a single experiment (4C5G, not-perm). "not-perm" refers to non-permeabilised cells (i.e. where SL-O was omitted).

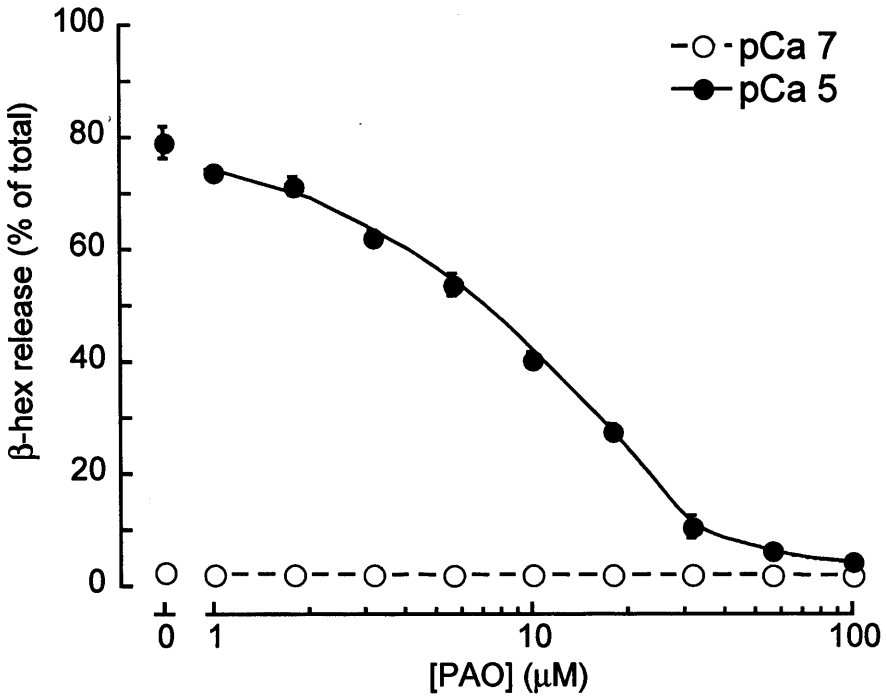

Figure 3.9: Effect of POA without runwdown. RPMC were permeabilised in the presence of 100 μM MgATP and the indicated concentration of POA, plus either 100 μM GTPγS and 3 mM Ca:EGTA (pCa 5), or 3 mM Ca:EGTA (pCa 7). After 10 min, cells were assayed for released β-hex as described in Materials and Methods. Data are from a representative experiment, and are means ± S.E.M. of 4 determinations.

Phenylarsine oxide (PAO) inhibits type II PI4K activity (Wiedemann *et al.*, 1996; Naveen *et al.*, 2005), although not as potently as its inhibition of type III PI4K (Balla *et al.*, 2002). This compound was found to completely abolish exocytosis with an IC_{50} of 10 μM (figure 3.9). However, this effect presented itself without rundown of the cells, i.e. when the drug was presented coincident with permeabilisation and stimulation. Since RPMC exocytosis occurs independently of ATP when stimulation is concurrent with permeabilisation (Howell *et al.*, 1987), this argues against the effect of PAO as inhibition of an ATP-dependent kinase activity. Indeed, whilst these experiments were being conducted, it was reported that PAO abolishes activity of Rho GTPases (Gerhard *et al.*, 2003). Since Rho has been reported to be absolutely required for exocytosis from RPMC (Price *et al.*, 1995; Sullivan *et al.*, 1999), the most parsimonious explanation is that PAO blocks exocytosis by blockade of Rho-mediated stimulation-secretion coupling. It was therefore impossible to use PAO to specifically probe a role for type II PI4K.

3.3. Conclusions

The results presented herein did not resolve the PIn isomer(s) required to maintain secretory competence in RPMC. However, several clues were furnished by these results. Firstly the inhibitory effects of PH-βARK^{WAA} were likely to be due to sequestration of an endogenous PPIn, since inhibition by this protein could be specifically reversed with short chain PPIn analogues (figure 3.3). However, use of the high affinity PtdIns(4,5)P_2 binding PH-domain from PLCδ_1 demonstrated that this was unlikely to be the endogenous lipid sequestered by PH-βARK^{WAA}. Secondly, a role for 3-phosphorylated PIn appears unlikely because rundown was insensitive to high affinity PtdIns3P, PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 binding proteins. The respective concentration-dependent acceleration of rundown by LY294002 and wortmannin were inconsistent with their concentration-dependent effects on PI 3-kinases; the observed effects may rather be due to inhibition of protein kinase activities at higher concentrations (Davies *et al.*, 2000). Therefore, it appears most unlikely that D-3 PIn are required to maintain secretory competence in mast cells.

In this light, the interaction of PH-βARK^{WAA} with monophosphorylated PIn isomers in a protein-lipid overlay assay (figure 3.4B) would implicate a role for PtdIns4*P* or PtdIns5*P*. No tools were available to test a role for the latter, since it was not possible to produce enough recombinant protein of the only published PtdIns5*P* binding protein to date, Ing52 (Gozani *et al.*, 2003). It was also not possible to provide conclusive evidence that PtdIns4*P* was required. However, several lines of evidence lead one to suspect that it may be important. Firstly, the PH domain from FAPP1, which binds to PtdIns4*P*, was seen to retard RPMC rundown (figure 3.7A). However, care must be taken in interpreting results using this protein, given its interactions with the Arf1 GTPase (Godi *et al.*, 2004) and PtdIns(4,5)P_2 (Roy and Levine, 2004). Secondly, type II PI 4-kinase is present in mast cells (figure 3.7C & D), and will operate under conditions of rundown to produce PtdIns4*P*.

Surprisingly, no direct evidence was found that PtdIns(4,5)P_2 is required to maintain secretory competence in mast cells. However, inhibition was observed with monoclonal antibody kt3g, which interacts with PtdIns(4,5)P_2 and PtdIns(3,4)P_2 (figures 3.5B and 4.10). Given that the latter relies on PI3K for its synthesis, the lack of effect of PI3K inhibitors at concentrations that block PI3K argue against a role for PtdIns(3,4)P_2. The inhibition observed with kt3g may therefore indeed be due to an interaction with PtdIns(4,5)P_2. Consistent with this observation, the inhibitory and maintaining effects of PH-βARK^{WAA} and PH-FAPP1 may be due to sequestration or protection of PtdIns4*P*, respectively. This pool of PtdIns4*P*, synthesized by a type II PI4K, may act independently, as well as being required for the synthesis of PtdIns(4,5)P_2 by PtdIns4*P*-5 kinases (Fruman *et al.*, 1998).

Chapter 4: Quantitative immunofluorescence analysis of phosphoinositides

4.1. Introduction

Much has been learnt about phosphoinositide localisation and metabolism through the use of specific lipid binding domains fused to green fluorescent protein (GFP) or its spectral derivatives expressed in living cells (Halet, 2005). Indeed, studies using the GFP-tagged PH domain from PLCδ1 have demonstrated the requirement for plasma membrane PtdIns(4,5)P_2 for exocytosis from chromaffin cells (Holz *et al.*, 2000) and the related PC12 cell line (Aikawa and Martin, 2003), as well as from pancreatic beta cells (Lawrence and Birnbaum, 2003). Furthermore, studies using this tool demonstrated that plasma membrane PtdIns(4,5)P_2 is required for synaptic vesicle endocytosis (Micheva *et al.*, 2001). However, this technique relies on heterologous expression of this fluorescent probe, an approach not feasible in RPMCs.

Other studies have employed immunofluorescence to follow phosphoinositide localisation and function in fixed cells, with variable results. Anti-PtdIns4*P* antibodies revealed a predominantly Golgi membrane localisation of this lipid in HeLa cells (Wang *et al.*, 2003), consistent with results from GFP-tagged PtdIns4*P*-binding PH domains (Godi *et al.*, 2004). Several studies have employed a monoclonal antibody against PtdIns(4,5)P_2, KT10 (Fukami *et al.*, 1988). It was found to stain both the plasma membrane and internal membrane compartments of HeLa cells (Wang *et al.*, 2004), or to produce a "patchy" labelling of the plasma membrane in COS-7 cells (Laux *et al.*, 2000). Notably, in the latter study such PtdIns(4,5)P_2 patches were found to be an artefact of paraformaldehyde fixation, and were not seen with glutaraldehyde. Staining exclusively at the plasma membrane was observed in hippocampal neurons after microwave fixation (Micheva *et al.*, 2001). Triton X-100 permeabilisation of fixed NRK cells produced a speckled stain in nuclei at inter-chromatin granule clusters (Boronenkov *et al.*, 1998), a localisation reported in HeLa cells with a different monoclonal anti-PtdIns(4,5)P_2 antibody, 2C11(Osborne *et al.*, 2001). Thus the subcellular location of PtdIns(4,5)P_2 reported with antibodies seems to vary greatly, depending on the fixation and permeabilisation conditions employed.

Alternative tools to monitor PIn in cells have also been developed. One example is FITC-conjugated neomycin (Arbuzova *et al.*, 2000), although this tool seemed to stain plasma membrane PtdIns(4,5)P_2 as well as other cellular compartments independently of the presence of PPIn (Holz *et al.*, 2000). Recently, specific PIn-binding PH domains have been used in an on-section labelling approach to report the localisation of PtdIns(4,5)P_2 and PtdIns(3,4)P_2 at an ultrastructural level (Watt *et al.*, 2002; Watt *et al.*, 2004).

In this chapter, results are presented that establish an accurate method for quantitative measurements of PtdIns(4,5)P_2 localisation in cells. This technique has then been employed to study the dynamics of this lipid during RPMC exocytosis. Finally, the approach is applied to another PtdInsP_2 isomer, PtdIns(3,4)P_2, to report this lipid's accumulation at the plasma membrane during challenge with an oxidative stress in HEK-293 cells.

4.2. Establishing conditions for detection of PtdIns(4,5)P_2 by immunofluorescence

To determine the subcellular localisation of PtdIns(4,5)P_2, monoclonal antibodies 2C11 (Thomas *et al.*, 1999) and kt3g (Matuoka *et al.*, 1988) were employed. Since 2C11 had revealed a punctate staining of nuclei after Triton X-100 permeabilisation (Osborne *et al.*, 2001; Thomas *et al.*, 2001), milder permeabilisation with SL-O or saponin was utilised in order to preserve membranes in fixed cells (see Materials and Methods). Under these conditions, both antibodies yielded a punctate staining of the cytoplasm, apparently excluded from the nucleus (figure 4.1A & B). The staining appeared specific for PtdIns(4,5)P_2, since it could be prevented by pre-absorbing 2C11 with glycerophosphoinositol (4,5)-*bis*phosphate (Gro*P*Ins(4,5)P_2), or kt3g with dioctanoyl-PtdIns(4,5)P_2 (figure 4.1A & B). Furthermore, sequestering endogenous PtdIns(4,5)P_2 with neomycin also prevented the staining, an observation made previously with monoclonal anti-PtdIns(4,5)P_2 antibody KT10 in NRK cells (Laux *et al.*, 2000). However, the signal obtained was not well co-localised with endogenous membranes, as assessed by staining with BODIPY-ceramide (Koffer *et al.*, 2002).

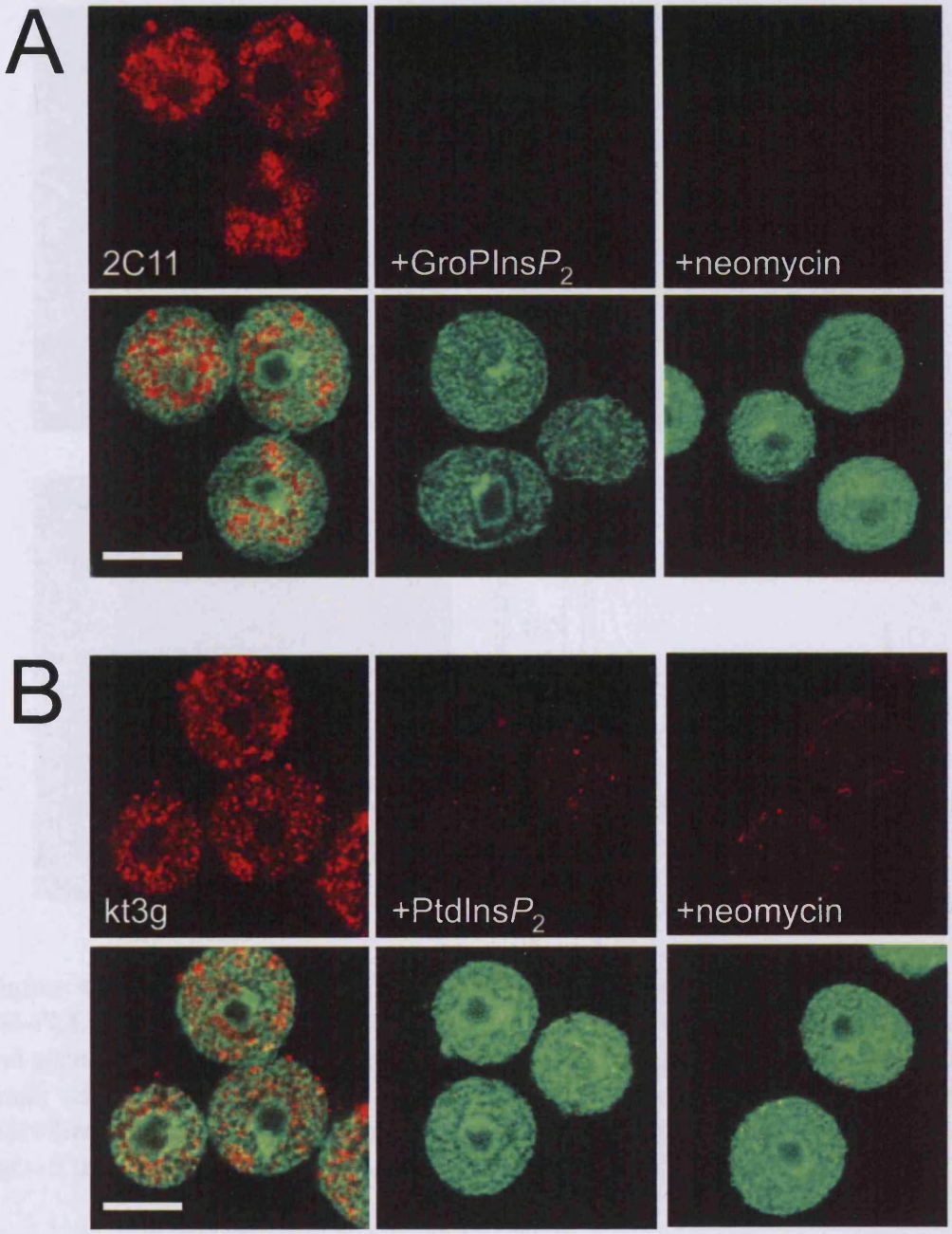

Figure 4.1: anti-PtdIns(4,5)P_2 antibodies produce a discontinuous stain of the cytoplasm at room temperature. RPMC were fixed and stained for PtdIns(4,5)P_2 with monoclonal antibodies 2C11 (A) or kt3g (B) at room temperature as described in Materials and Methods. Left panels show antibody staining, centre panels show antibody preabsorbed with either 0.2 mg/ml GroPIns(4,5)P_2 (A) or 0.5 mg/ml diC$_8$-PtdIns(4,5)P_2 (B); panels at right show cells preabsorbed and stained in the continuing presence of 100 μM neomycin. Top panels show anti-PtdIns(4,5)P_2 antibodies alone (red), whereas bottom panels show this channel merged with BODIPY-ceramide stained membranes (green). Scale bars = 10 μm.

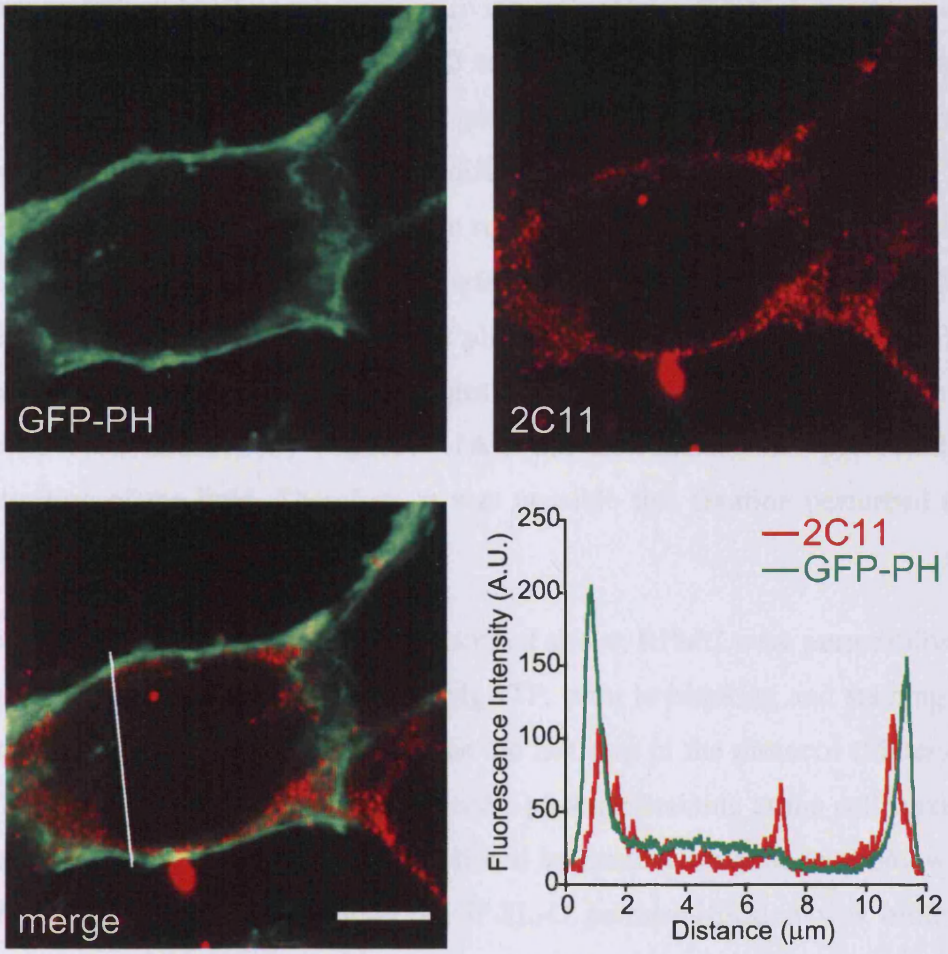

Figure 4.2: anti-PtdIns(4,5)P_2 antibodies do not co-localise with expressed GFP-PH-PLCδ1 at room temperature. HEK cells expressing GFP-PH-PLCδ1 were fixed and stained with 2C11 at room temperature as described in Materials and Methods. The graph refers to the fluorescence intensity (in arbitrary units) of the GFP (green) and 2C11 (red) channels along the line through the cell depicted in the merged image. Scale bar = 5 μm.

To determine the validity of the signal observed with 2C11, the staining was compared with that given by GFP-PH-PLCδ1, a probe commonly used to determine PtdIns(4,5)P_2 localisation in living cells (Halet, 2005). Upon transient transfection of this probe into HEK-293 cells, a plasma membrane staining was observed; in contrast, 2C11 produced a punctate staining of the cytoplasm in the same cells, which displayed a grossly different distribution to that of GFP-PH-PLCδ1 (figure 4.2). Although it has been suggested that GFP-tagged lipid binding domains may not reveal all pools of phosphoinositides in cells (Balla *et al.*, 2000), the inability of 2C11 to detect an intact plasma membrane pool of PtdIns(4,5)P_2 observed with the expressed probe suggested that the 2C11 staining, apparently specific for PtdIns(4,5)P_2 (figure 4.1A), did not reflect the pre-fixation localisation of the lipid. Therefore, it was possible that fixation perturbed the localisation of PtdIns(4,5)P_2.

To circumvent the fixation problems described above, RPMC were permeabilised with SL-O in the presence of 100 μM MgATP, prior to blocking and staining of the cells; fixation was then performed as the last step in the protocol (Materials and Methods). Such an approach produced a punctate staining at the cell surface (figure 4.3A); this signal was again abolished by preabsorbing the antibody with Gro*P*Ins(4,5)P_2 and was not observed if SL-O permeabilisation was omitted (figure 4.3B & C). These data were therefore consistent with localisation of PtdIns(4,5)P_2 to the inner leaflet of the plasma membrane. Interestingly, the majority of cells displayed this distribution so long as MgATP was provided at ≥ 10 μM (figure 4.4); such concentrations of MgATP are sufficient to maintain exocytosis in permeabilised cells (Howell *et al.*, 1989; Pinxteren *et al.*, 2001). A similar set of observations were also made recently using antibody KT10 in mechanically permeabilised PC12 cells (Grishanin *et al.*, 2004).

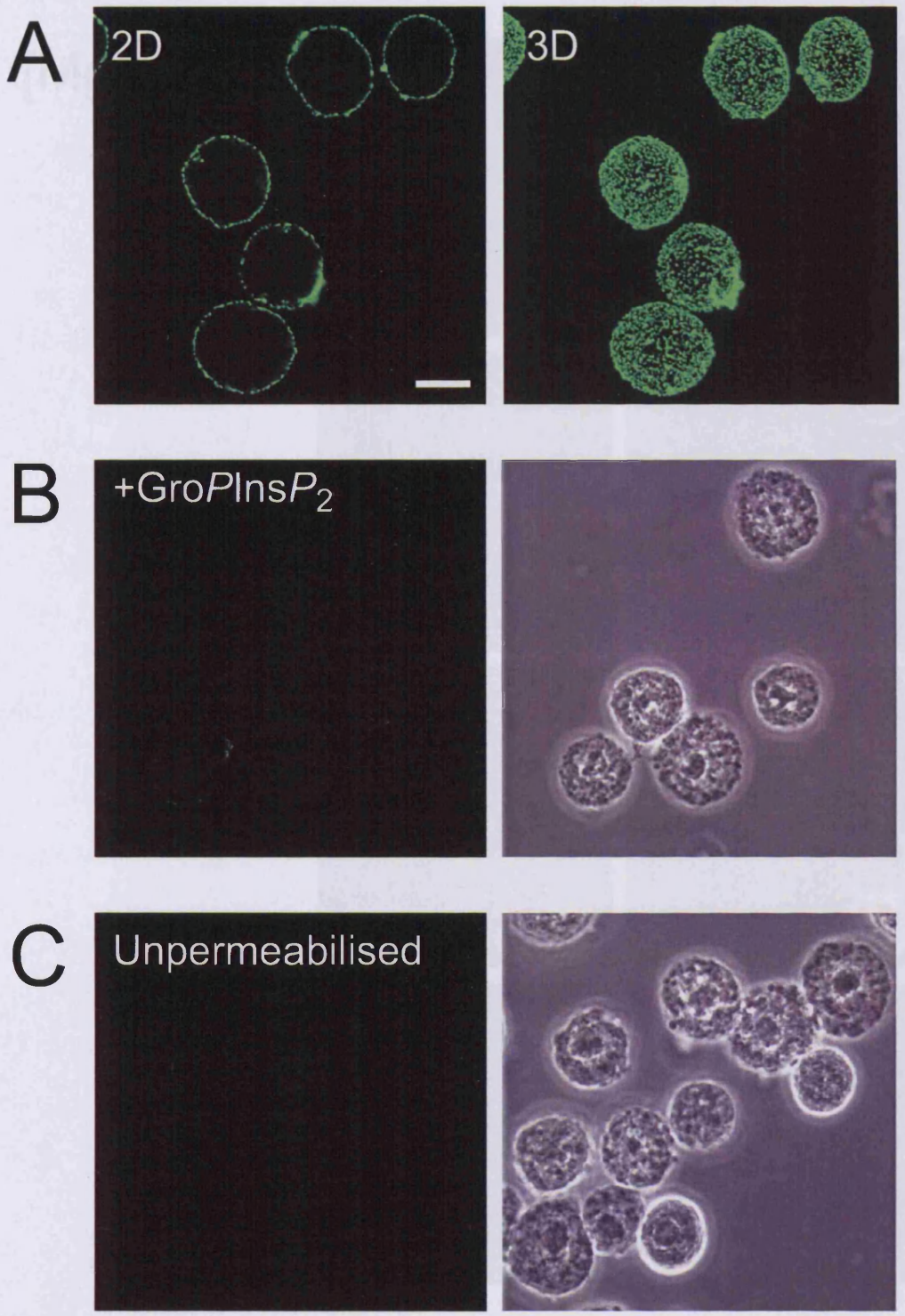

Figure 4.3: 2C11 stains the cell periphery in permeabilised cells before fixation. (A) RPMC were permeabilised with SL-O in the presence of 100 µM MgATP and 3 mM EGTA, before blocking and staining with 2C11 as described in Materials and Methods. A single confocal section (2D) or z-stack of several confocal sections (encompasing the whole cell) taken at 1 µm intervals (3D) are shown. (B) as A, except 2C11 was preabsorbed with 0.2 mg/ml Gro*P*InsP_2. (C) as A with omission of SL-O. Scale bar = 10 µm.

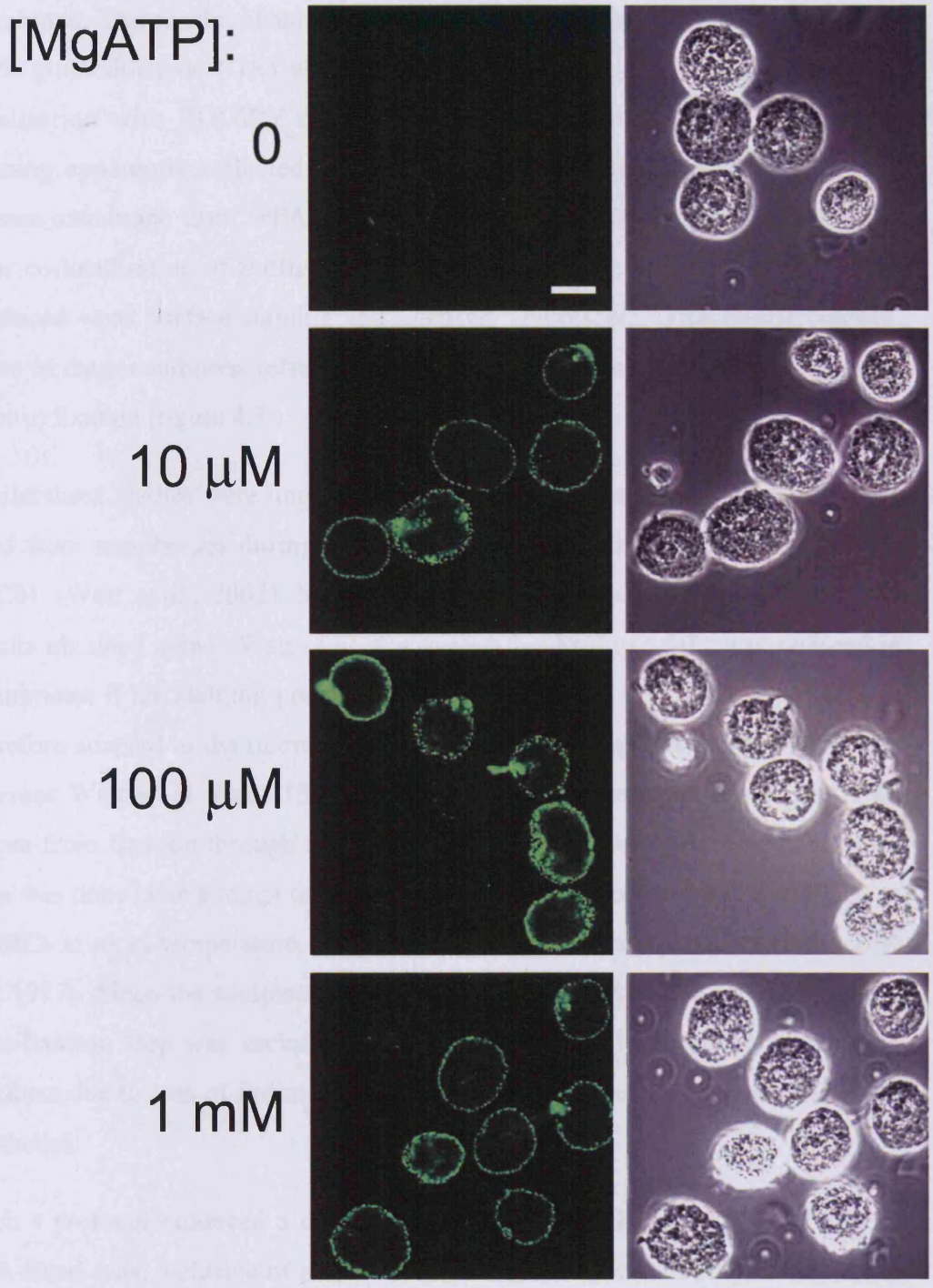

Figure 4.4: 2C11 staining is dependent on MgATP. RPMC were permeabilised with SL-O in the presence of 3 mM EGTA and the indicated concentration of MgATP before staining with 2C11 as described in figure 4.3. Images at left show 2C11 staining (green), those at right the same field by phase contrast. Scale bar = 10 μm.

Taken together, these results indeed seemed to indicate that fixation could perturb the endogenous localisation of PtdIns(4,5)P_2 in cells. Different fixation conditions were therefore tested in an attempt to preserve PtdIns(4,5)P_2 at the plasma membrane. The results of this study are presented in figure 4.5A: inclusion of 0.2% glutaraldehyde (GA) with 2% paraformaldehyde (PFA) led to good co-localisation with BODIPY-ceramide stained membranes. In most cells, the staining apparently reflected plasma-membrane proximal granules and/or the plasma membrane itself. PFA alone or with a lower concentration of GA led to poor co-localisation of PtdIns(4,5)P_2 with membranes, whereas 3% GA alone produced weak surface staining and intensely fluorescent extracellular patches. None of these conditions reflected closely the localisation observed with staining prior to fixation (figure 4.3).

Whilst these studies were underway, it was reported that PtdIns(4,5)P_2 could be shed from membranes during staining of cryo-sections with GST-tagged PH-PLCδ1 (Watt *et al.*, 2002). Such a phenomenon would be consistent with the results obtained above. Watt *et al.* discovered that PtdIns(4,5)P_2 was retained in membranes if the staining protocol was performed at ≤ 4°C. This principle was therefore adapted to the immunofluorescent protocols reported herein. However, whereas Watt *et al.* fixed 1321N1 astrocytoma cells at room temperature, all stages from fixation through staining to post-fixation were performed at ≤ 4°C. This was done in an attempt to avoid cell lysis, which is observed after fixation of RPMCs at room temperature, but prevented at reduced temperatures (Lawson *et al.*, 1977). Since the samples would need to be imaged at room temperature, a post-fixation step was included to preserve antibody localisation, preventing artefacts due to loss of PtdIns(4,5)P_2 after warming the cells (see Materials and Methods).

Such a protocol produced a discontinuous staining of the plasma membrane in PFA-fixed cells; inclusion of ≥ 0.05% GA yielded a continuous plasma membrane stain (figure 4.5B). For subsequent experiments, 3% GA was employed as a fixative since this reduced non-specific binding to exposed granule cores. When 3-dimensional projections of confocal sections were observed, 2C11 was seen to produce a threadlike staining of the PM, co-localised with the cortical F-actin

network (figure 4.6), and consistent with that observed after staining permeabilised cells prior to fixation (figure 4.3). Such a distribution was also observed with the anti-PtdIns(4,5)P_2 antibodies 10F8 (Thomas *et al.*, 1999) and kt3g, or with the PH domain from PLCδ1 (figure 4.6). The PH domain from GRP1, which is selective for PtdIns(3,4,5)P_3 (Gray *et al.*, 1999; Klarlund *et al.*, 2000), produced no staining. The thread-like staining at the cell surface appeared to reflect plasma membrane morphology when viewed either by field emission scanning electron microscopy or with the generic PM dye, CM-Dil$_{18}$ (figure 4.6). Therefore, these data are consistent with a uniform distribution of PtdIns(4,5)P_2 at the PM of RPMCs, although local heterogeneity of PtdIns(4,5)P_2 distribution may exist but be undetectable at this level of resolution.

Two different control experiments were performed to address the specificity of the staining with 2C11. Firstly, the antibody was preabsorbed prior to staining with a 10,000-fold molar excess (or 1000-fold, assuming 10 PtdIns(4,5)P_2 binding sites per 2C11 IgM) of various inositol phosphates, corresponding to the headgroup of various inositol lipids. Figure 4.7A shows that of the inositol phosphates tested, only those of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were able to compete with the cellular antigen for antibody binding. Thus, it could be concluded that the cellular antigen is either PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3. However, given that the PH domain from GRP1 did not stain cells, it seemed unlikely that the cellular antigen could correspond to the latter. To formally exclude this possibility, a second control experiment was performed: cells were pre-incubated with high concentrations of agents that would specifically sequester the endogenous lipid, preventing antibody binding. Figure 4.7B demonstrates that sequestering endogenous PtdIns(4,5)P_2 with 1 mM neomycin (Schacht, 1978) or 50 μM PH-PLCδ1 (Lemmon *et al.*, 1995) prevented staining with 2C11, whereas sequestering endogenous PtdIns(3,4,5)P_3 with PH-GRP1 (Klarlund *et al.*, 2000) did not. The cellular antigen must, therefore, correspond PtdIns(4,5)P_2. Interestingly, the PH domain from FAPP1, which binds to PtdIns4*P*, as well as to PtdIns(4,5)P_2 with a lower affinity (Roy and Levine, 2004), seemed to produce a partial competition with 2C11 (figure 4.7B). No competition was observed after sequestering endogenous PtdIns(3,4)P_2 with the PH domain from TAPP1 (Dowler *et al.*, 2000).

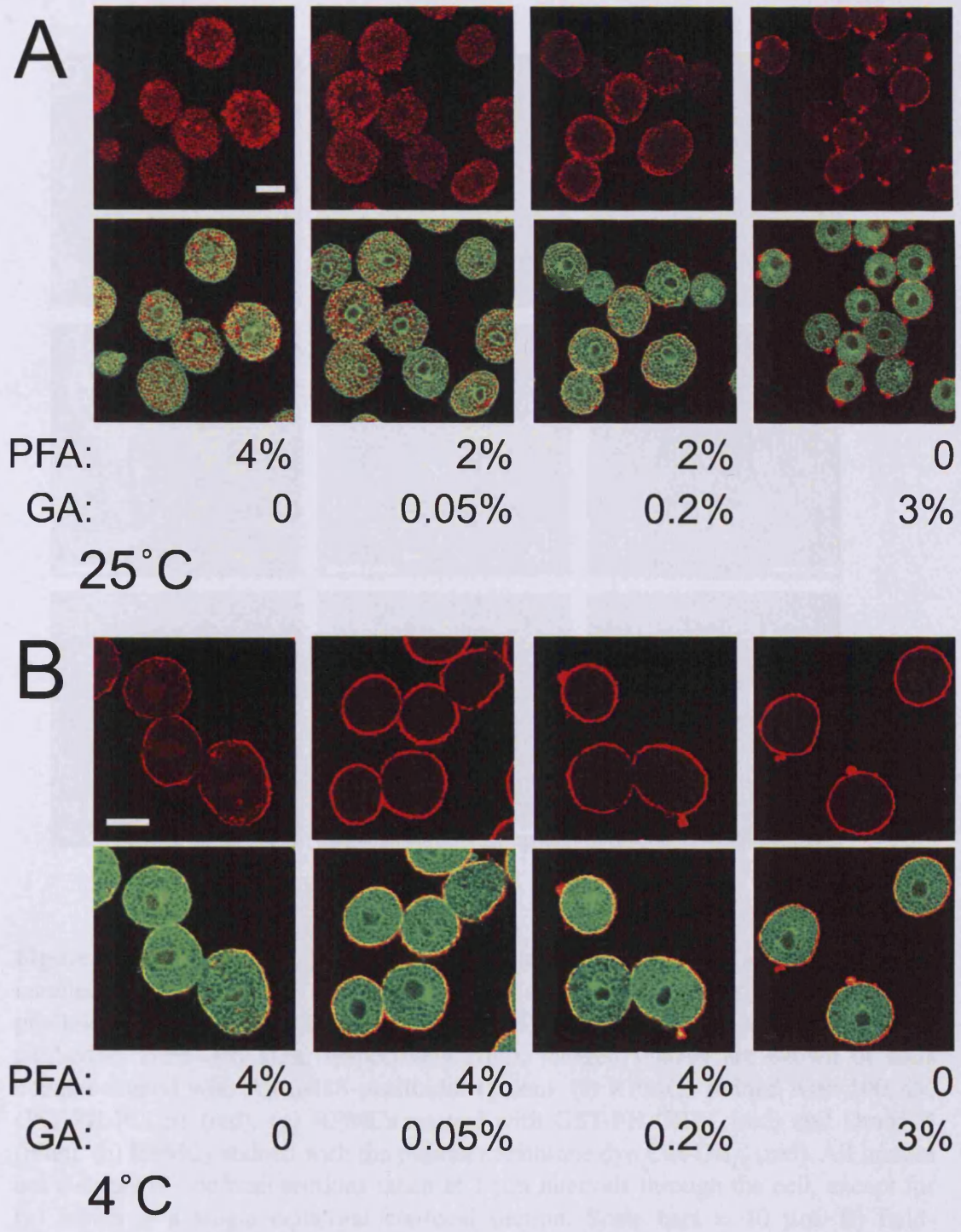

Figure 4.5: Effect of temperature and fixation on anti-PtdIns(4,5)P_2 staining. RPMC were fixed with the indicated concentrations of paraformaldehyde (PFA) and glutaraldehyde (GA) and stained with anti-PtdIns(4,5)P_2 antibodies 2C11 (A) or 10F8 (B). Fixation and staining was performed at 25°C (A) or 4°C (B) as described in Materials and Methods. Top panels show anti-PtdIns(4,5)P_2 (red), bottom panel a merge between 2C11 and BODIPY-ceramide stained membranes (green). Scale bars = 10 μm.

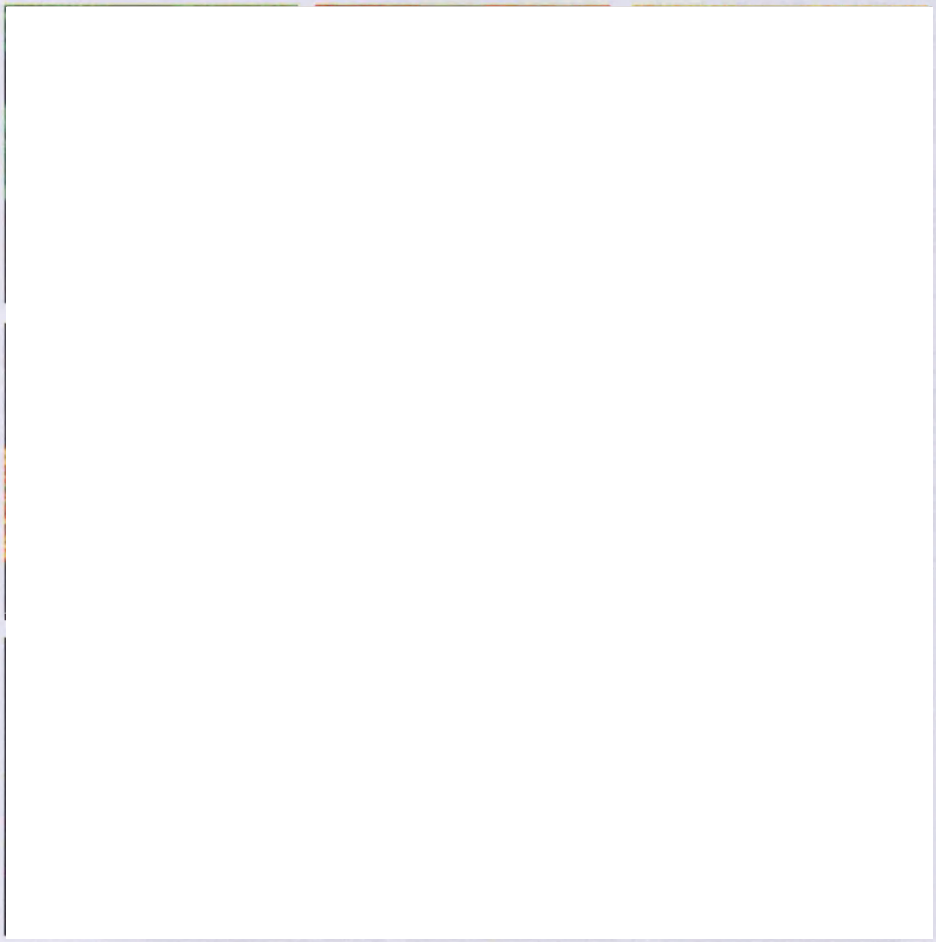

Figure 4.6: PtdIns(4,5)P_2 probes label the plasma membrane. (a-c) RPMCs were labelled with monoclonal anti-PtdIns(4,5)P_2 antibody 2C11 (red) and Alexa488-phalloidin (green). (d-e) merged images of RPMCs stained with anti-PtdIns(4,5)P_2 antibodies 10F8 and kt3g, respectively (red); merged images are shown of cells counter-stained with Alexa488-phalloidin (green). (f) RPMCs stained with 100 nM GST-PH-PLCδ1 (red). (g) RPMCs stained with GST-PH-GRP1 (red) and Draq5™ (blue). (h) RPMCs stained with the plasma membrane dye CM-DiI$_{18}$ (red). All images are z-stacks of confocal sections taken at 1 μm intervals through the cell, except for (g) which is a single equatoral confocal section. Scale bars = 10 μm. (i) field-emission scanning electron micrograph of a fixed RPMC (image courtesy of Dr. Stephen Gschmeissner).

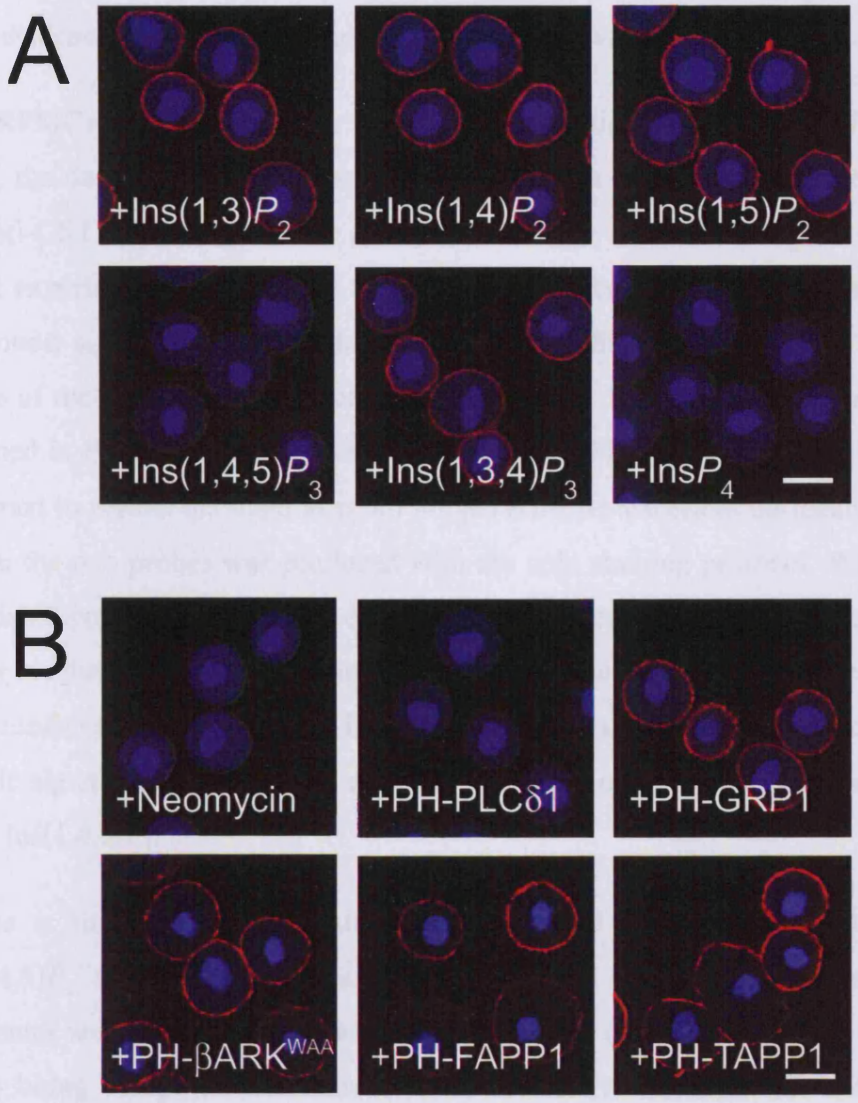

Figure 4.7: specificity of anti-PtdIns(4,5)P_2 antibody 2C11. RPMC were fixed and stained with 2C11 (red) and Draq5™ (blue) at 4°C as described in Materials and Methods. (A) Antibodies were applied after preabsorption with 213 μM of the indicated inositol phosphates (a 1000-fold molar excess assuming 10 PtdIns(4,5)P_2 binding sites/IgM). Images show equatorial confocal sections. (B) Cells were either pre-absorbed with 50 μM of the indicated PH domain, or stained in the presence of 1 mM neomycin. Scale bars = 10 μm.

Notably, it was concluded in chapter 3 that the inhibition of exocytosis by the mutant PH domain from βARK (PH-βARK^{WAA}) must be due to an interaction with an inositol lipid other than PtdIns(4,5)P_2. Indeed, 50 μM of this PH domain (a concentration 20-fold that required to produce substantial inhibition of exocytosis, figure 3.2B) did not compete with 2C11, demonstrating that PH-βARK^{WAA} did not sequester endogenous PtdIns(4,5)P_2 (figure 4.7B). This lends further evidence to support the original conclusion drawn in chapter 3.

When RPMCs were stained with a lower concentration (10 μM) of GST-PH-PLCδ1, the domain did not compete with 2C11; if its localisation was revealed with anti-GST antibodies, it was seen to co-localise with 2C11 (figure 4.8B). Control experiments revealed that the anti-GST antibody, despite giving a weak background signal, did not produce a similar localisation when used in the absence of the GST-tagged PH domain (figure 4.8A). Similar experiments were performed in HEK-293 cells transiently expressing GFP-PH-PLCδ1 (figure 4.9). In contrast to results obtained at room temperature, an excellent co-localisation between the two probes was produced with the cold staining protocol. When 3-dimensional projections of several confocal sections were viewed, 2C11 produced a higher resolution image of the surface structures picked out by both probes. The poorer resolution observed with GFP-PH-PLCδ1 is likely caused by the diffuse cytosolic signal, corresponding to unbound PH domain, or PH domain bound to soluble Ins(1,4,5)P_3.

Because a similar staining pattern was observed with monoclonal anti-PtdIns(4,5)P_2 antibodies 10F8 and kt3g, inositol phosphate competition experiments were also performed with these antibodies (figure 4.10). However, as well as being competed with Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4, both antibody stainings were prevented with Ins(1,3,4)P_2. In fact, Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4 were better competitors for kt3g staining than Ins(1,4,5)P_3. Taken together with the observation that kt3g displays a higher affinity interaction with PtdIns(3,4)P_2 on protein-lipid overlay assays (figure 3.4), it appears that kt3g forms a higher affinity interaction with PtdIns(3,4)P_2 than PtdIns(4,5)P_2.

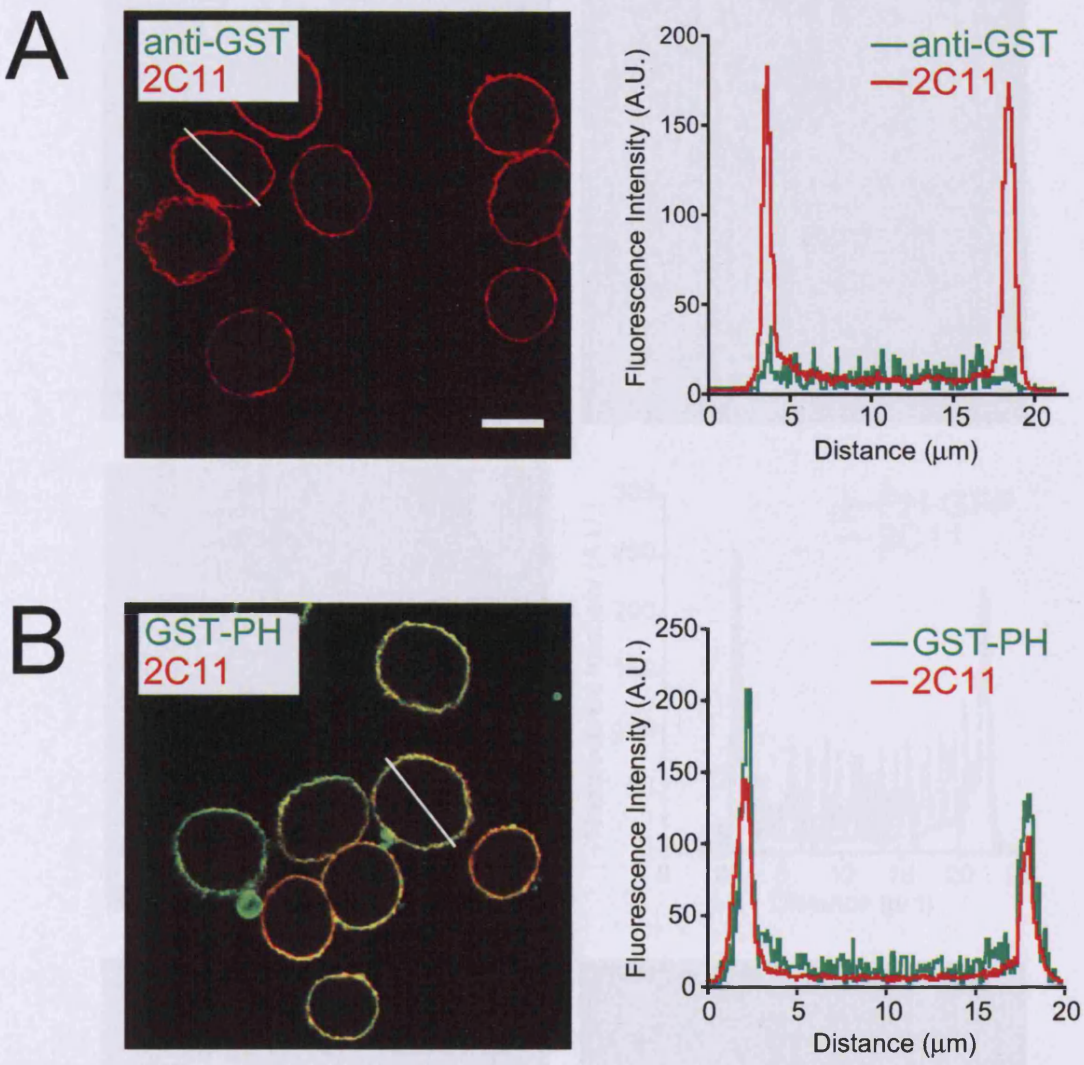

Figure 4.8: 2C11 co-localises with GST-PH-PLCδ1 at the plasma membrane. RPMC were fixed and stained with 2C11 (red) and polyclonal anti-GST antibody (green) after incubation during blocking in the presence (B) or absence (A) of 10 μM GST-PH-PLCδ1. Left panels show a merged image from the red and green channel. Co-localisation appears yellow. The graphs on the right show the fluorescence intensity profiles for both chanels along the lines depicted in the images. Scale bar = 10 μm.

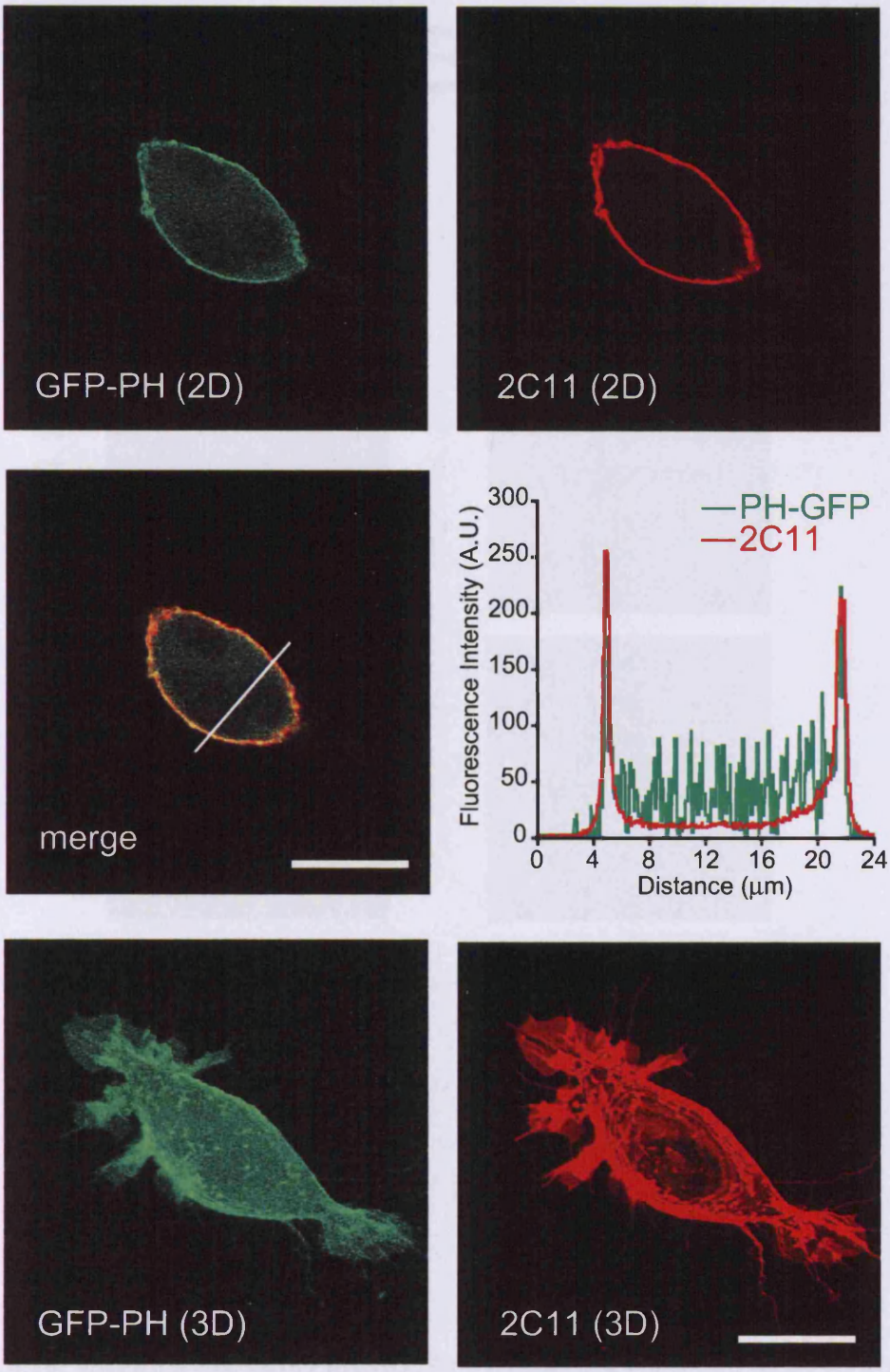

Figure 4.9: 2C11 co-localises with expressed GFP-PH-PLCδ1 at 4°C. HEK cells expressing GFP-PH-PLCδ1 were fixed and stained with 2C11 at 4°C as described in Materials and Methods. The graph refers to the fluorescence intensity (in arbitrary units) of the GFP (green) and 2C11 (red) channels along the line through the cell depicted in the merged image. 2D indicates a single confocal section; 3D denotes a z-stack of confocal sections acquired at 1 µm intervals through the same field. Scale bars = 20 µm.

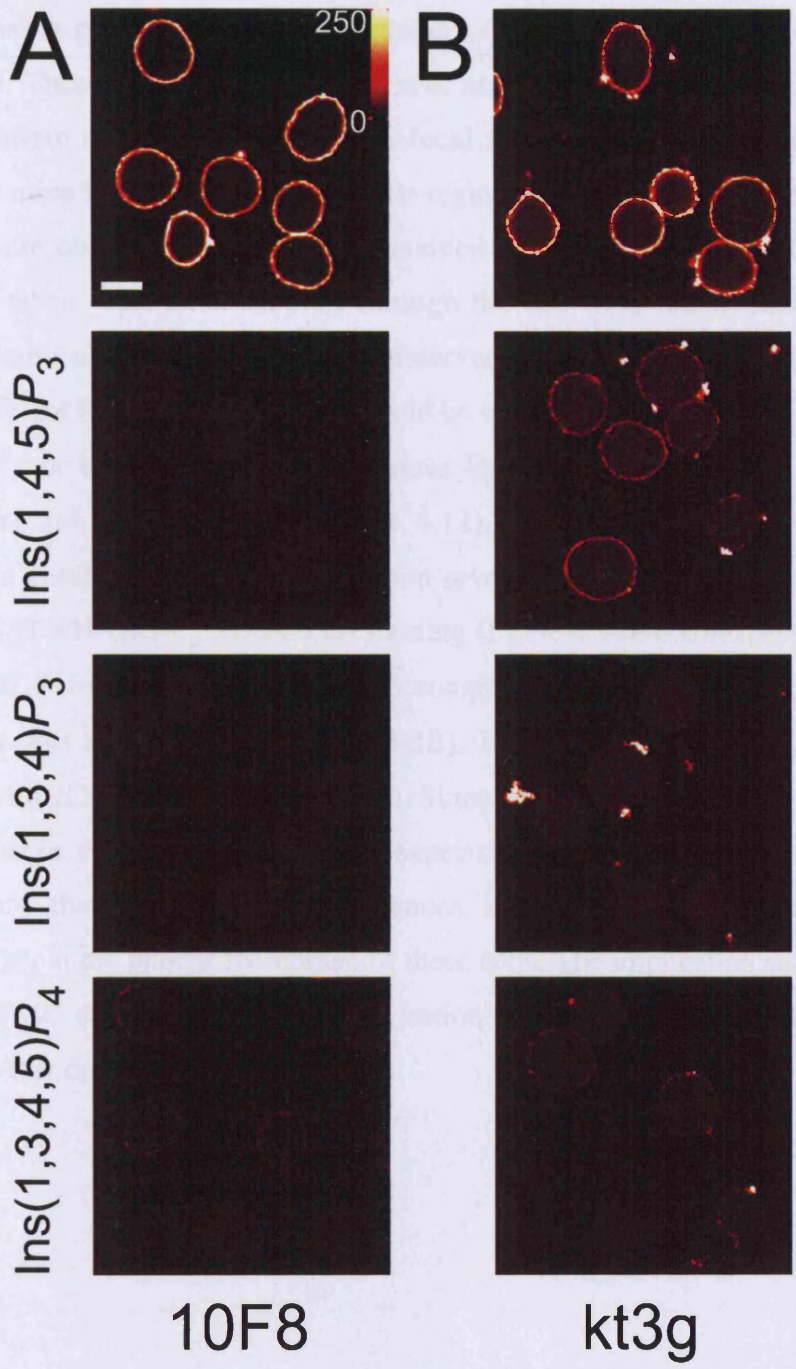

Figure 4.10: specificity of anti-PtdIns(4,5)P_2 antibodies 10F8 and kt3g. RPMC were fixed and stained for PtdIns(4,5)P_2 with monoclonal antibodies 10F8 (A) or kt3g (B) at 4°C as described in Materials and Methods. Antibodies were applied either alone (top pannels) or after preabsorption with 300 μM of the indicated inositol polyphosphates. Images show fluorescence intensity scales for equatorial confocal sections, proceeding from black at 0, through red to 250 (arbitrary units) in white, as indicated in the inset. Laser power and CCD camera gain and offset were set such that background signal (in the absence of secondary antibody) and the maximum intensity fell within the range of detection. Scale bar = 10 μm.

2C11 thus gives a reliable localisation of PtdIns(4,5)P_2 in fixed cells after a cold fix and staining protocol in RPMC and HEK-293 cells. To address whether this protocol has a general application, staining of NIH-3T3 fibroblasts was also performed. These cells are rather flattened, and 2C11 produced a continuous staining pattern across the cell when confocal sections were views, which was apparently more intense in the juxtanuclear regions (figure 4.11). Similar staining patterns were observed in COS-7 cells stained with KT10 (Laux *et al.*, 2000). However, when orthogonal sections through the cell were reconstructed from stacks of confocal sections, staining was observed at the cell periphery. The signal was specific for PtdIns(4,5)P_2, since it could be competed with Gro*P*Ins(4,5)P_2 or Ins(1,4,5)P_3, or by sequestering endogenous lipid with neomycin or GST-PH-PLCδ1, but not GST-PH-GRP1 (figure 4.12). Furthermore, GST-PH-PLCδ1 produced a similar staining to 2C11 when revealed with anti-GST antibodies, whereas GST-PH-GRP1 produced no staining (figure 4.12B). Unexpectedly, 10 μM of GST-PH-PLCδ1 was sufficient to compete with 2C11 in NIH-3T3 cells, whereas it was not in RPMC (figure 4.8B). Indeed, 50 μM was required to compete with 2C11 in RPMC (figure 4.7). Since identical protocols and antibody dilutions were employed in all these experiments, it seems likely that this discrepancy therefore reflects differences in the local concentration of PtdIns(4,5)P_2 at the plasma membrane of these cells. The implication would thus be that RPMC contain a higher concentration of PtdIns(4,5)P_2 at the plasma membrane that do NIH-3T3 cells.

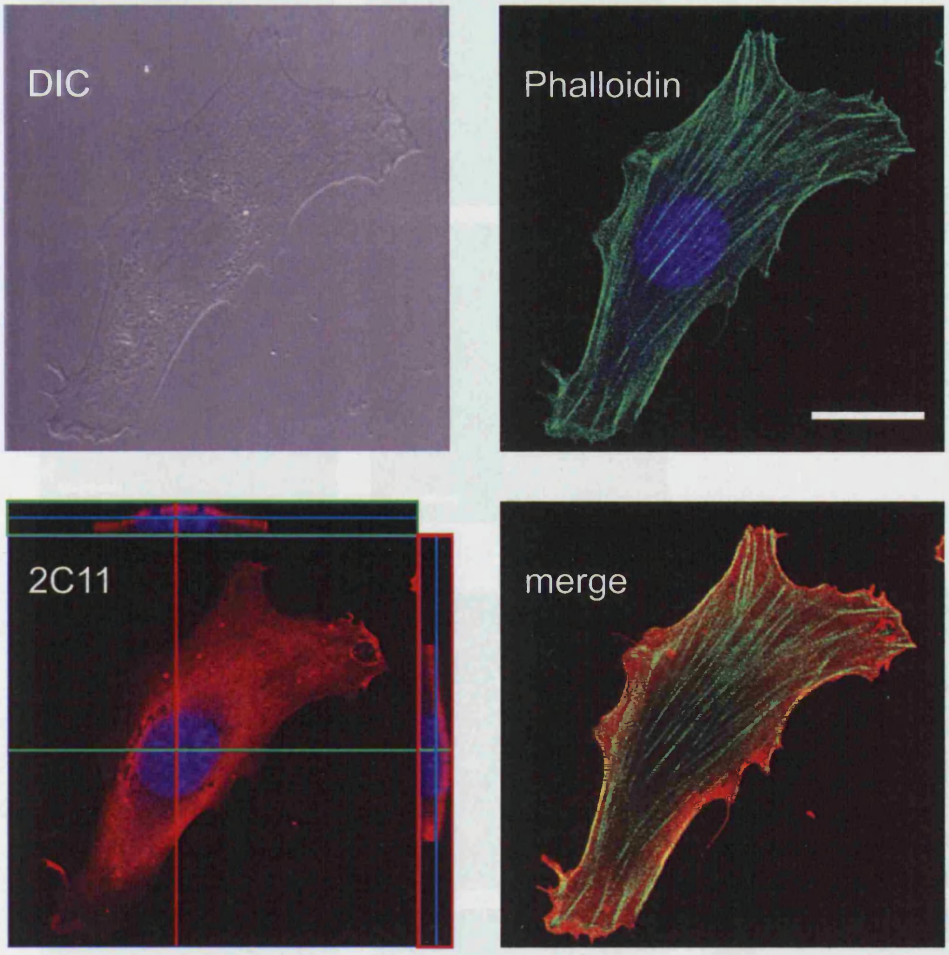

Figure 4.11: Surface staining of NIH-3T3 cells with 2C11 at 4˚C. 3T3 cells were fixed and stained with 2C11 (red), Draq5™ (blue) and Alexa488-phalloidin (green) as indicated at 4˚C (Materials and Methods). For the image depicting 2C11, orthogonal views through the indicated horizontal and vertical axes are also shown. The blue line in the orthogonal views represents the position chosen for the optical section shown in the other panels. Scale bar = 20 μm.

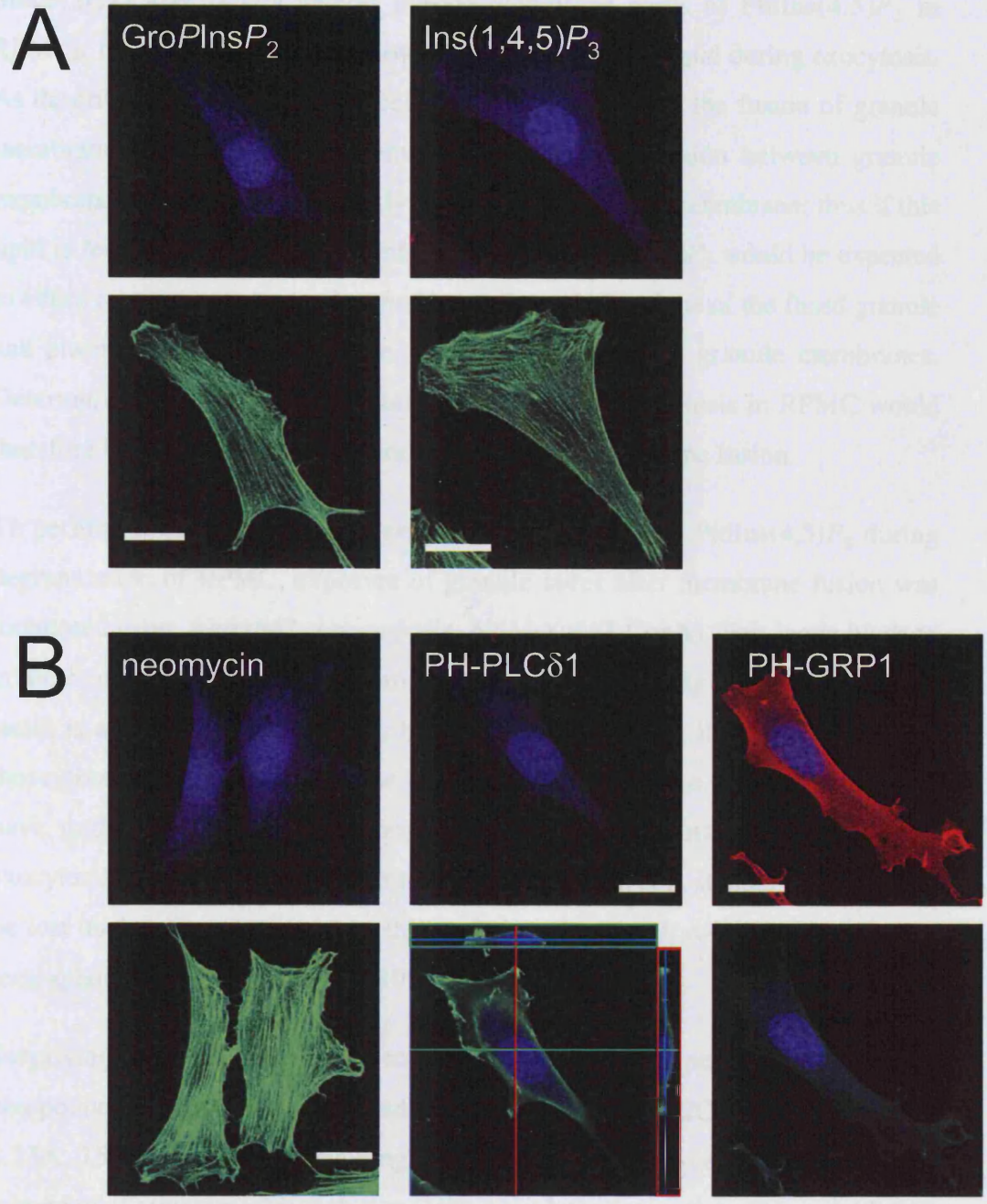

Figure 4.12: 2C11 detects PtdIns(4,5)P_2 at the surface of NIH-3T3 cells. 3T3 cells were fixed and stained for PtdIns(4,5)P_2 with monoclonal antibody 2C11 (red), nuclei with Draq5™ (blue) and either F-actin with Alexa488-phalloidin (green) or GST-tagged proteins with anti-GST (green) as indicated. (A) 2C11 was preabsorbed with 0.2 mg/ml Gro*P*Ins(4,5)P_2 or a 2000-fold molar excess of Ins(1,4,5)P_3. (B) Cells were preabsorbed with 100 μM neomycin (cells counter-stained with phalloidin) or 10 μM of the indicated GST-PH domain fusion proteins, which were revealed with anti-GST (green). Single confocal sections are shown, with orthogonal views for PH-PLCδ1 stained cells as in figure 4.11. Scale bars = 20 μm.

4.3. PtdIns(4,5)P_2 dynamics during exocytosis

Since 2C11 specifically detects plasma membrane pools of PtdIns(4,5)P_2 in RPMCs, this tool was used to follow the dynamics of this lipid during exocytosis. As described in chapter 1, mast cell exocytosis occurs via the fusion of granule membranes with the plasma membrane, followed by fusion between granule membranes. PtdIns(4,5)P_2 was only detected at the plasma membrane; thus if this lipid is required at the site of membrane fusion, PtdIns(4,5)P_2 would be expected to either relocate to the granule membranes through the plane of the fused granule and plasma membranes, or to be synthesized *de novo* at granule membranes. Determining the localisation of PtdIns(4,5)P_2 during exocytosis in RPMC would therefore have important implications for its role in membrane fusion.

To permit the simultaneous observation of exocytosis and PtdIns(4,5)P_2 during degranulation of RPMC, exposure of granule cores after membrane fusion was monitored using Alexa647 concanavalin-A (Alexa647-ConA). This lectin binds to granule cores with high affinity, producing an intense staining pattern. Thus if this lectin is applied to the fixed cells before permeabilisation, it only has access to those granule cores exposed to the extracellular medium, i.e. those granules that have undergone exocytosis (Norman *et al.*, 1996). Notably, since RPMC exocytosis involves extrusion of granule cores from the cell, stained granules may be lost during exocytosis and so this technique has the drawback of being only semi-quantitative (Norman *et al.*, 1996).

Surprisingly, RPMC stimulated to degranulate with the polycationic agonist, compound 48/80 (48/80) displayed a severe reduction in 2C11 staining (figure 4.13A, 15 s). This loss of staining was apparent in most cells during the first minute of stimulation, although it reappeared at later time points (figure 4.13, 150 and 300 s), well after exocytosis is complete (Penner, 1988). At these later time points, some staining was also apparent on internal membranes from fused granules, although the majority was at the plasma membrane. It thus seemed that exocytosis was accompanied by the removal of plasma membrane PtdIns(4,5)P_2.

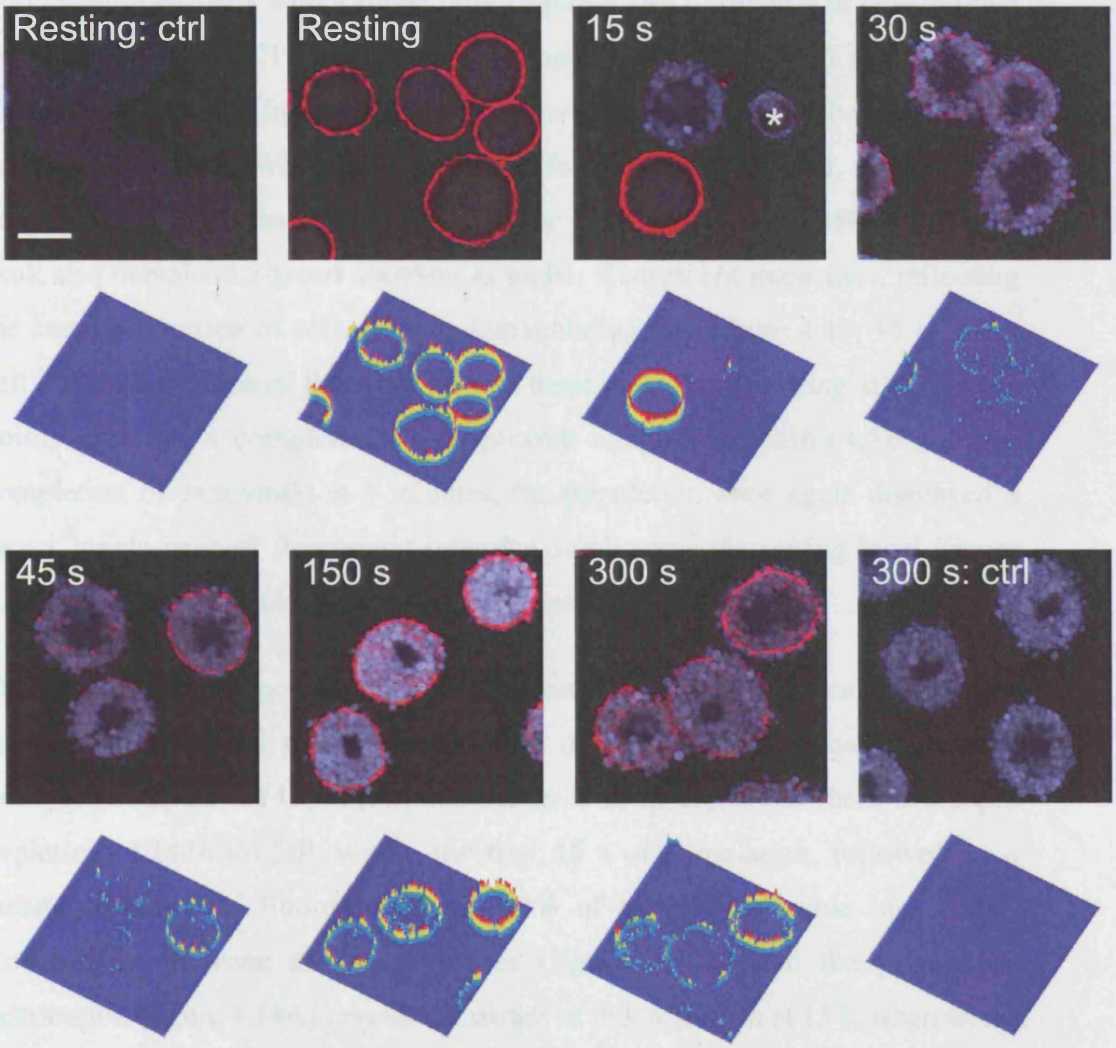

Figure 4.13: Transient depletion of plasma membrane PtdIns(4,5)P_2 staining after activation of exocytosis. RPMC were stimulated with 10 µg/ml 48/80 for the indicated time periods, before fixing and staining with 2C11 (red) and Alexa647-ConA (blue). Top panels show merged images between the red and blue channels, bottom panels a fluorescence intensity plot for the same field in the red channel. "ctrl" indicates stainings where 2C11 has been omitted, in order to determine the background fluorescence. The asterisk denotes a contaminating neutrophil. Scale bar = 10 µm.

To provide quantitative analysis of this reduction in PtdIns(4,5)P_2-staining, metamorph software was used to provide an automated analysis of low-magnification image stacks of RPMC (see Materials and Methods). Such analysis revealed that the population of resting RPMC displayed a broad distribution of fluorescent intensities with a single peak (figure 4.14A). Analysis of cells stained in the absence of 2C11 yielded a much narrower peak centred at a reduced fluorescent intensity (figure 4.14A, ctrl), providing a measure of the background noise of this system. When cells stimulated for 15 s were analysed, a single peak was observed that substantially overlaid the background signal. However, this peak also contained a broad shoulder at higher fluorescent intensities, reflecting the small proportion of cells not yet degranulating (see figure 4.13, 15 s, lower cell). The implication of these data is that those cells degranulating at early time points undergo a complete loss of plasma membrane PtdIns(4,5)P_2. After completion of exocytosis at 5 minutes, the population once again displayed a broad, single peak of fluorescent intensity overlapping the resting level (figure 4.14A, 300 s), representing the recovery of staining.

From these data, the population mean fluorescence value was calculated at each time point, and used to plot the kinetics of PtdIns(4,5)P_2 dynamics during exocytosis (figure 4.14B). From this curve, it is apparent that there is a rapid depletion of PtdIns(4,5)P_2 within the first 15 s of stimulation, followed by a steady recovery of fluorescence to ~90% of the resting value in ~3 min. Comparison between the mean values (figure 4.14B) and the population distribution (figure 4.14A) reveals the nature of this depletion at 15 s: whereas the population mean drops by 73%, the fluorescence distribution reveals that 82% of the cells overlap with the background peak. Therefore, the mean value represents the majority of cells having undergone almost complete depletion of PtdIns(4,5)P_2.

The simplest interpretation for these observations is that PtdIns(4,5)P_2 itself is being transiently depleted from the plasma membrane during exocytosis. However, alternative explanations are feasible. For example, the decrease could represent binding of a high affinity effector protein to PtdIns(4,5)P_2 in an interaction that survives fixation, and thus prevents access of antibody to the lipid.

To test this possibility, radiolabelling of inositol lipids in RPMCs was performed. Notably, since this extraction procedure uses 1 M HCl (see Materials and Methods), any interactions between protein and lipid are unlikely to survive. RPMC were labelled in low-inositol Medium 199, containing 1.6 μM inositol; parallel cultures contained either unlabelled inositol, or [2-^{3}H]-inositol at 25 μCi/ml. After loading for 19 h, cells were rinsed and then stimulated for 0, 15, 30 or 60 s with 48/80 (figure 4.15).

After fixation and staining with 2C11, a number of differences between cells incubated for 19 h and those used immediately after purification could be observed (figure 4.15A). Firstly, the resting cells contained two peaks of fluorescence: the major peak containing 74% of the cells, and a minor peak overlapping that of background containing 26% of the cells. This latter peak presumably reflected cells that had not survived the 19 h incubation. Secondly, although a similar number of cells (79%) overlapped with the background peak after stimulation for 15 s, the population mean dropped only by 54% (figure 4.15B). This most likely reflects the fact that the resting population contained cells with no fluorescence above background, decreasing the population mean and thus accounting for the relative decrease observed at 15 s. Finally, recovery of PtdIns(4,5)P_2-staining appeared faster after incubating the cells for 19 h in culture, completing to ~80% of control in ~ 1 min (figure 4.15).

Importantly, when the mean fluorescence values were plotted along with the biochemical measurements of [2-^{3}H]-PtdIns(4,5)P_2, excellent agreement was found between the two estimates (figure 4.15B). Both displayed the transient depletion within 15 s (54 ± 6 % for 2C11, 52% for [2-^{3}H]-PtdIns(4,5)P_2), and recovery within ~ 60s. Therefore, the fluorescence measurement appeared to reflect changes in [2-^{3}H]-PtdIns(4,5)P_2 during exocytosis. However, fluorescence measurements had a tendency to slightly underestimate the biochemical analysis. Although this may be due to an undetermined experimental error in either technique, the disparity could also be due to the existence of a second, minor pool of PtdIns(4,5)P_2 which is detected by [2-^{3}H]-inositol labelling, but not by 2C11.

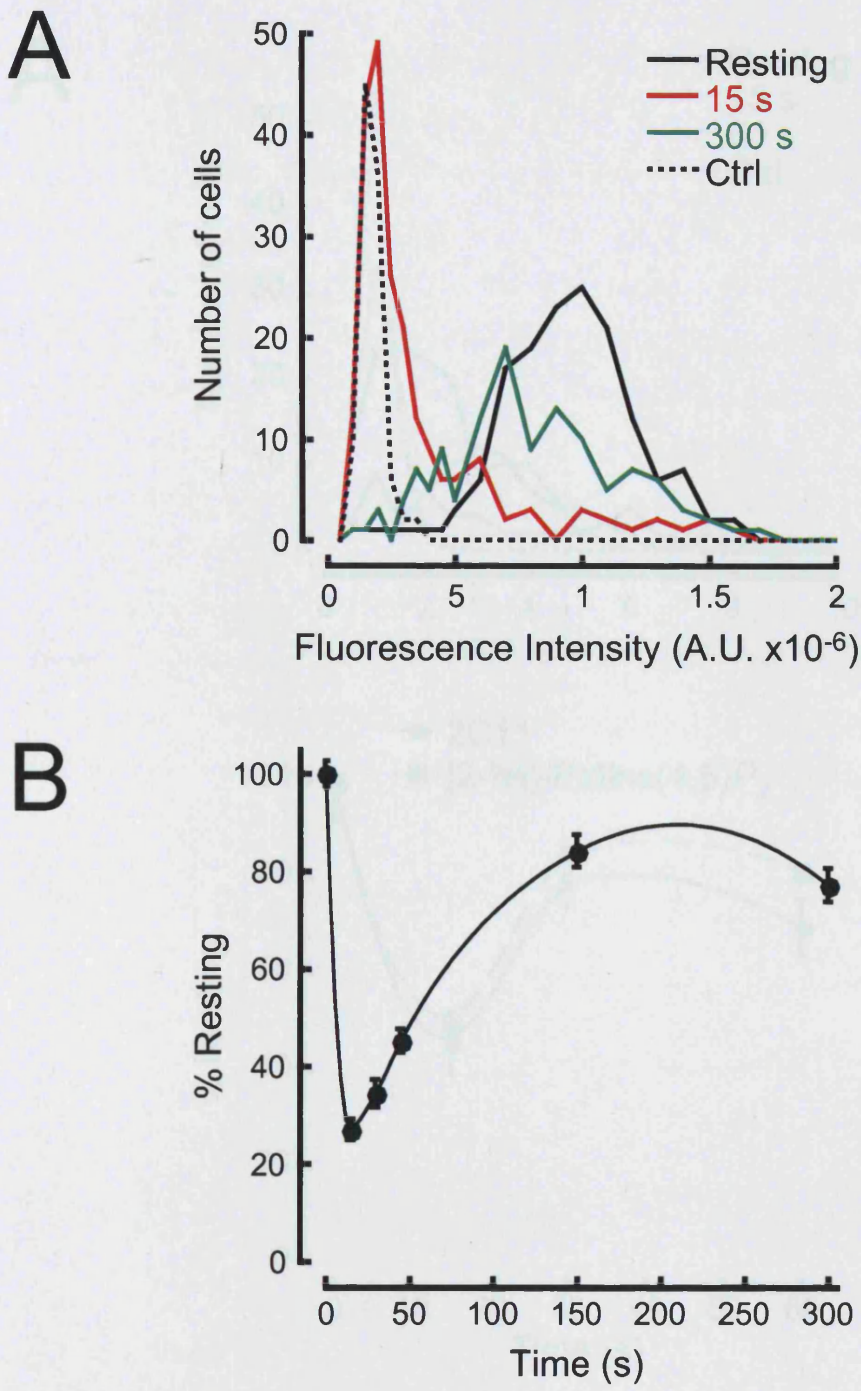

Figure 4.14: quantitative analysis of 2C11 staining during exocytosis. Low magnification images from the experiment presented in figure 4.13 were analysed using an automated programme (written by A. Nicol and D. Zicha, CR-UK Light Microscopy Laboratory) to determine total fluorescence intensity for the red channel as described in Materials and Methods. (A) The distribution of fluorescence intensity across a resting population, or across a population stimulated for 15 and 300 s with 10 µg/ml 48/80 is plotted as indicated. "ctrl" refers to a population stimulated for 300s, where 2C11 was omitted from the staining procedure. (B) The population means (± S.E.M., $n \geq 100$) for the indicated time points, calculated after subtraction of the ctrl fluorescence and normalisation to the resting (0 s) level.

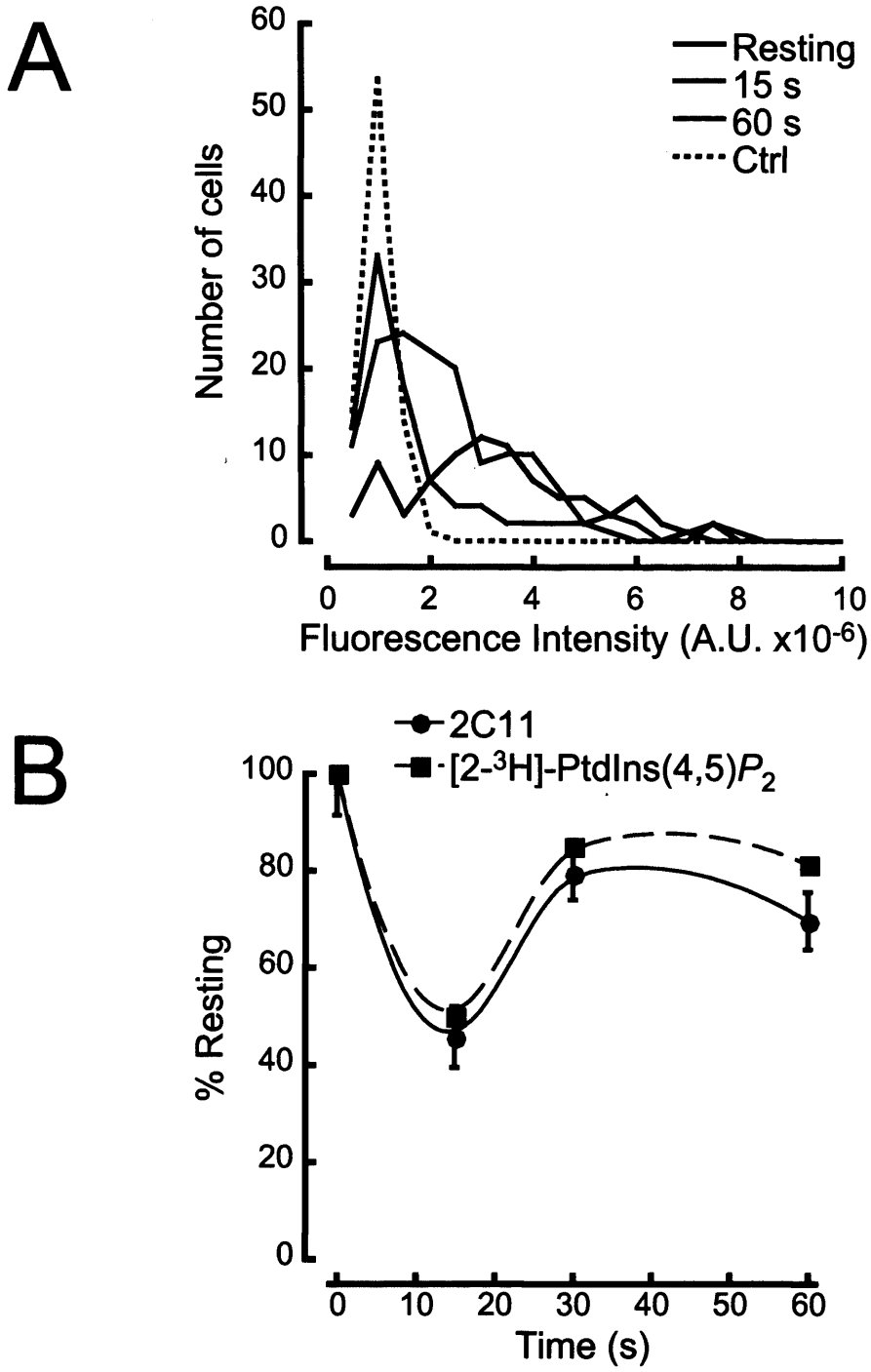

Figure 4.15: 2C11 accurately measures PtdIns(4,5)P_2 levels during exocytosis. RPMC were cultured for 19 hours in Medium 199 with 1.6 μM unlabelled Ins (A & B), or 25 μCi/ml [2-^{3}H]-Ins (B) before stimulation with 10 μg/ml 48/80 for the indicated time periods. (A) The distribution of fluorescence intensities as described for figure 14.14A. (B) The mean fluorescent intensities (± S.E.M., $n \geq 98$) for the population stimulated for the indicated time point are plotted as in figure 4.14B. For comparison, the counts in [2-^{3}H]-PtdIns(4,5)P_2 are plotted on the same graph; both data sets are normalised to the levels observed in resting cells (time 0). [2-^{3}H]-PtdIns(4,5)P_2 was determined after extraction of the lipids and analysis using HPLC (S.K. Dove, University of Birmingham) as described in Materials and Methods.

As discussed at the beginning of this chapter, 2C11 was previously found to stain nuclear speckles in HeLa cells (Osborne *et al.*, 2001). In this study, Triton X-100 was used to permeabilise all cellular membranes. Since saponin was used in the experiments presented hitherto, the nuclear envelope is most likely not permeabilised. To investigate whether mast cells contain a detergent resistant nuclear pool of PtdIns(4,5)P_2, Triton X-100 was used to extract membranes during staining with 2C11. Indeed, RPMC contained a weak speckled staining in the nucleus (figure 4.16, resting). Notably, after stimulation with 48/80 the fluorescence intensity was seen to increase dramatically, increasing by 5-fold within 30 s, before dropping and eventually reaching a plateau at 4-fold after ~ 3 min (figure 4.16). The total fluorescence intensity was compared to cells imaged using the standard protocol used for plasma membrane PtdIns(4,5)P_2, and revealed that the resting nuclear staining was ~1% the intensity of the plasma membrane staining. The size of the nuclear PtdIns(4,5)P_2 pool therefore seems relatively minor compared to the plasma membrane pool in RPMCs, and may therefore reflect the difference between fluorescence and radiolabelled estimates of PtdIns(4,5)P_2.

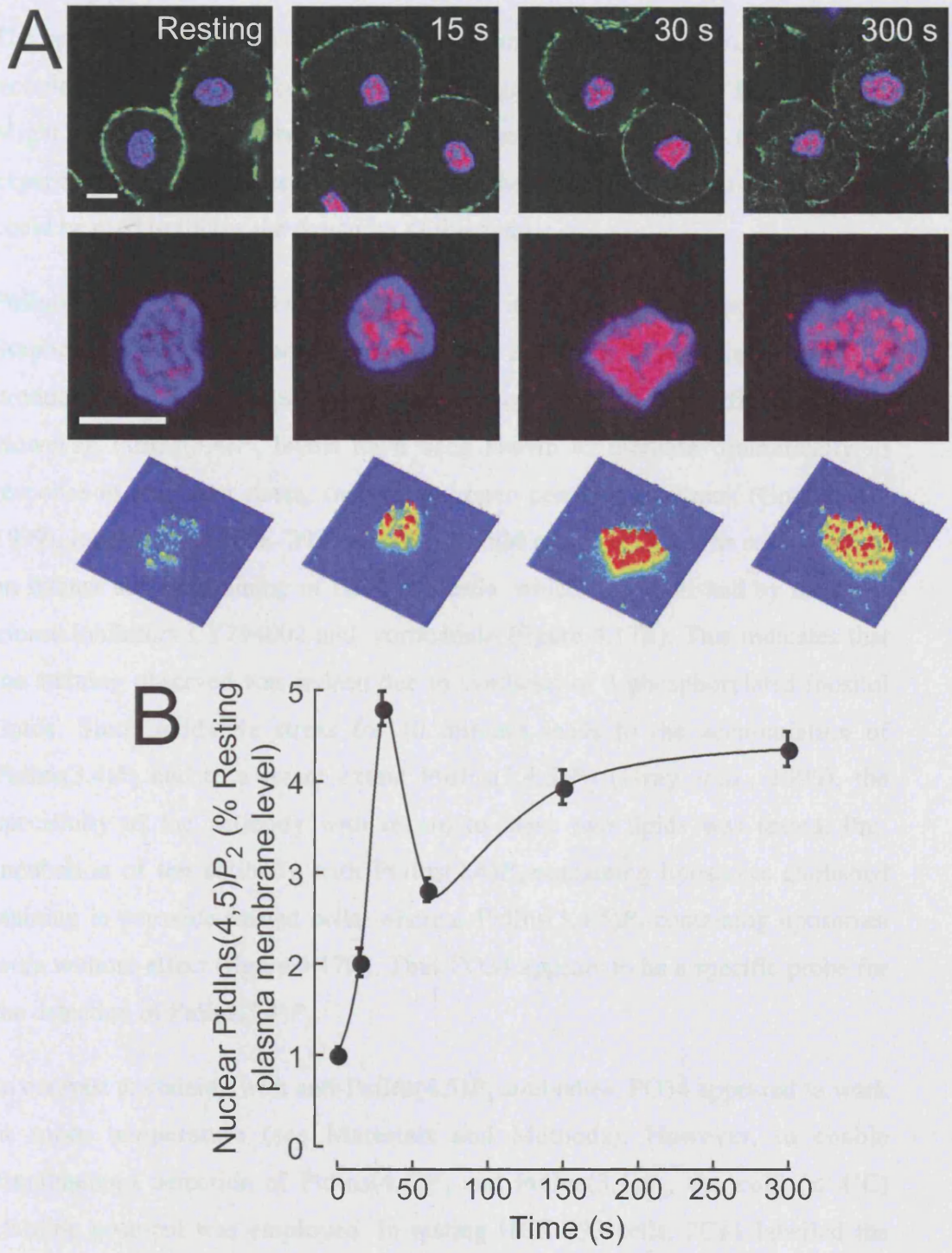

Figure 4.16: nuclear PtdIns(4,5)P_2 levels increase in response to 48/80. RPMC were stimulated with 10 µg/ml 48/80 as indicated, fixed and extracted with 0.2% Triton X-100. They were stained with 2C11 (red), Alexa488-phalloidin (green) and Draq5™ (blue) as described in Materials and Methods. (A) Centre panels show an enlarged view of one of the nuclei from the top panel; bottom panels show fluorescence intensity profiles in the red channel for the nucleus shown in the centre panel. Scale bars = 5 µm. (B) Mean fluorescence intensity (± S.E.M., n ≥ 100) of nuclei were calculated, and expressed as the % of the fluorescence of plasma membrane staining.

PtdIns(3,4)P_2 dynamics during oxidative stress

The results presented thus far indicate that immunofluorescence is an accurate technique with which to follow the dynamics of localised pools of PtdIns(4,5)P_2. Might such a technique be applicable to other PIn? To address this question, experiments were conducted to establish whether antibodies against PtdIns(3,4)P_2 could be used to follow the dynamics of this lipid.

PtdIns(3,4)P_2 is present at undetectable levels in resting cells (Auger *et al.*, 1989; Stephens *et al.*, 1991). Indeed, staining with anti-PtdIns(3,4)P_2 antibody PO34 produced only a very faint, diffuse staining of HEK 293 cells (figure 4.17A). However, PtdIns(3,4)P_2 levels have been shown to increase dramatically in response to oxidative stress, such as hydrogen peroxide treatment (Gray *et al.*, 1999). Incubation of HEK-293 cells with 10 mM peroxide led to the emergence of an intense surface staining of HEK 293 cells, which was abolished by the PI 3-kinase inhibitors LY294002 and wortmannin (figure 4.17B). This indicates that the staining observed was indeed due to synthesis of 3-phosphorylated inositol lipids. Since oxidative stress for 10 minutes leads to the accumulation of PtdIns(3,4)P_2 and to a lesser extent PtdIns(3,4,5)P_3 (Gray *et al.*, 1999), the specificity of the antibody with regard to these two lipids was tested. Pre-incubation of the antibody with PtdIns(3,4)P_2-containing liposomes abolished staining in peroxide-treated cells, whereas PtdIns(3,4,5)P_3-containing liposomes were without effect (figure 4.17C). Thus PO34 appears to be a specific probe for the detection of PtdIns(3,4)P_2.

In contrast to staining with anti-PtdIns(4,5)P_2 antibodies, PO34 appeared to work at room temperature (see Materials and Methods). However, to enable simultaneous detection of PtdIns(4,5)P_2 and PtdIns(3,4)P_2, the cold ($\leq$ 4°C) staining protocol was employed. In resting HEK-293 cells, 2C11 labelled the plasma membrane as noted previously (figure 4.9); in contrast to cells labelled at room temperature, PO34 also produced a plasma membrane staining, albeit rather weak (figure 4.18A). It seems likely that this is caused by cross-reactivity of PO34 with PtdIns(4,5)P_2: this would explain why only a very weak, diffuse signal is seen with PO34 at room temperature (figure 4.17A), given that a similar localisation of PtdIns(4,5)P_2 is also seen under these conditions (figure 4.1).

Upon a 10 minute oxidative shock with 10 mM peroxide, an intense staining with PO34 appeared, whereas the staining with 2C11 was not appreciably altered (figure 4.18B). Notably, the two signals displayed an identical localisation, demonstrating synthesis of PtdIns(3,4)P_2 at the plasma membrane in response to oxidative shock. Given that the co-localisation was near perfect, it was important to check that this was not due to cross-reactivity among the fluorescent secondary antibodies. Indeed, Alexa488 anti-IgG_3 did not cross-react with the 2C11 IgM, and the Alexa555 anti-IgM did not cross-react with the PO34 IgG_3 (figure 4.18C).

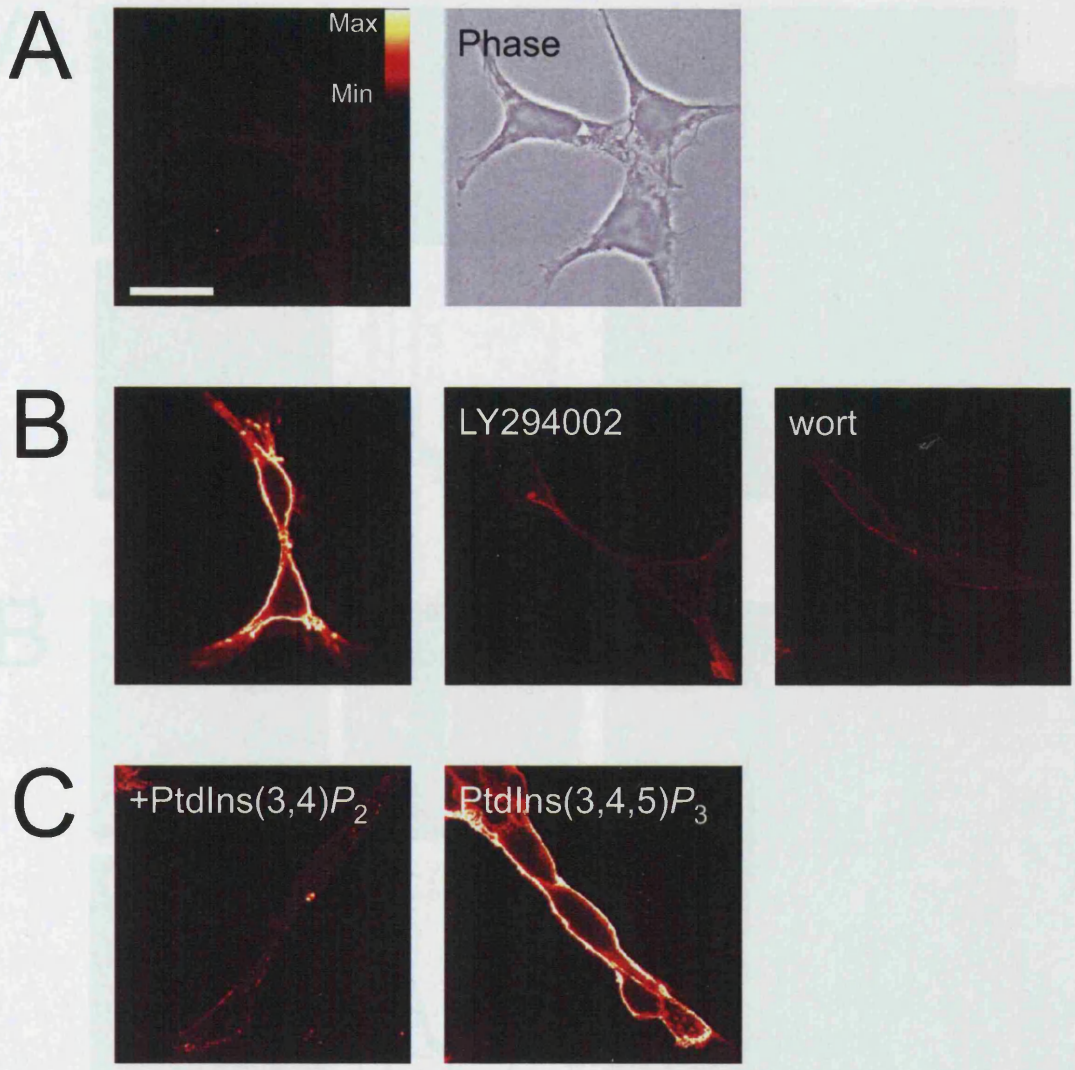

Figure 4.17: antibody PO34 specifically stains PtdIns(3,4)P_2. HEK 293 cells were treated for 10 min in the presence (B & C) or absence (A) of 10 mM H_2O_2. They were then fixed, permeabilized with 0.5% saponin and stained for PtdIns(3,4)P_2 with monoclonal antibody PO34 at room temperature as described in Materials and Methods. Images show fluorescence intensity scales, with signal strength increasing through red to white, as indicated in A. (B) cells were pre-incubated for 20 min with 50 μM LY294002 or 100 nM wortmannin (wort) before stimulation in the continuing presence of LY294002, as indicated. (C) PO34 was preabsorbed with liposomes containing 5 mole % dipalmitoyl-PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 as indicated. Scale bar = 10 μm.

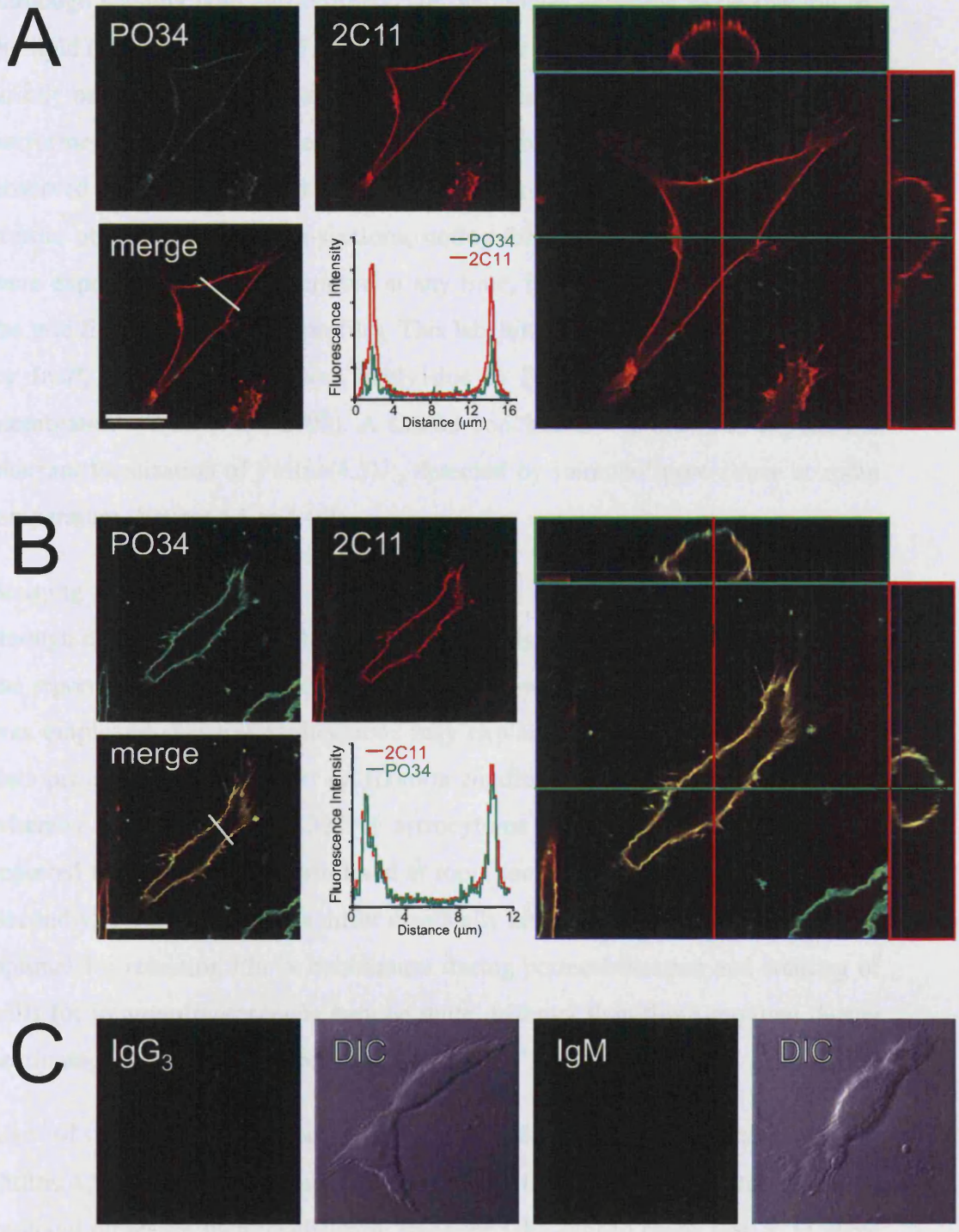

Figure 4.18: anti-PIn antibodies can detect multiple PIn isomers in the same cell. HEK 293 cells were treated for 10 min in the presence (B, C) or absence (A) of 10 mM H_2O_2 prior to fixation and staining with PO34 and Alexa488-anti-IgG_3 (green) plus 2C11 and Alexa555-anti-IgM (red). The enlarged image on the right of A and B shows orthogonal views along the depicted axes at top and right. (C) shows cells stained with 2C11 and Alexa488-anti-IgG_3 (green) or with PO34 and Alexa555-anti-IgM (red); DIC images from the same field are shown on the right. Scale bar = 20 μm.

4.5. Conclusions

Although staining with anti-PtdIns(4,5)P_2 antibodies appeared to be specific for the lipid (figures 4.1, 4.3, 4.7, 4.10 and 4.12), the localisation observed depended strictly on the fixation conditions used and the temperature at which staining was performed (figure 4.5). Indeed, membrane association of the signal was only observed when fixation and staining was performed ≤ 4°C. Watt *et al.* made similar observations on cryo-sections, noting that "when grid-mounted sections were exposed to room temperature at any time, there was significant staining of the grid film outside the cell profiles. This labelling could be inhibited specifically by InsP_3 headgroups and was likely due to PtdIns(4,5)P_2 release from the membranes" (Watt *et al.*, 2002). A similar conclusion was drawn to explain the aberrant localisation of PtdIns(4,5)P_2 detected by immunofluorescence at room temperature (figures 4.1 and 4.2).

Staining was also sensitive to the temperature of fixation, and was improved through the use of glutaraldehyde as a fixative (figure 4.5). This is in contrast with the report by Watt *et al.*, wherein paraformaldehyde fixation at room temperature was employed. Several explanations may explain this discrepancy. Firstly, the data presented in this chapter on fixation conditions were performed in RPMC, whereas Watt *et al.* used 1321N1 astrocytoma cells; RPMC were previously reported to undergo lysis when fixed at room temperature (Lawson *et al.*, 1977). Secondly, the two procedures differ drastically after fixation, therefore conditions optimal for retaining PIn in membranes during permeabilisation and staining of cells for immunofluorescence may be quite different than those required during sectioning and on-section labelling.

Use of GFP-PH-PLCδ1 as a reporter for the pre-fixation localisation of PtdIns(4,5)P_2 provides compelling evidence that the cold fixation-staining protocol preserves such localisation, since the GFP-tagged probe and 2C11 show a near perfect overlap (figure 4.9). The immunofluorescence protocol also possesses the advantage of a lower cytosolic background. Furthermore, by comparing fluorescence measurements with biochemical measurements (figure 4.15), 2C11 was shown to accurately reflect the relative PtdIns(4,5)P_2 content of

cells. Immunofluorescence thus provides a reliable, accurate and quantitative tool with which to observe PtdIns(4,5)P_2 content in fixed cells.

In addition, monoclonal antibody PO34 was shown to be a reliable tool to detect PtdIns(3,4)P_2 in fixed cells (figures 4.17 and 4.18). Using this tool, PtdIns(3,4)P_2 was shown to be synthesized exclusively at the plasma membrane in response to a 10 minute oxidative stress. This is in contrast with the results reported in two other studies, which noted that as well as appearing at the plasma membrane, PtdIns(3,4)P_2 could also be observed on the nuclear envelope (Yokogawa *et al.*, 2000), endoplasmic reticulum (ER) and luminal vesicles of the multivesicular body (Watt *et al.*, 2004) under similar conditions. Some of these differences may be explained by the use of saponin to permeabilise cells for the work presented in this chapter: saponin will probably not permeabilise the membranes of multi-vesicular bodies or the nuclear envelope, preventing staining of the luminal vesicles and inner membrane of the nuclear envelope, respectively. It is harder to explain the lack of fluorescence on ER membranes, but it worth noting again the differences between the immunofluorescence protocol and on-section labelling of cryosections (Watt *et al.*, 2002; Watt *et al.*, 2004).

It was surprising that PtdIns(3,4)P_2-staining did not require reduced temperatures for retention at the plasma membrane (figure 4.17). One reason why PtdIns(3,4)P_2 may be more readily retained in the membrane than is PtdIns(4,5)P_2 may be a difference in physical/chemical interactions with the membranes, possibly through different fatty-acid compositions of the lipids. Indeed, PPIn were shown to be enriched in stearic and palmitic acids (at the expense of arachidonate) relative to PtdIns in liver (Augert *et al.*, 1989) and myocardium (Lamers *et al.*, 1993). Furthemore, PtdIns(3,4)P_2 has been shown to be synthesised via PtdIns3*P* (Banfic *et al.*, 1998a; Banfic *et al.*, 1998b; Zhang *et al.*, 1998); using kinase mutants, it was recently reported that PtdIns3*P* and PtdIns4*P*-deficient yeast displayed different changes in fatty-acid composition (Wenk *et al.*, 2003). There is thus precedent in the literature for differential fatty acid profiles among PIn.

An alternative explanation is that peroxide treatment may lead to stabilisation of the lipids in the bilayer. There is evidence for cytoskeletal rearrangement after oxidative stress (Zhu *et al.*, 2005), with the formation of a large number of

protrusions (figure 4.18). PIn have been found to be strongly associated with the actin cytoskeleton (Fukami *et al.*, 1992; Fukami *et al.*, 1996), which may thus stabilise the lipids after fixation. Because PtdIns(4,5)P_2 staining was not tested at room temperature in the presence of oxidative stress, this therefore remains a possibility. However, using the cold fixation-staining protocol, it was possible to image both PIn in single cells (figure 4.18). Together, these results demonstrate that immunofluorescence can be applied to monitor the localisation of PPIn pools, as well as enabling dynamic changes in lipid levels to be observed.

Chapter 5: Phosphoplipase C activity is crucial for exocytosis

5.1. Introduction

In the preceding chapter, a transient decrease in PtdIns(4,5)P_2 levels was recorded after activation of exocytosis with compound 48/80. Previous studies showed that the turnover of PIn was accelerated after activation of RPMC with 48/80 or antigen (Cockcroft and Gomperts, 1979). It thus seems that a change in PIn metabolism is associated with exocytosis in RPMCs. The experiments presented in this chapter aimed to define which metabolic pathway(s) are responsible for this accelerated PIn metabolism, as well as if and how this metabolism affects exocytosis.

Notably, phospholipase C-mediated breakdown of PtdIns(4,5)P_2 has already been implicated in the regulation of mast cell exocytosis. Bone-marrow derived mast cells (BMMCs) devoid of PLCγ2 do not activate the requisite InsP_3/Ca^{2+} pathway and fail to degranulate in response to antigen (Wang *et al.*, 2000). Furthermore, antibodies against PLCβ3, but not against PLCγ1, blocked degranulation in response to 48/80 (Ferry *et al.*, 2001). Therefore, phospholipase C activity appears a likely candidate to mediate PtdIns(4,5)P_2 depletion.

5.2. Defining the pathway of PtdIns(4,5)P_2 depletion

There are three defined metabolic pathways that may lead to PtdIns(4,5)P_2 depletion: removal of the D-4 and D-5 phosphates by inositol phosphatases, phosphorylation of the D-3 position by PI3K to produce PtdIns(3,4,5)P_3, or PLC mediated hydrolysis at the phosphodiester bond to produce diaclylgcerol (DAG) and Ins(1,4,5)P_3. Notably, the first two pathways will not alter total PIn levels, whereas activation of PLC will lead to depletion of total PIn, ultimately at the expense of PtdIns as PtdIns(4,5)P_2 is resynthesized by phosphorylation (Augert *et al.*, 1989).

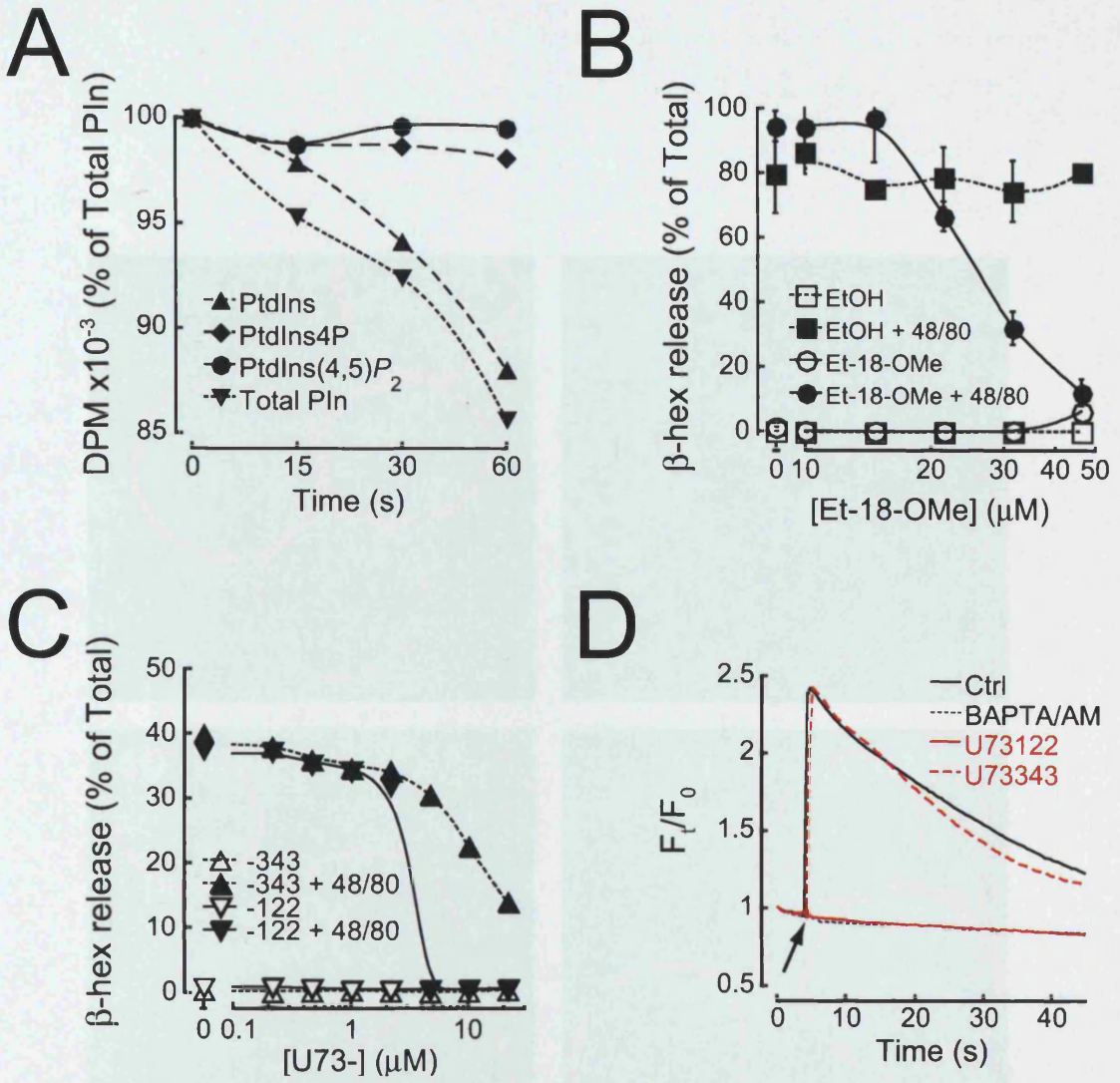

Figure 5.1. Phospholipase C activity in RPMC is required for exocytosis and Ca^{2+} signalling. (A) Stimulation with 48/80 causes a decrease in bulk phosphoinositide content. Cells labelled with [2-^{3}H]-inositol as in figure 4.15 were stimulated for the time indicated on the abscissa; the ordinate shows the change in radioactivity of PtdIns, PtsIns(4)*P*, PtdIns(4,5)P_2 or their sum (total PIn) relative to resting levels of total PIn. (B, C) PLC inhibitors prevent mast cell degranulation. RPMCs were pre-incubated with Et-18-OMe (or EtOH, as vehicle control) for 20 min (B), or U73-122/-343 for 5 min (C), at the indicated concentration before stimulation with 48/80 at 25 °C. After 10 min, the medium was assayed for released β-hexosaminidase activity. (D) PLC inhibitors block Ca^{2+} signalling. RPMCs were loaded for 20 min with Fluo3, pre-incubated with the indicated compound as described for (B-C) or for 20 min with BAPTA-AM, and activated with 48/80 (arrow). Normalised fluorescence intensity traces are shown from a single representative cell for each condition.

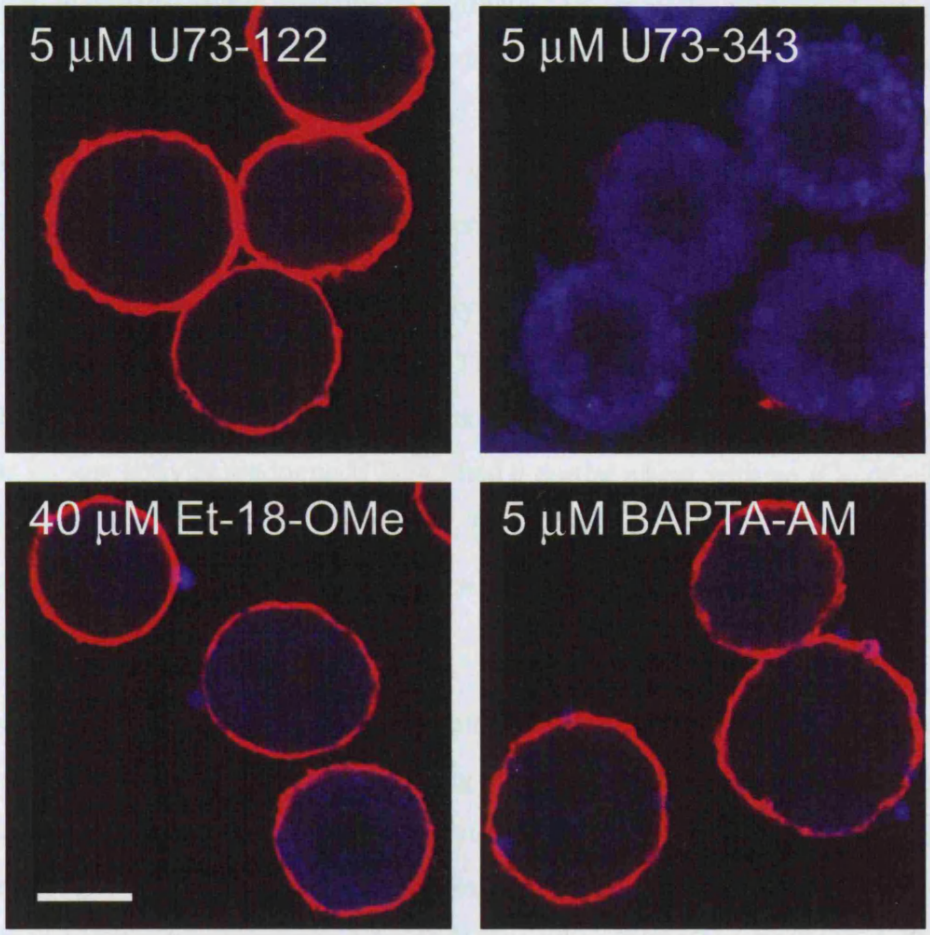

Figure 5.2: PLC causes depletion of PtdIns(4,5)P_2 from the plasma membrane. RPMC were incubated with the indicated compounds as described in figure 5.1. Subsequently, degranulation was evoked with 48/80 for 30 s. Cells were fixed and stained with 2C11 (red) and Alexa647-ConA (blue). Scale bar = 10 μM.

Could any of these pathways be discerned in RPMC? To answer this question, data on the levels of all PIn detected after metabolic labelling of cells for 19 hours with [2-^{3}H]-inositol were analysed (figure 5.1A). From these data, it is apparent that PtdIns underwent depletion such that approximately 15% of the total labelled PIn was depleted within 60 seconds of stimulation with 48/80 (figure 5.1A). This result suggests that there is a sustained increase in PLC activity and that this is likely to account for the majority of the decrease in PtdIns(4,5)P_2. The drastic decreases in PtdIns levels relative to PtdIns(4,5)P_2 may be explained by an increase in the rate of PtdIns(4,5)P_2 synthesis from PtdIns, whereas re-synthesis of PtdIns from DAG and inositol does not keep up; PtdIns(4,5)P_2 levels may therefore be ultimately maintained at the expense of PtdIns, as observed after receptor activation in hepatocytes (Augert *et al.*, 1989).

To address whether this PLC activity was required for exocytosis, a pharmacological approach was employed. The PLC inhibitor U73122 (Thompson *et al.*, 1991; Yule and Williams, 1992) blocked exocytosis with an IC_{50} of ~ 2 μM, whereas its low activity analogue U73343 had a partial effect with an IC_{50} of ~20 μM (figure 5.1C). Another PLC inhibitor, Et-18-OMe (Powis *et al.*, 1992), also blocked exocytosis with an IC_{50} of ~27 μM (figure 5.1B). PLC activity thus seemed to be mandatory for exocytosis.

As discussed above, PLCγ2 activity has already been shown to be requisite for Ca^{2+} signalling in cultured BMMCs (Wang *et al.*, 2000). Calcium has also been implicated as being important for exocytosis from RPMCs (Kagayama and Douglas, 1974; Gomperts *et al.*, 1983; Howell and Gomperts, 1987). To test whether PLC activity was required for Ca^{2+} signalling, RPMCs were loaded with the calcium indicator Fluo 3, incubated with pharmacological agents, and challenged with 48/80. As previously reported (Penner, 1988; Mori *et al.*, 2000), RPMCs displayed a rapid increase in intracellular Ca^{2+} levels, which then declined gradually (figure 5.1D). This transient was not observed if the cells were loaded with the calcium chelator BAPTA-AM; 5 μM U73122 also abolished the Ca^{2+} transient, whereas U73343 was without effect. PLC inhibitors also appeared to block the bulk depletion of plasma membrane PtdIns(4,5)P_2. As shown in figure 5.2, 5 μM U73122 or 40 μM Et-18-OMe abolished exocytosis and the depletion

of PtdIns(4,5)P_2, whereas U73343 was without effect. BAPTA also abolished PtdIns(4,5)P_2 depletion, most likely by reducing basal Ca^{2+} levels and inhibiting PLC, which is a calcium-dependent enzyme (Rhee *et al.*, 1989). These data are therefore consistent with a requirement for PLC in the generation of a calcium signal that initiates degranulation.

However, conditions are known whereby exocytosis can be triggered from mast cells in the effective absence of calcium (Penner, 1988; Churcher and Gomperts, 1990; Koffer and Churcher, 1993). Furthermore, calcium release occurs within 2 s of stimulation with 48/80 (figure 5.1), whereas breakdown of PtdIns(4,5)P_2 may take several seconds (figure 4.13). Therefore, it seemed possible that there may be other functions associated with PtdIns(4,5)P_2 breakdown beyond the InsP_3/Ca^{2+} pathway. Mast cells permeabilised with SL-O undergo robust exocytosis when stimulated with GTPγS if exogenous Ca^{2+} is provided in the μM range. Since the calcium concentration is buffered with EGTA, InsP_3-triggered calcium release from stores will not lead to an elevation of intracellular [Ca^{2+}], therefore this pathway is bypassed.

Figure 5.3 shows the results of an experiment whereby RPMCs were permeabilised with SL-O in the presence of 100 μM MgATP (so that PtdIns(4,5)P_2 levels are not depleted without stimulation) for 3 minutes, before fixation and staining with Alexa647-ConA and 2C11. When calcium was buffered to a resting level (100 nM) in the absence of GTPγS, a pattern of plasma membrane PtdIns(4,5)P_2 staining identical to that of resting, intact cells was observed (see figure 4.6). Furthermore, no exocytosis was evident. On the other hand, when calcium was buffered to 10 μM in the presence of GTPγS, the cells exhibited "all-or-none" behaviour (Hide *et al.*, 1993), with some cells showing no sign of exocytosis, whereas others had undergone substantial (and in most cases, complete) degranulation. Notably, those cells with no clear signs of exocytosis were replete with PtdIns(4,5)P_2 staining; in fact, plasma membrane PtdIns(4,5)P_2 levels may even be elevated after the provision of Ca^{2+} and GTPγS. In contrast, the degranulated cells showed an almost complete ablation of PtdIns(4,5)P_2, similar to that observed in the first few seconds of activation with 48/80 (figure

4.13). So, it was apparent from these experiments that PtdIns(4,5)P_2 hydrolysis was associated with exocytosis from permeabilised cells, as it was in intact cells.

As with intact cells, it was reasoned that loss of PtdIns(4,5)P_2 could be due to the action of phospholipases, phosphatases or PI 3-kinases. Once again, a pharmacological approach was employed. U73122 and Et-18-OMe were used to inhibit PLC, and β-glycerophosphate was used as a generic phosphatase inhibitor. LY294002 (Vlahos *et al.*, 1994) was used to inhibit PI3K; a high concentration (100 μM) was used in order to inactivate the relatively insensitive PI3K-C2α (Domin *et al.*, 1997). Neomycin was also used; although this compound was previously reported to inhibit PLC (Sagawa *et al.*, 1983), it most likely does so by preventing access of the enzyme via its high affinity interaction with PtdIns(4,5)P_2 (Schacht, 1978). This makes it equally likely that neomycin may block phosphatases and PI 3-kinases. Results obtained with neomycin must thus be interpreted only in conjunction with comparative data from other inhibitors.

When applied to permeabilised cells in conjunction with MgATP, Ca^{2+} and GTPγS, 10 μM U73122 blocked degranulation along with the reduction in plasma membrane PtdIns(4,5)P_2 (figure 5.4). Neomycin displayed a similar effect, although PtdIns(4,5)P_2 levels appeared to be reduced in several cells. However, this may be due to the ability of neomycin to prevent access of the lipid to 2C11 during staining (figure 4.7). Like control cells (figure 5.3), all of the other compounds tested displayed a mixture of degranulated, PtdIns(4,5)P_2 depleted cells and non-degranulated, PtdIns(4,5)P_2 replete cells (figure 5.4). Occasionally, cells were observed that had not degranulated but had lost their PtdIns(4,5)P_2 staining. These were always a minority (< 5%) for most of the conditions shown in figure 5.3. The presence of U73122 increased this proportion to approximately 7% at 5 μM, and as high as 12% at 10μM. This is possibly due to the action of phosphatases in the absence of PLC-mediated PtdIns(4,5)P_2 hydrolysis. However, cells with signs of compound exocytosis that retained resting plasma membrane PtdIns(4,5)P_2 levels were not observed under any experimental condition tested.

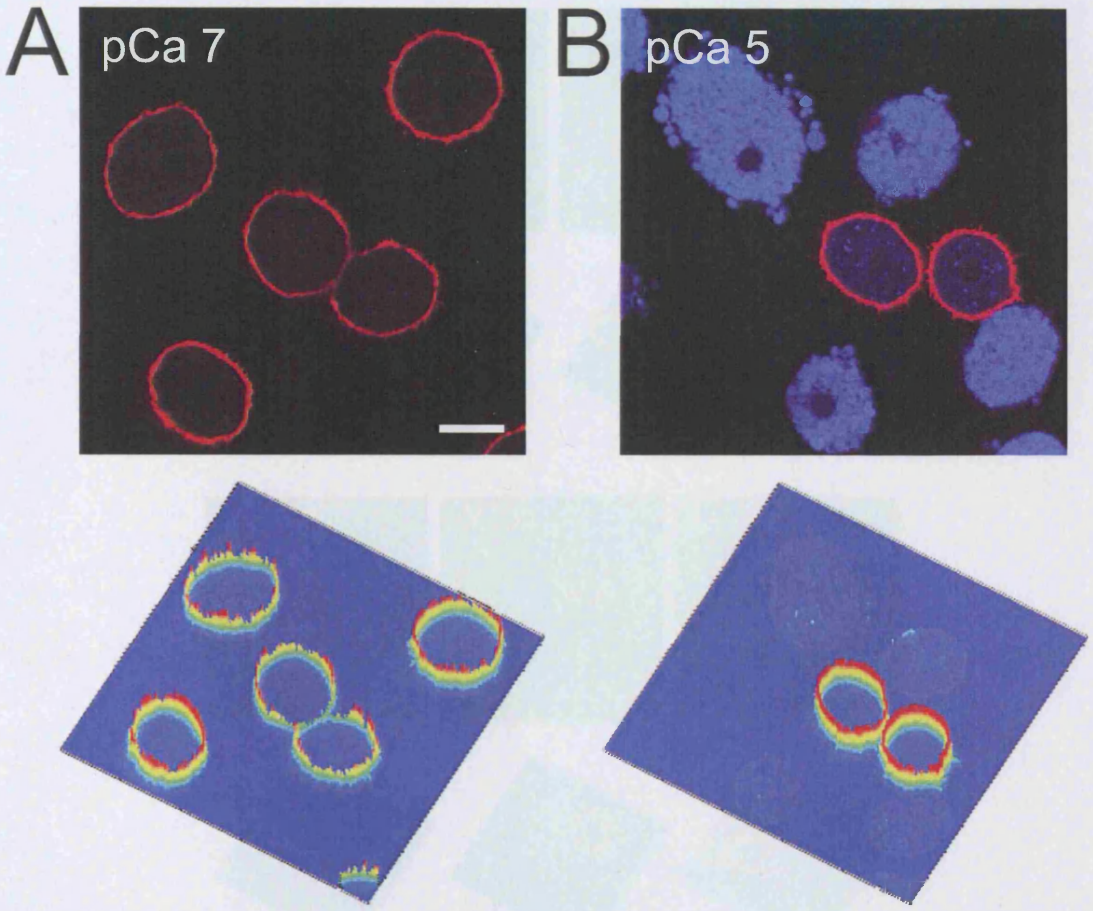

Figure 5.3: PtdIns(4,5)P_2 becomes depleted from the plasma membrane of permeabilised mast cells that undergo exocytosis. RPMC were permeabilised with SL-O in the presence of 100 µM MgATP and 3 mM Ca:EGTA at pCa 7 (A), or 100 µM MgATP, 10 µM GTPγS and 3 mM Ca:EGTA at pCa 5 (B) for 3 min at 30°C. Cells were then fixed and stained with 2C11 (red) and Alexa647-ConA (blue). Upper panels show merged images of 2C11 and ConA; bottom panels show the fluorescence intensity profile for 2C11. Scale bar = 10 µm.

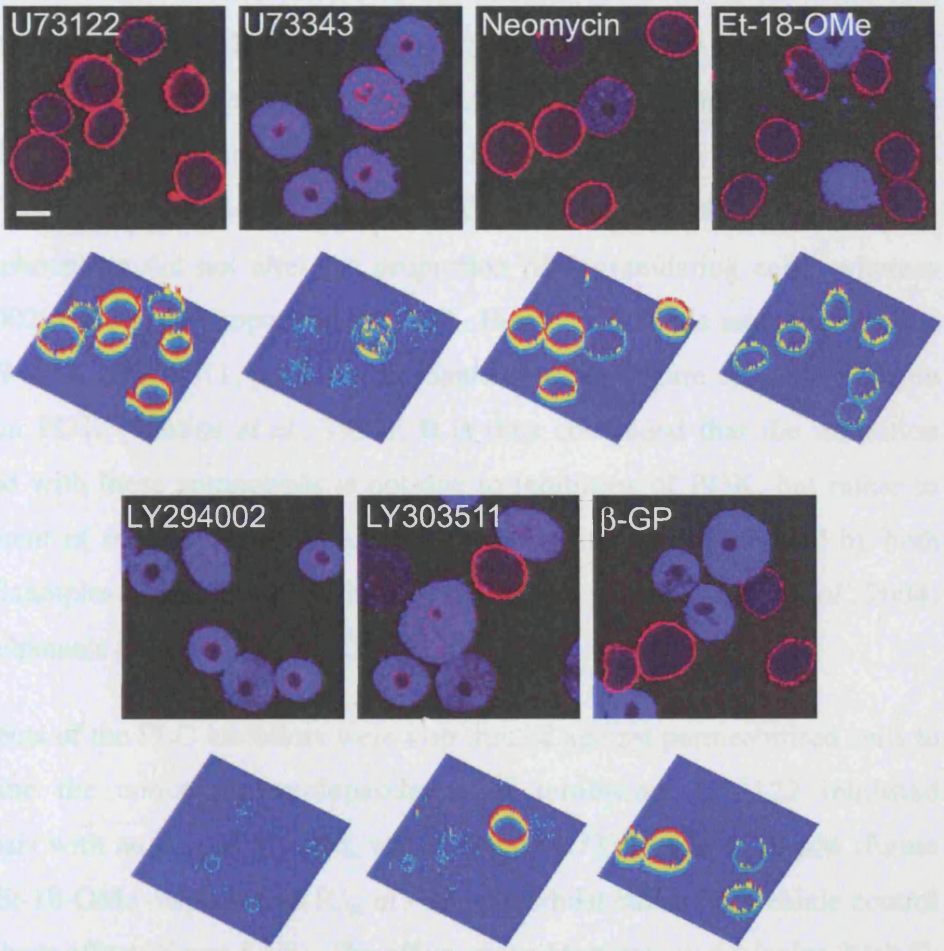

Figure 5.4: Effect of antagonists of PtdIns(4,5)P_2 metabolism on PtdIns(4,5)P_2 and exocytosis. RPMC were permeabilised with SL-O in the presence of 100 μM MgATP, 10 μM GTPγS and 3 mM Ca:EGTA at pCa 5 for 3 min at 30°C in the presence of 10 μM U73-122 or -343, 3 mM neomycin, 40 μM Et-18-OMe, 100 μM LY294002, 100 μM LY303511 or 5 mM β-GP. Cells were then fixed and stained with 2C11 (red) or Alexa647-ConA (blue). Top panels show merged images of 2C11 and concanavalin A; bottom panels show the fluorescence intensity profile for 2C11. Scale bar = 10 μm.

The number of degranulated cells observed in the control experiment (figure 5.3) varied widely. Since signs of degranulation were unambiguous (figures 5.3 and 5.4), degranulated cells were simply counted. In 10 experiments, the proportion of degranulated cells in stimulated controls ranged from 44-100%, with a mean ± S.D. of 79 ± 20%. For each of the treatments outlined in figure 5.4, the number of degranulated cells was normalised to the number observed in the untreated, stimulated controls. As shown in figure 5.5, U73122 (figure 5.5A), neomycin (figure 5.5B) and Et-18-OMe (figure 5.5C) all substantially reduced the proportion of degranulating cells, whereas U73343 (5.5A) or 0.4% ethanol (as vehicle control for Et-18-OMe, figure 5.5C) were without effect. Notably, β-glycerophosphate did not alter the proportion of degranulating cells, whereas LY294002 reduced the proportion by ~20%. However, a single atom substitution of LY294002, LY303511, produces an identical effect (figure 5.5C), yet has no effect on PI3K (Vlahos *et al.*, 1994). It is thus concluded that the inhibition observed with these compounds is not due to inhibition of PI3K, but rather to impairment of other proteins required for exocytosis that are targeted by both drugs. Examples identified to date include the NF-κB pathway (Choi *et al.*, 2004) and K_v channels (El-Kholy *et al.*, 2003).

The effects of the PLC inhibitors were also titrated against permeabilised cells to determine the concentration-dependence of inhibition. U73122 inhibited exocytosis with an IC_{50} of 1.6 μM, whilst that of U73343 was > 10 μM (figure 5.6A). Et-18-OMe displayed an IC_{50} of ~20 μM, whilst ethanol as vehicle control was without effect (figure 5.6B). The effect of the Myristoylated Alanine-Rich C-Kinase Substrate effector domain ($MARCKS_{151-175}$) was also tested, since this peptide was shown to inhibit PLC in vitro (Wang *et al.*, 2002). $MARCKS_{151-175}$ blocked exocytosis with an apparent IC_{50} of ~100 μM, although this dropped to ~10 μM when the peptide was allowed to equilibrate into cells for 5 minutes before stimulation (figure 5.6C).

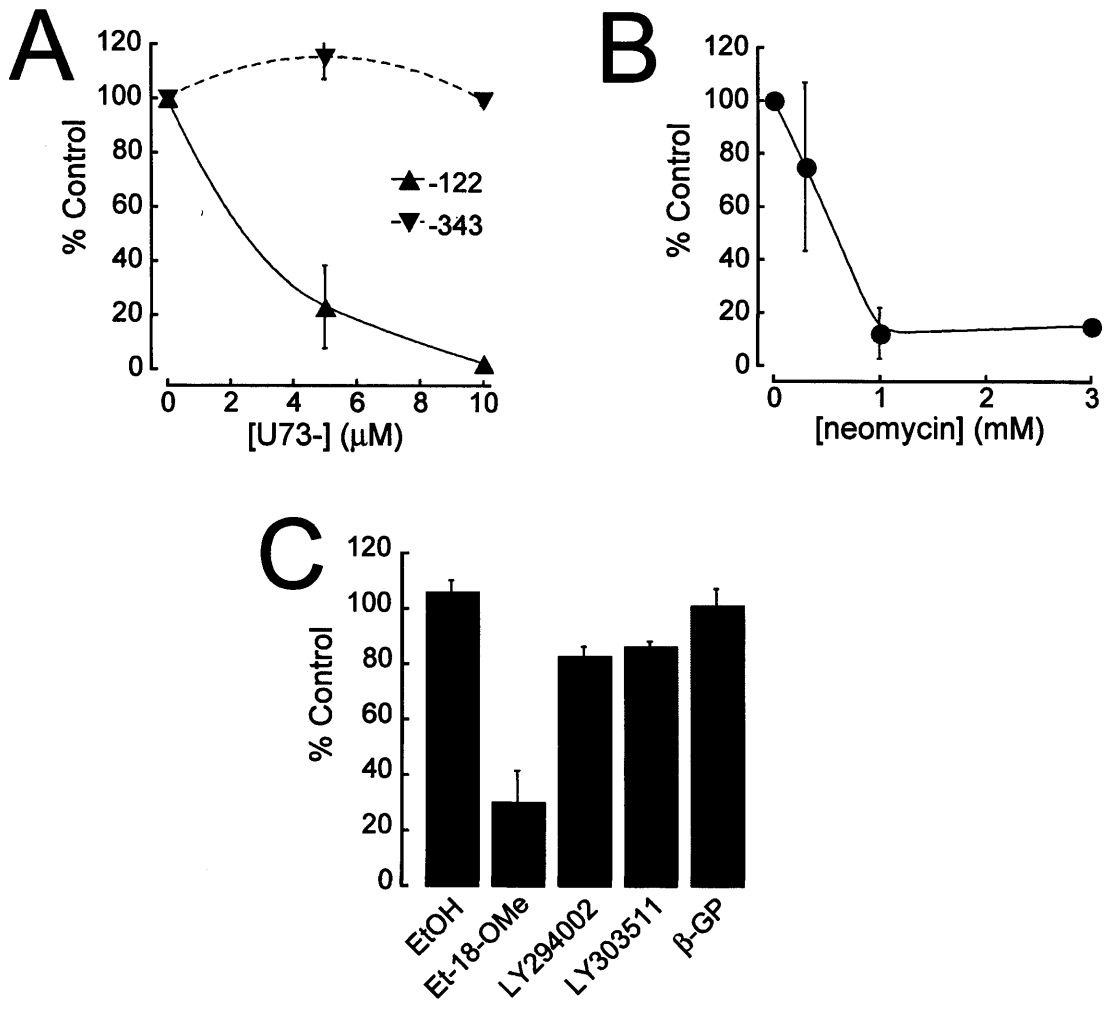

Figure 5.5: Effect of antagonists of PtdIns(4,5)P_2 metabolism on exocytosis. RPMC were permeabilised as described in (A) at pCa 5, 100 μM MgATP and 10 μM GTPγS, in the presence of U73-122 or U73-343 (B), neomycin (C), 40 μM Et-18-OMe, 100 μM LY294002 or LY303511, and 5 mM β-GP (D) as indicated. The numbers of degranulated cells were counted, and the numbers normalised to the control value for each experiment. Values represent the means of three or more independent experiments ± S.E.M., with the exceptions of U73-343 and LY303511, which are means ± range of duplicate experiments. 0.4% EtOH is the vehicle control for 40 μM Et-18-OMe.

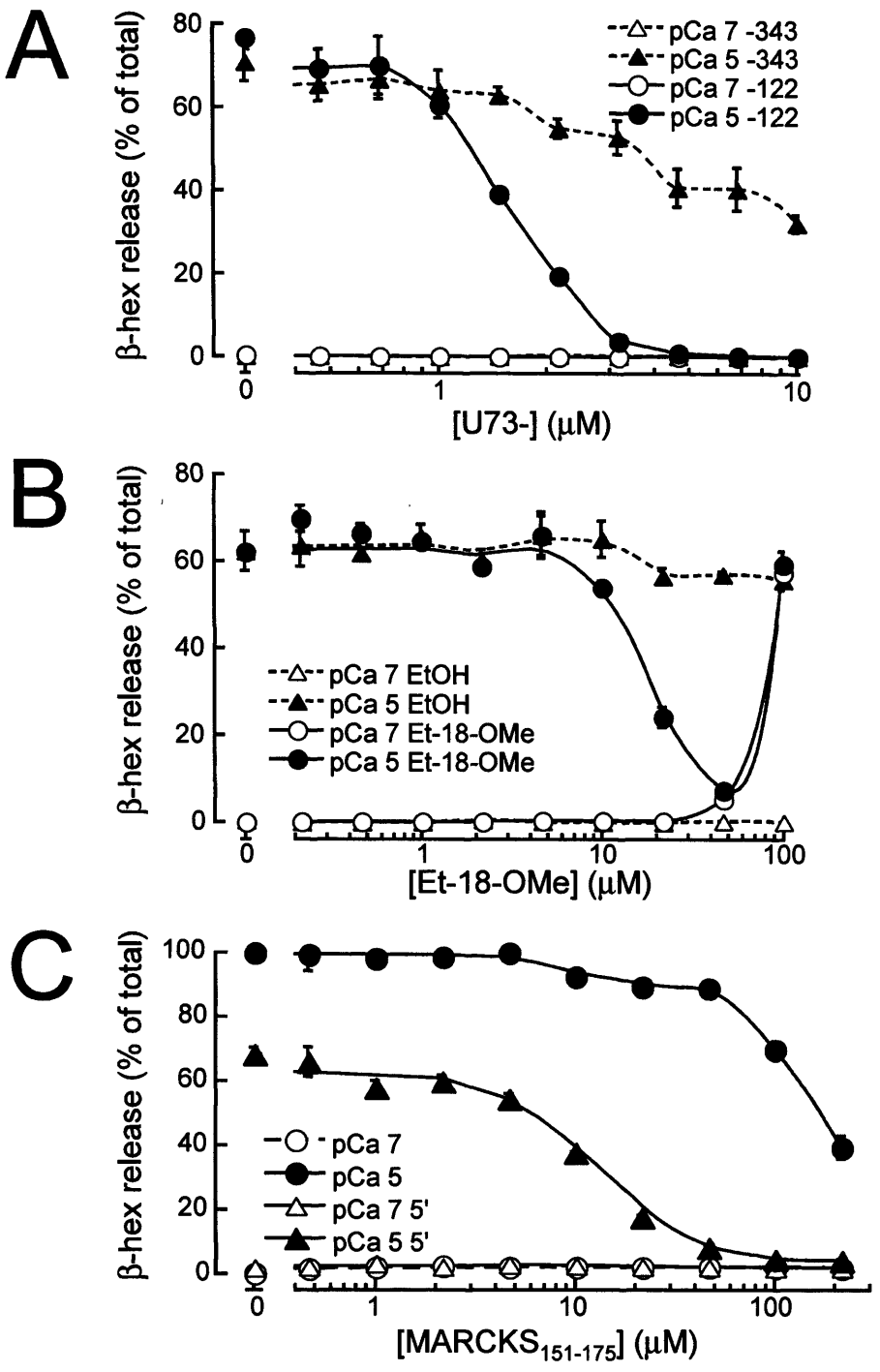

Figure 5.6: Concentration-dependent inhibition of exocytosis by PLC inhibitors. RPMC were permeabilised in the presence of 100 μM MgATP and 3 mM Ca:EGTA at pCa 7 or 100 μM MgATP, 100 μM GTPγS and 3 mM Ca:EGTA at pCa 5 in the presence of the indicated concentrations of U73122 and U73343 (A), Et-18-OMe and EtOH as vehicle control (B) or MARCKS effector domain peptide (residues 151-175) (C). For the curves designated 5' in C, RPMC were permeabilised in the presence of the indicated concentration of peptide, 100 μM MgATP and 300 μM Ca:EGTA at pCa 8. After 5 minutes, they were stimulated at pCa 7 or pCa 5 with GTPγS as above. For pCa 7, single determinations are shown; for pCa 5, data are means ± S.E.M. (n = 3, A & B) or means ± range (n = 2, C).

5.3. Discrepancies emerging from data in the literature

Taken together, these results support the conclusion that PLC catalyses the breakdown of PtdIns(4,5)P_2 from permeabilised cells stimulated with Ca^{2+} and GTPγS, as is the case in intact cells stimulated with 48/80. This breakdown appears to be required for exocytosis, independently of a requirement for Ca^{2+} signalling. Such a conclusion is in stark contrast to previous studies, which reported that activation of PLC was not required for exocytosis from RPMC (Cockcroft *et al.*, 1987; Aridor *et al.*, 1990). Such evidence has even been used to demonstrate "PLC-independent" effects of U73122 on exocytosis (Gloyna *et al.*, 2005). These inconsistencies warrant resolution with the current data presented herein.

One study demonstrated that neomycin did not affect exocytosis in permeabilised cells at concentrations that abolish PLC activity (Cockcroft *et al.*, 1987). As a read-out for PLC activity, this study measured the release of inositol phosphates (a product of PLC). These were isolated after separation from inositol lipids through addition of specific quantities of chloroform, methanol and water, which forms a dense, organic layer (containing the lipids) and an upper, aqueous layer containing inositol phosphates (Bligh and Dyer, 1959). Subsequently, phosphates were purified on Dowex anion exchange columns. One concern regarding this experimental strategy is that neomycin could bind to inositol phosphates and interfere with their extraction from the aqueous layer. To test this possibility, 1 μM Ins(1,4,5)P_3 was spiked with 3 nCi of [2-^{3}H]-Ins(1,4,5)P_3 in a buffer identical to that used by Cockcroft et al, and extracted as described in the same study. As shown in figure 5.7, addition of 300 μM neomycin (the maximum concentration used by Cockcroft et al.) caused the Ins(1,4,5)P_3 to be retained at the interface between the aqueous and organic layers, unless the extraction mixture was acidified with ~0.1M HCl. Since Cockcroft et al specifically state that water was used for extraction, it seems possible that this study failed to report bona fide PLC activity; it may thus be possible that mM concentrations of neomycin are required to inhibit PLC in permeabilised cells, as is the case for exocytosis (Pinxteren *et al.*, 2001).

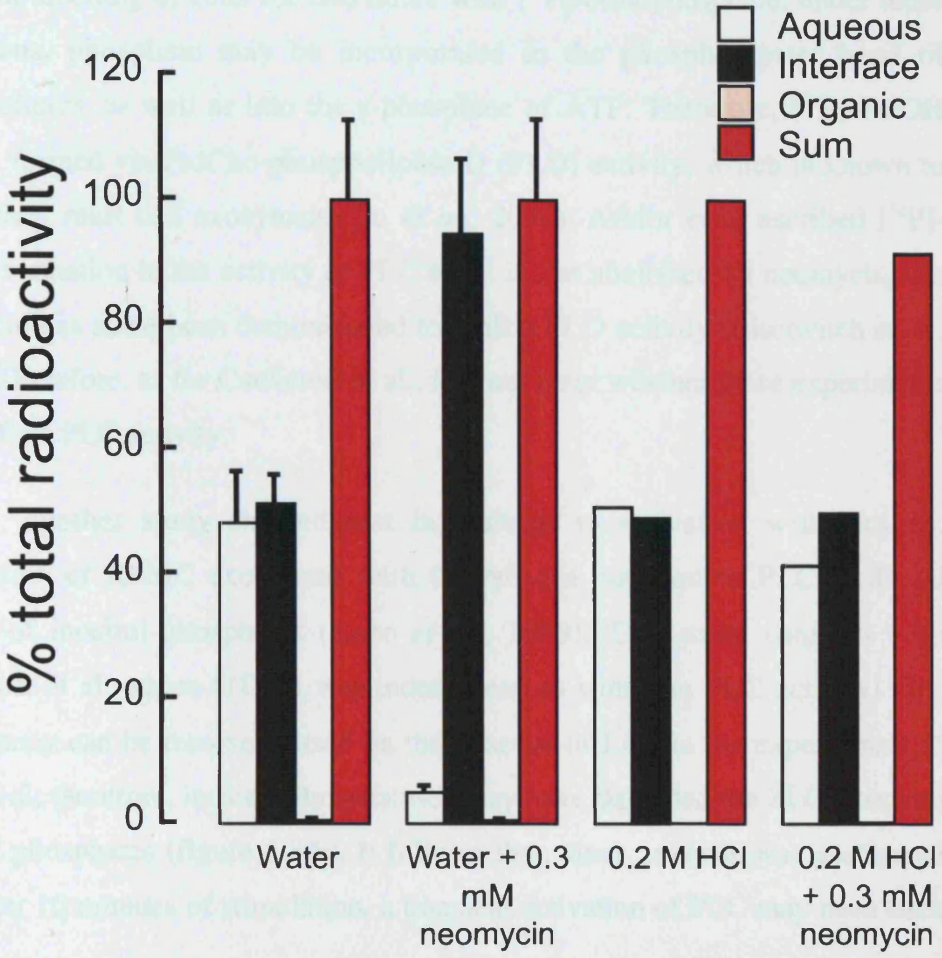

Figure 5.7: Effect of neomycin on the extraction of Ins(1,4,5)P_3. Solutions of 1 μM Ins(1,4,5)P_3 spiked with 3 nCi [2-^{3}H]-Ins(1,4,5)P_3 were mixed with or without 0.3 mM neomycin in buffer containing 1 mg/ml BSA (see Materials and Methods) in a volume of 0.4 ml. A two-phase system was then generated with a final water (or 0.2 M HCl):MeOH:$CHCl_3$ ratio of 0.9:1:1. Aliquots of the aqueous and organic phases were sampled, and the remainder saved for counting ("interface"). All three fractions were dried under a stream of nitrogen gas to remove $CHCl_3$ and MeOH, then subjected to liquid scintillation counting. Data are means ± range of duplicates.

A second study claimed that exocytosis could occur from RPMC without activation of PLC under certain conditions (Aridor *et al.*, 1990). This study utilised formation of PtdOH as a readout of PLC activity, since PtdOH is formed by the activity of DAG-kinase on the PLC-generated DAG. However, this study relied on labelling of cells for two hours with [^{32}P]-orthophosphate; under these conditions, phosphate may be incorporated in the phosphodiester bond of phospholipids, as well as into the γ-phosphate of ATP. Therefore, [^{32}P]-PtdOH may be formed via PtdCho-phospholipase D (PLD) activity, which is known to accompany mast cell exocytosis (Lu *et al.*, 2004). Aridor et al ascribed [^{32}P]-PtdOH formation to the activity of PLC since it was abolished by neomycin, but neomycin has since been demonstrated to inhibit PLD activity (Liscovitch *et al.*, 1991). Therefore, as for Cockcroft et al., it is not clear whether these experiments truly reflect PLC activity.

Finally, another study showed that in contrast to activation with antigen, stimulation of RPMC exocytosis with GTPγS did not lead to PLC-mediated release of inositol phosphates (Saito *et al.*, 1989). This study conflicts with Cockcroft et al, where GTPγS was indeed seen to stimulate PLC activity. This discrepancy can be resolved based on the absence of LiCl in the experiments of Saito et al; therefore, inositol phosphatases may have degraded the PLC-liberated inositol phosphates (figure 5.8A). It follows that, since analysis was performed only after 10 minutes of stimulation, a transient activation of PLC may have been missed.

The experiments reported in this and the preceding chapter report PtdIns(4,5)P_2 levels directly. Although this may not be a direct indicator of PLC activity, only antagonists of PLC are seen to prevent the depletion of PtdIns(4,5)P_2. That these antagonists also abolish exocytosis is the evidence presented that PLC is required for exocytosis. This does not discount the possibility that there may be certain conditions under which the requirement for hydrolysis of plasma membrane PtdIns(4,5)P_2 may be bypassed. Such conditions have not, however, been discovered during the course of this thesis.

5.4. Requirement for PtdIns(4,5)P_2 depletion during exocytosis

Although Ins(1,4,5)P_3 appears to be required for calcium signalling, the results with PLC inhibitors in permeabilised cells point to other functions for PtdIns(4,5)P_2 hydrolysis. Inositol phosphates mediate a plethora of cellular functions aside from regulating intracellular calcium levels; notably, Ins(1,4,5)P_3 can be phosphorylated by Ins(1,4,5)P_3 3-kinase, and the resulting Ins(1,3,4,5)P_4 enters a complex metabolic pathway of functionally distinct inositol polyphosphates (Irvine and Schell, 2001). However, given that exocytosis from RPMC can occur in the effective absence of ATP, roles for higher inositol phosphates seem unlikely to explain the requirements for PtdIns(4,5)P_2 hydrolysis. On the other hand, Ins(1,4,5)P_3 or one of its catabolites may perform a critical function for exocytosis. Figure 5.8A shows the catabolic pathway for Ins(1,4,5)P_3, along with the two enzymatic steps inhibited by mM concentrations of Li^+. With this in mind, it can be predicted that if Ins4*P* or Ins itself were required, then mM Li^+ should prevent their synthesis and also exocytosis; figure 5.8B shows that this is not the case. This leaves possible functions for Ins(1,4)P_2 or Ins(1,4,5)P_3 itself. Introduction of either molecule into permeabilised mast cells at ~10^{-4} M did not alter the level of exocytosis, nor did they restore exocytosis from cells in which PLC activity was abolished with U73122 (figure 5.8A & B). Together, these data argue against a direct role for inositol phosphates during exocytosis, beyond a role in Ca^{2+} signalling.

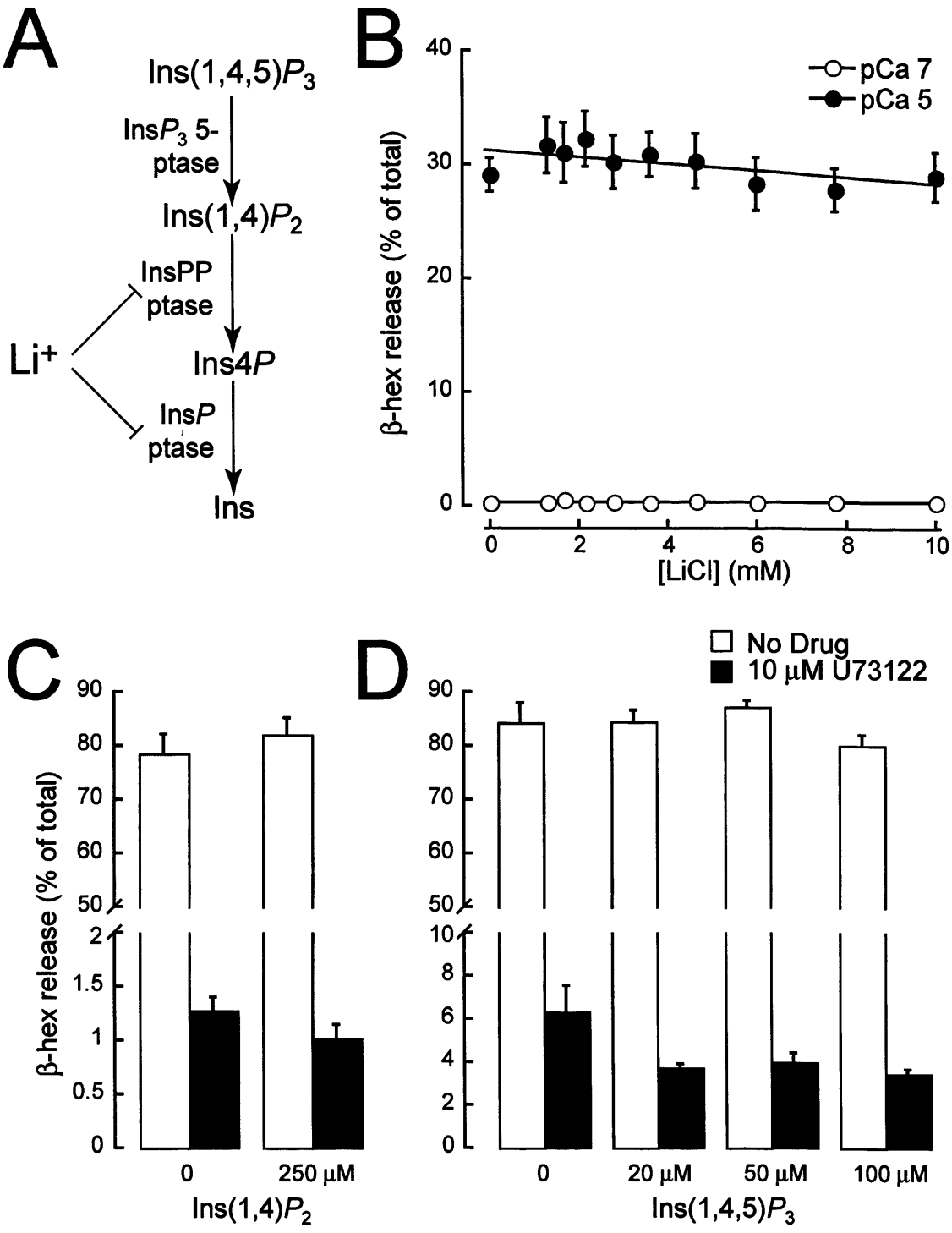

Figure 5.8: Generation of inositol phosphates is not sufficient for exocytosis in permeabilised cells. (A) Pathway of Ins(1,4,5)P_3 catabolism; "ptase" refers to phosphatase. (B) RPMCs were permeabilised in the presence of 100 μM MgATP, 0.3 mM Ca:EGTA at pCa 8 and the indicated concentrations of LiCl. After 10 minutes, cells were activated in the continuing presence of 100 μM MgATP with either 3 mM Ca:EGTA at pCa 7, or 3 mM Ca:EGTA at pCa 5 and 100 μM GTPγS. Data are means ± S.E.M. (n = 4). (C and D) RPMC were permeabilised in the presence of 10 μM U73122 and inositol phosphates as indicated, along with 100 μM MgATP, 100 μM GTPγS and 3 mM Ca:EGTA at pCa 5. Data are means ± S.E.M., n = 3 (C) or 4 (D).

The other product of PtdIns(4,5)P_2 hydrolysis is DAG. Several studies have noted the ability of the DAG analogues (the phorbol esters) to prolong secretory competence in permeabilised RPMC (Howell *et al.*, 1989; Pinxteren *et al.*, 2001). Indeed, the phorbol esters phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate (PDBu), as well as the DAG analogue 1-oleoyl 2-acetyl-sn-glycerol (OAG) all reduced the extent of rundown after 10 minutes' permeabilisation (figure 5.9A). Despite this positive effect, the most potent analogue (PMA) was not able to restore exocytosis from cells treated with U73122 (figure 5.9B). From this, it is concluded that DAG itself is not sufficient for exocytosis in the absence of PLC activity. One possible explanation for this could be that a metabolite of DAG is required for exocytosis. In order to introduce authentic DAG into permeabilised RPMC, PtdIns-specific PLC purified from Bacillus cereus (PtdIns-PLC) was employed. This enzyme was far less sensitive to PLC inhibitors in vitro, and retained activity at concentrations that abolished the endogenous PLC (figure 5.10A). Figures 5.10 shows that, like DAG analogues, PtdIns-PLC retarded the extent of rundown at 10 minutes, but was not sufficient to restore exocytosis when endogenous PLC was blocked with Et-18-OMe or U73122. PtdIns-PLC was previously reported to inhibit chromaffin cell exocytosis by depleting endogenous PtdIns4*P* and PtdIns(4,5)P_2 (Eberhard *et al.*, 1990); figure 5.10D shows that this was not the case for PtdIns(4,5)P_2 in RPMC. Together, these results indicate that DAG synthesis cannot be the sole functional requirement for PLC-mediated PtdIns(4,5)P_2 hydrolysis.

PtdIns(4,5)P_2 hydrolysis is required for actin cytoskeletal rearrangements necessary for phagocytosis (Scott *et al.*, 2005), raising the possibility that removal of PtdIns(4,5)P_2 per se could be the requirement for exocytosis. Removal of plasma membrane PtdIns(4,5)P_2 is also required for invasion of cells by pathogenic Salmonella strains; rather than employing PLC activation, this parasite relies instead on an injected inositol phosphatase, SigD (Terebiznik *et al.*, 2002). Recombinant SigD introduced into permeabilised RPMCs abolished plasma membrane PtdIns(4,5)P_2 (figure 5.11A), but inhibited exocytosis (figure 5.11B). This depended on the catalytic activity of the protein, since the catalytically inactive C462S point mutant (Marcus *et al.*, 2001) affected neither PtdIns(4,5)P_2

(figure 5.11A) nor exocytosis (figure 5.11B). Thus elimination of plasma membrane PtdIns(4,5)P_2 prior to activation was not favourable for exocytosis.

What if depletion of PtdIns(4,5)P_2 by SigD occurred concurrent with (as opposed to before; figure 5.11) stimulation? Figure 5.12 shows the effect of SigD in cells permeabilised in the presence of stimulus for 10 minutes (sufficient time for PtdIns(4,5)P_2 depletion by the enzyme). Under these conditions, SigD did not block exocytosis but was sufficient to restore exocytosis from cells where PLC was blocked with U73122 (figure 5.12A). However, this did not depend on the catalytic activity as the C462S mutant displayed an almost identical effect. Recombinant GST-2xFYVE (which should not impinge acutely on PtdIns(4,5)P_2 metabolism) also restored exocytosis. The reason for this is not clear, but when compared with the inactive analogue U73343, it can be seen that the functional group of U73122 is a maleimide. Therefore, the restoration by these exogenous proteins may simply be due to reaction of U73122 with cysteine residues, preventing blockade of PLC. In contrast, when PLC was inhibited with 40 μM Et-18-OMe, no effect of PtdIns(4,5)P_2 depletion with SigD on exocytosis was observed. This indicates that dephosphorylation of PtdIns(4,5)P_2 does not compensate for hydrolysis of PtdIns(4,5)P_2 at the phosphodiester bond.

The failure of DAG, or PtdIns(4,5)P_2 depletion by dephosphorylation, to restore exocytosis indicates that neither are sufficient by themselves. However, it is possible that both hydrolysis of PtdIns(4,5)P_2 and concomitant generation of DAG could be required. To mimick this under conditions whereby endogenous PLC was inhibited (with Et-18-OMe), permeabilised RPMC were incubated with SigD and PtdIns-PLC together (figure 5.12C). The tendency of PtdIns-PLC to retard, and of SigD to accelerate rundown could be seen under these conditions: in fact, accelerated rundown with SigD and retarded rundown with PtdIns-PLC were additive, such that the increase in secretion due to PtdIns-PLC was still apparent after inhibition of exocytosis with SigD. This indicates that removal of PtdIns(4,5)P_2 and generation of DAG affect separate, parallel pathways required for exocytosis to occur.

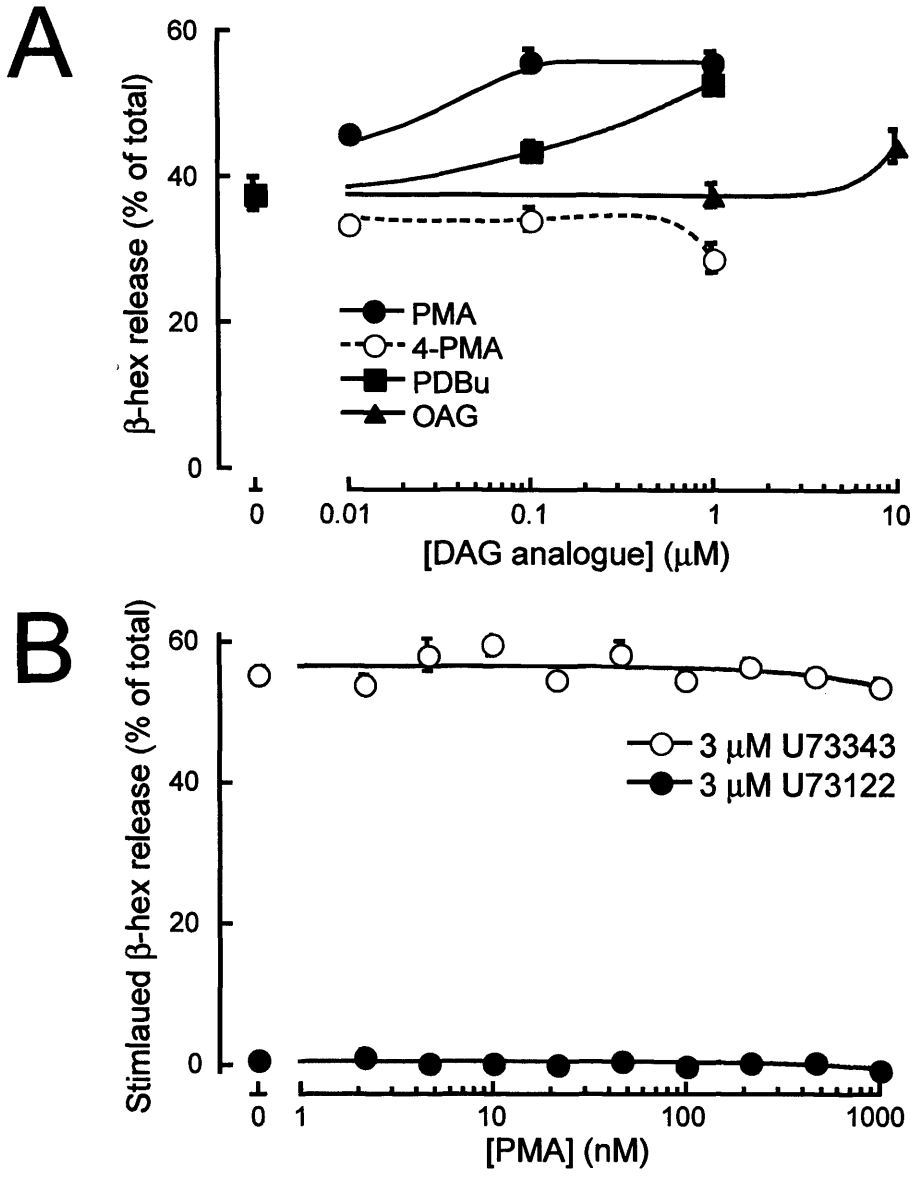

Figure 5.9: Phorbol esters are not sufficient to restore exocytosis in permeabilised cells. (A) RPMC were permeabilised in the presence of 100 μM MgATP, 300 μM Ca:EGTA at pCa 8 and the indicated concentrations of phorbol ester or OAG. After 10 minutes, cells were stimulated with 100 μM GTPγS and 3 mM Ca:EGTA at pCa 5 in the continued presence of 100 μM MgATP. (B) RPMC were permeabilised in the presence of 3 μM U73122/343, the indicated concentration of PMA, 100 μM MgATP, 100 μM GTPγS and 3 mM Ca:EGTA at pCa 5. Data are means ± S.E.M. (n = 3).

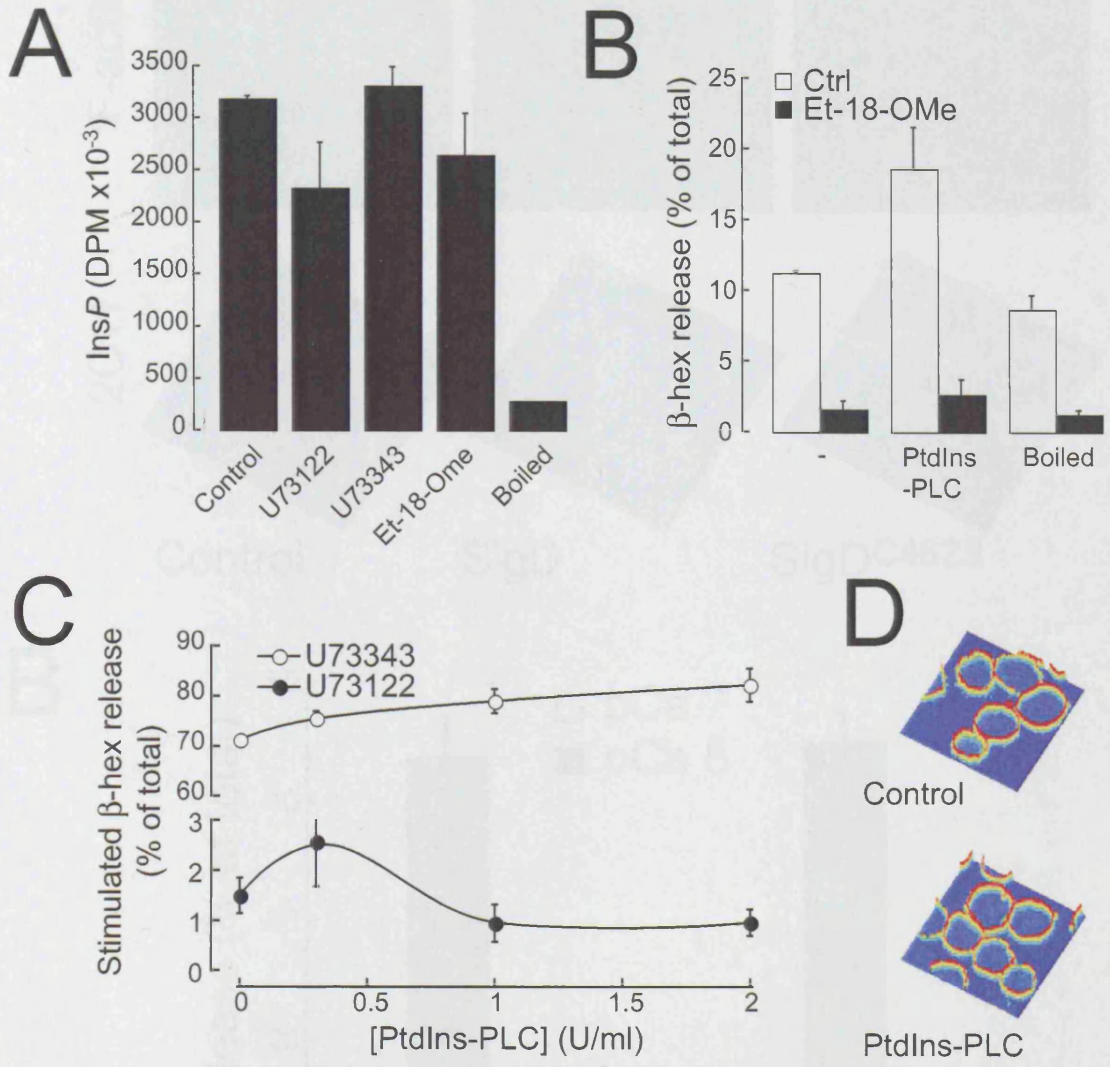

Figure 5.10: DAG generation is not sufficient to restore exocytosis in permeabilised cells. (A) *In vitro* activity of 0.3 U/ml PtdIns-PLC against PtdIns micelles in the presence of 5 μM U73122/343 or 40 μM Et-18-OMe. "Boiled" refers to enzyme heat inactivated for 15 min at 110°C. Data are means of duplicates ± range. (B) RPMCs were permeabilised in the presence of 300 μM Ca:EGTA at pCa 8, 100 μM MgATP and 0.3 U/ml PtdIns-PLC as indicated, in the presence or absence of 20 μM Et-18-OMe. After 10 minutes, they were stimulated with 100 μM GTPγS and 3 mM Ca:EGTA at pCa5 in the continuing presence of 100 μM MgATP. Data are means of triplicates, ± S.E.M; unstimulated cells released > 2% total β-hex activity. (C) Mast cells were permeabilised in the presence of 3 μM U73122/343, 100 μM GTPγS and 3 mM Ca:EGTA at pCa 5 (or pCa 7 without GTPγS, subtracted to give stimulated release) with the indicated concentration of PtdIns-PLC. MgATP was not present in this experiment. Data are means ± S.E.M. (n = 3). (D) PtdIns-PLC does not decrease PtdIns(4,5)P_2 levels. Adherent mast cells were permeabilised in the presence of 100 μM MgATP, 300 μM Ca:EGTA at pCa 8 in the presence or absence of 0.3 U/ml PtdIns-PLC. After 10 minutes at 30°C, cells were fixed and stained with 2C11.

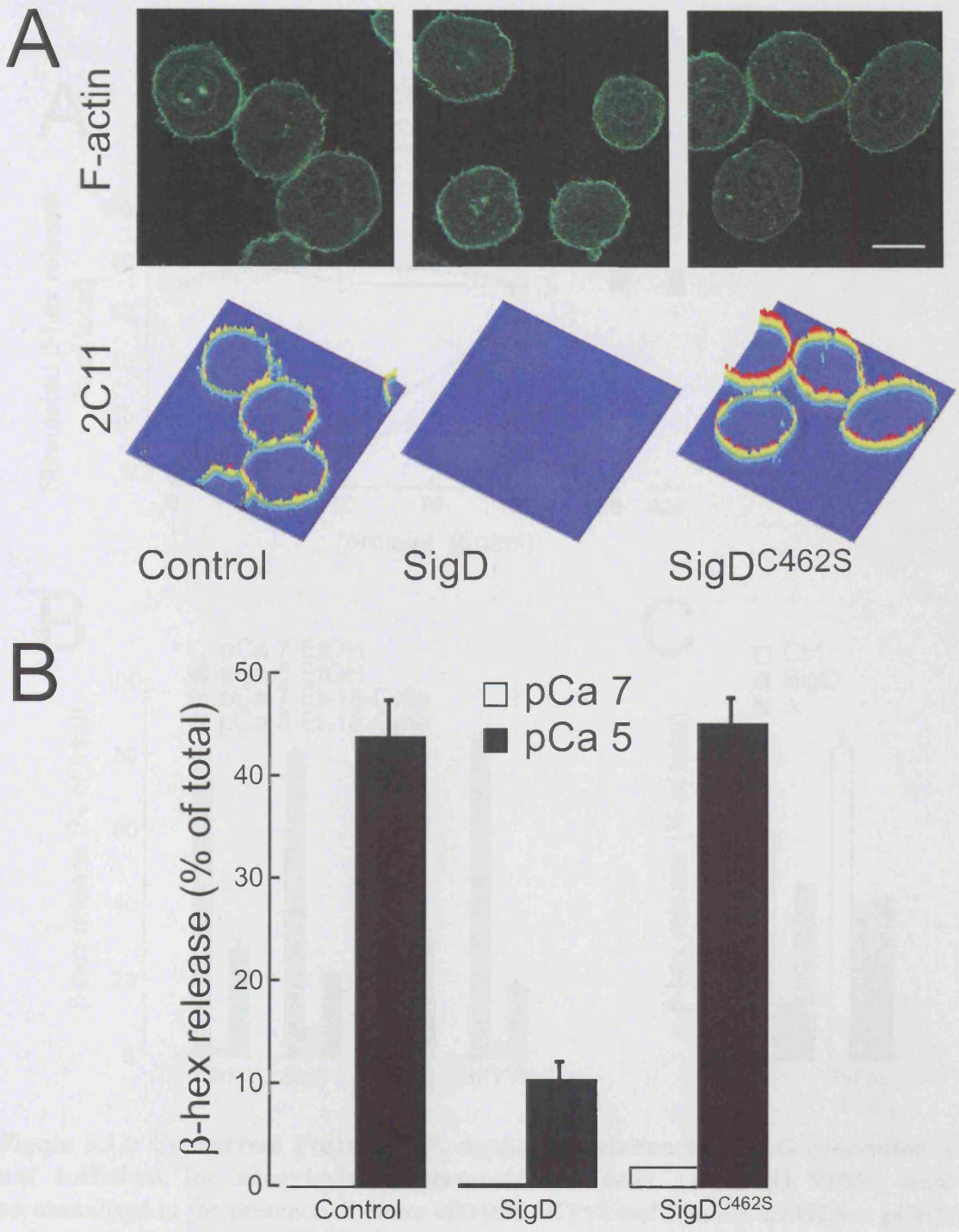

Figure 5.11: Removal of PtdIns(4,5)P_2 is not sufficient for exocytosis from permeabilised cells. (A) RPMC were permeabilised in the presence of 100 µM MgATP and 300 µM Ca:EGTA at pCa 8 in the absence (control) or presence of 150 µg/ml wild-type or catalytically inactive C462S mutant SigD. After 10 min at 30°C, cells were fixed and stained with 2C11 and Alexa488-phalloidin. Top panels show phalloidin staining, bottom panels intensity profiles for 2C11. (B) RPMC in suspension were otherwise treated as in A. After 10 min at 30 °C, they were either maintained at rest with 3 mM Ca:EGTA at pCa 7, or stimulated with 100 µM GTPγS and 3 mM Ca:EGTA at pCa 5, as indicated. Data are single determinations (pCa 7) or means ± S.E.M. of triplicates (pCa 5).

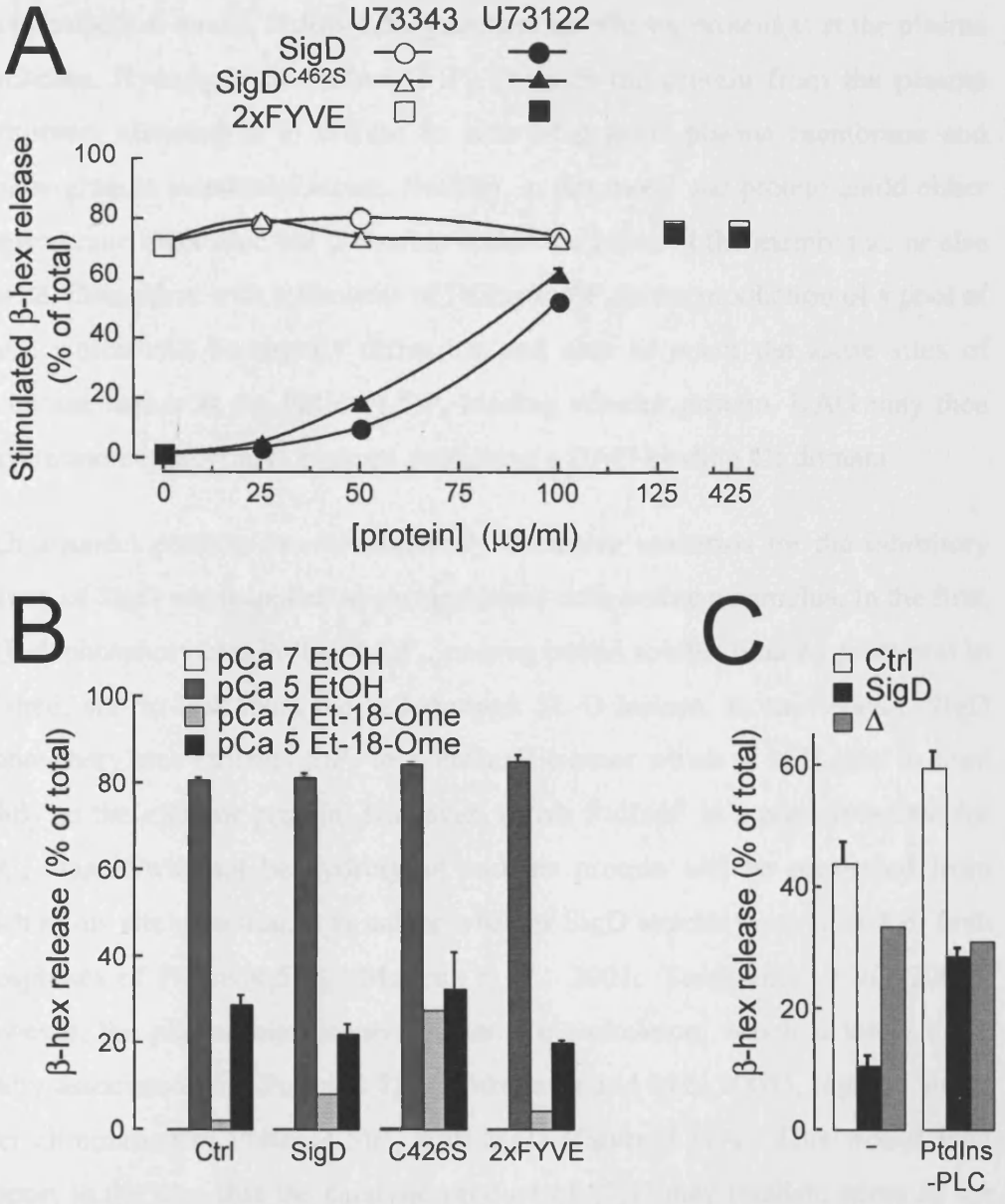

Figure 5.12: Concurrent PtdIns(4,5)P_2 dephosphorylation and DAG generation is not sufficient for exocytosis in permeabilised cells. (A & B) RPMC were permeabilised in the presence of either 100 μM GTPγS and 3 mM Ca:EGTA at pCa 5, or 3 mM Ca:EGTA at pCa 7. The following reagents were included: (A) 3 μM U73122 or U73343 and the indicated concentration of protein. (B) 40 μM Et-18-OMe or 0.4% EtOH as vehicle control, 100 μg/ml SigD wild-type or C462S mutant, and 430 μg/ml GST-2xFYVE. (C) RPMC were permeabilised in the presence of 100 μM MgATP and 300 μM Ca:EGTA at pCa 8 in the presence of 20 μM Et-18-OMe or 0.3 U/ml PtdIns-PLC as indicated. After 10 minutes, exocytosis was initiated with 100 μM GTPγS and 3 mM Ca:EGTA at pCa 5 in the continuing presence of 100 μM MgATP (control cells at pCa 7 without GTPγS secreted < 2% total β-hex). "Δ" refers to the difference in observed secretion in the presence or absence (Ctrl) of SigD, calculated by subtracting the former from the latter. Data are means of triplicate determinations ± S.E.M. for stimulated cells; unstimulated cells (pCa 7) were single determinations.

Such an observation would still be reconcilable with a model whereby both elimination of PtdIns(4,5)P_2 and DAG generation are required (figure 5.13). In this hypothetical model, PtdIns(4,5)P_2 anchors an effector protein(s) at the plasma membrane. Hydrolysis of PtdIns(4,5)P_2 liberates the protein from the plasma membrane, allowing it to diffuse to sites of granule-plasma membrane and granule-granule membrane fusion. Notably, in this model the protein could either be membrane associated but diffusible within the plane of the membrane, or else soluble. Coincident with hydrolysis of PtdIns(4,5)P_2 is the production of a pool of DAG, which will be rapidly diffusible and able to reach the same sites of membrane fusion as the PtdIns(4,5)P_2-binding effector protein. DAG may then activate another protein(s), perhaps containing a DAG-binding C1 domain.

Such a model predicts two non-mutually exclusive scenarios for the inhibitory activity of SigD when applied to permeabilised cells before a stimulus. In the first, SigD dephosphorylates PtdIns(4,5)P_2, causing bound soluble binding protein(s) to be shed, and to leak from the cell through SL-O lesions. In the second, SigD dephosphorylates PtdIns(4,5)P_2 to a PtdIns$P$ isomer which is still able to bind tightly to the effector protein. However, if this PtdIns$P$ is a poor substrate for PLC, then it will not be hydrolysed and the protein will be prevented from reaching its site of action. It is unlear whether SigD attacks the D-4, D-5 or both phosphates of PtdIns(4,5)P_2 (Marcus *et al.*, 2001; Terebiznik *et al.*, 2002). However, the plasma membrane-associated cytoskeleton, which is know to be tightly associated with PtdIns(4,5)P_2 (Takenawa and Itoh, 2001), remains intact after elimination of PtdIns(4,5)P_2 with SigD (figure 5.11A). This would lend support to the idea that the catalytic product of SigD may mediate some of the functions associated with PtdIns(4,5)P_2.

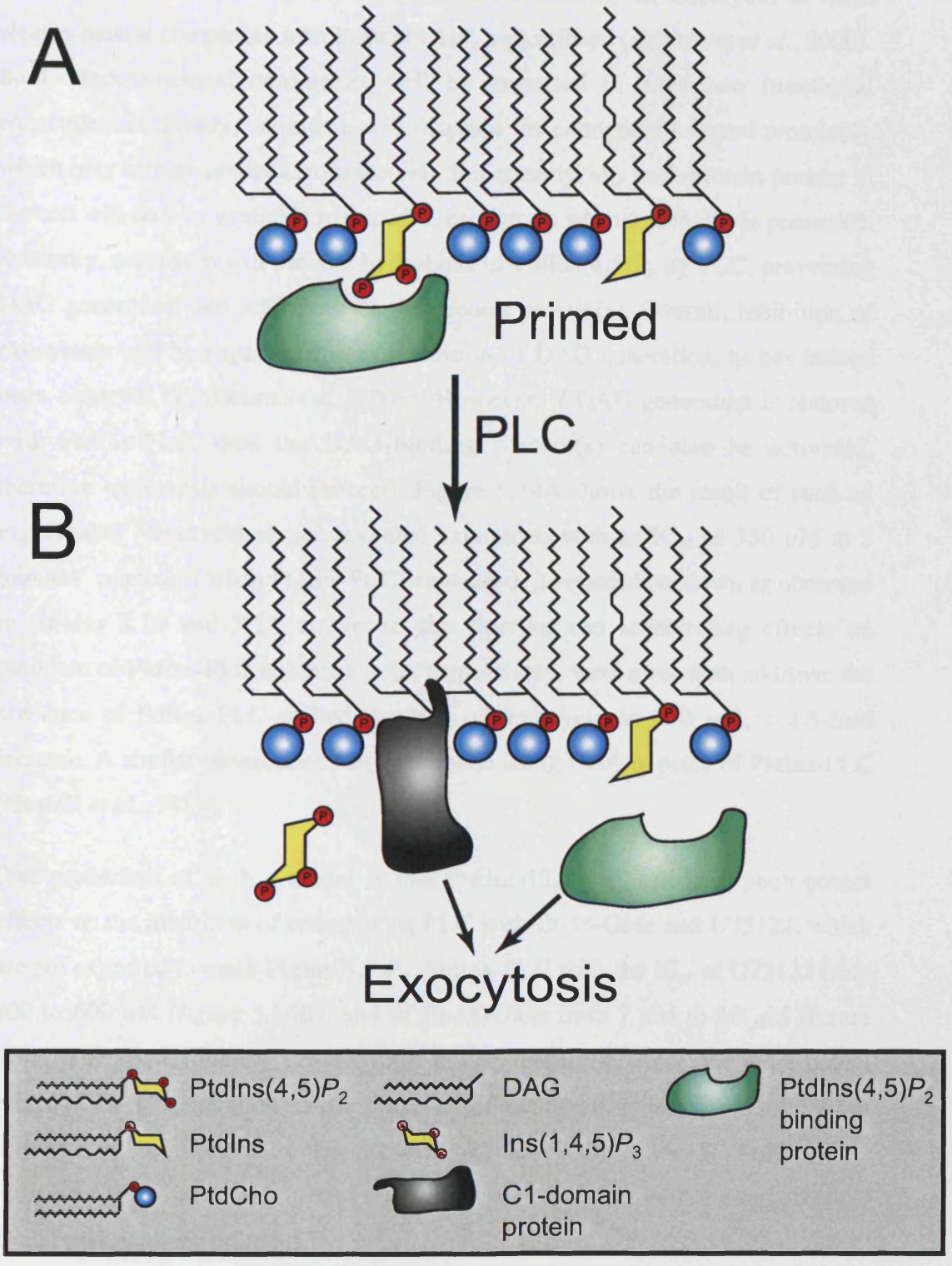

Figure 5.13: A model for PLC-mediated activation of exocytosis. (A) PtdIns(4,5)P_2 binds an endogenous protein(s) at the inner leaflet of the plasma membrane, ready to expedite membrane fusion after stimulation. (B) On stimulation, PLC becomes activated and cleaves PtdIns(4,5)P_2, releasing the endogenous activator protein so that it may diffuse to sites of membrane fusion both at the plasma membrane and between granule membranes. Furthermore, the diaclyglycerol (DAG) generated may diffuse in the plane of the membrane to similar sites of action, and there activate endogenous binding proteins containing, for example, a DAG-binding C1 domain.

To circumvent some of these uncertainties, the ability of neomycin to form electro-neutral complexes with PtdIns(4,5)P_2 was utilised (Arbuzova *et al.*, 2000). Such electro-neutral complexes will be expected to have two functional consequences. Firstly, neomycin will displace the endogenous bound protein(s), which may or may not leak from the cell. Importantly, any such protein present in the cell will now be available to expedite exocytosis when a stimulus is presented. Secondly, neomycin will prevent hydrolysis of PtdIns(4,5)P_2 by PLC, preventing DAG generation and activation of the second protein(s). Overall, inhibition of exocytosis will be expected due to inhibition of DAG generation, as has indeed been observed (Pinxteren *et al.*, 2001). However, if DAG generation is restored with PtdIns-PLC, then the DAG-binding protein(s) can also be activated, therefore exocytosis should proceed. Figure 5.14A shows the result of such an experiment. Neomycin alone prevented exocytosis with an IC_{50} of 350 μM at 5 minutes' rundown; when PtdIns-PLC was added, it retarded rundown as observed in figures 5.10 and 5.12. However, the slowing and accelerating effects on rundown of PtdIns-PLC and neomycin, respectively, were more than additive: the presence of PtdIns-PLC shifted the IC_{50} of neomycin to 900 μM, a 2.5-fold increase. A similar observation has been made using PMA in place of PtdIns-PLC (Howell *et al.*, 1989).

One prediction of such a model is that PtdIns-PLC will not have such potent effects on the inhibition of endogenous PLC with Et-18-OMe and U73122, which are not expected to mask PtdIns(4,5)P_2. PtdIns-PLC shifs the IC_{50} of U73122 from 400 to 600 nM (figure 5.14B), and of Et-18-OMe from 7 μM to 10 μM (figure 5.14C), an approximately 1.5-fold shift in each case. Therefore, the more potent effect of DAG generation in the presence of neomycin (when PtdIns(4,5)P_2 is sequestered) compared to the presence of Et-18-Ome and U73122 (when PtdIns(4,5)P_2 will be free to bind its effector) is consistent with the hypothetical model presented in figure 5.13.

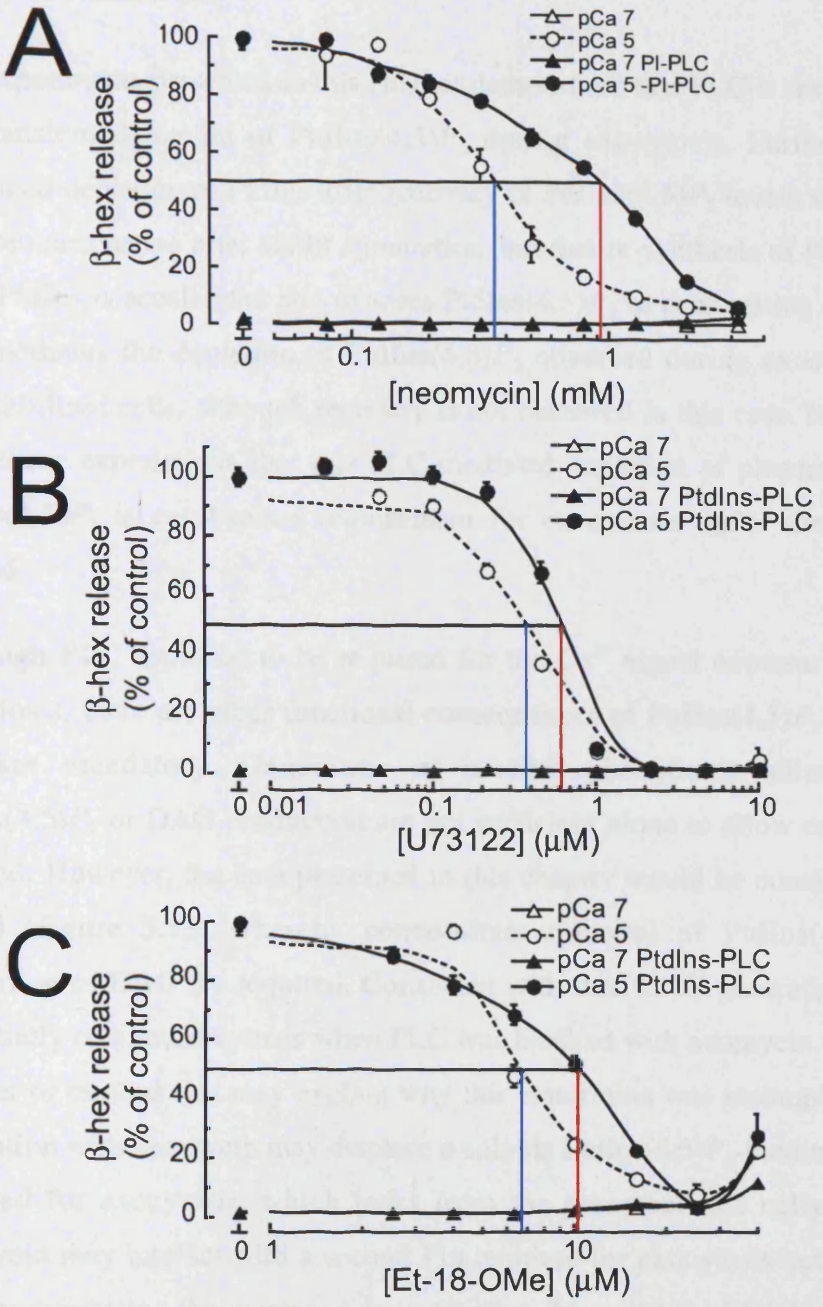

Figure 5.14: Effect of DAG generation on the IC_{50} of PLC inhibitors. RPMC were permeabilised in the presence of 100 μM MgATP, 300 μM Ca:EGTA at pCa 8 and the indicated concentrations of neomycin (A), U73122 (B) or Et-18-OMe (C), in the presence or absence of 0.3 U/ml PtdIns-PLC. After 5 min, cells were stimulated in the continuing presence of 100 μM MgATP with 100 μM GTPγS and 3 mM Ca:EGTA at pCa5, or with 3 mM Ca:EGTA at pCa 7 without GTPγS as control. The vertical lines indicate the IC_{50} of each compound in the presence (red) or absence (blue) of PtdIns-PLC. Data are means ± S.E.M. of triplicates (pCa 5) or single determinations (pCa 7). Data are normalised to the control values (i.e. those in the absence of PLC inhibitor compounds, 0), and were 38% (58% with PtdIns-PLC) for (A), 42% (64% with PtdIns-PLC) for (B), and 72% (92% with PtdIns-PLC) for (C) of total β-hexosaminidase.

5.5. Conclusions

The experiments presented in this chapter demonstrate that PLC is responsible for the transient depletion of PtdIns(4,5)P_2 during exocytosis. Furthermore, the continued depletion of PtdIns after recovery of PtdIns(4,5)P_2 levels suggests that PLC remains active after 48/80 stimulation, but that re-synthesis of PtdIns(4,5)P_2 from PtdIns is accelerated and restores PtdIns(4,5)P_2 to near resting levels. PLC also mediates the depletion of PtdIns(4,5)P_2 observed during exocytosis from permeabilised cells, although recovery is not observed in this case. It is apparent from these experiments that this PLC-mediated depletion of plasma membrane PtdIns(4,5)P_2 is an absolute requirement for exocytosis under the conditions applied.

Although PLC appeared to be required for the Ca^{2+} signal necessary to initiate exocytosis, there are other functional consequences of PtdIns(4,5)P_2 breakdown that are mandatory. Generation of inositol phosphates, elimination of PtdIns(4,5)P_2 or DAG production are not sufficient alone to allow exocytosis to proceed. However, the data presented in this chapter would be consistent with a model (figure 5.13) whereby concomitant removal of PtdIns(4,5)P_2 and generation of DAG are required. Consistent with this, DAG generation was able to partially restore exocytosis when PLC was blocked with neomycin. There are a number of reasons that may explain why this restoration was incomplete. Firstly, incubation with neomycin may displace a soluble PtdIns(4,5)P_2-binding protein(s) required for exocytosis, which leaks from the permeabilised cells. Secondly, neomycin may interact with a second PIn required for exocytosis but with lower affinity, producing the increased concentration-dependence of inhibition. Such a candidate PIn could be PtdIns4P, as discussed in Chapter 3.

Chapter 6: Discussion

6.1. Quantitative immunofluorescence of phosphoinositides

Fusion of specific lipid binding domains such as those listed in table 1.1 to GFP and its spectral derivatives has enabled the direct visualisation of PIn and their turnover in living cells (Halet, 2005). In pioneering studies, Varnai and Balla used the PH-domain from PLCδ1 fused to GFP to study the turnover of PtdIns(4,5)P_2 (Varnai and Balla, 1998). Activation of PLC was seen to induce translocation of the probe from the plasma membrane to the cytosol; measurement of the plasma membrane to cytosol ratio correlated well with PtdIns(4,5)P_2 mass from equilibrium-labelled cells. However, since this study was conducted, controversy has arisen over whether GFP-PH-PLCδ1 truly reflects PtdIns(4,5)P_2 levels. This has arisen due the high-affinity binding of the probe to the product of PtdIns(4,5)P_2 hydrolysis, Ins(1,4,5)P_3 (Lemmon *et al.*, 1995). In epithelial cells, the translocation from the plasma membrane has been suggested to more closely report generation of Ins(1,4,5)P_3, as opposed to depletion of PtdIns(4,5)P_2 (Hirose *et al.*, 1999). Indeed, this technique is routinely used to follow Ins(1,4,5)P_3 production in neurons (Nahorski *et al.*, 2003). In contrast, another study demonstrated that translocation of GFP-PH-PLCδ1 more accurately reflects PtdIns(4,5)P_2 depletion, and not Ins(1,4,5)P_3 or Ins(1,4,5)P_3-mediated calcium signalling in neuroblastoma cells (van der Wal *et al.*, 2001). Yet another study conducted in astrocytoma cells revealed that the dynamics of GFP-PH-PLCδ1 after activation of PLC was influenced by both PtdIns(4,5)P_2 and Ins(1,4,5)P_3 (Xu *et al.*, 2003). Together, these reports highlight the variable behaviour of expressed lipid-binding domains, depending on the cell type and stimulus applied. Therefore, the possibility that Ins(1,4,5)P_3 interferes with the localisation of GFP-PH-PLCδ1 must be taken into account when using this probe, and is a serious caveat to its use.

Xu *et al* also highlighted a second major drawback of the use of GFP-PH-PLCδ1: this probe was unable to detect the rapid, transient synthesis of PtdIns(4,5)P_2 prior to PLC activation (Xu *et al.*, 2003), despite the probe's ability to report the depletion of PtdIns(4,5)P_2 by PLC (Varnai and Balla, 1998; van der Wal *et al.*, 2001; Xu *et al.*, 2003). Another potential problem is that expressed GFP-PH-PLCδ1 is not always a passive tool. At high expression levels, it can sequester

PtdIns(4,5)P_2 and so inhibit PtdIns(4,5)P_2-dependent processes (Holz *et al.*, 2000; Lawrence and Birnbaum, 2003; Micheva *et al.*, 2003). This latter point can be an advantage, however, since it can be used to reduce free PtdIns(4,5)P_2 levels to investigate the function of this lipid.

A more general point with the use of expressed lipid-binding domains is that there may be another determinant within the probe that will bias its localisation to a particular membrane, independently of PIn binding (Balla *et al.*, 2000; Roy and Levine, 2004). Indeed, this is the case with the majority of PH domains from the yeast *S. cerevisiae* (Yu *et al.*, 2004). Bearing this in mind, it is particularly concerning that GFP-PH-PLCδ1 is usually reported to detect only plasma membrane pools of PtdIns(4,5)P_2 (Halet, 2005): the plasma membrane is enriched in cholesterol, which is able to influence the membrane binding activity of GFP-PH-PLCδ1 (Flesch *et al.*, 2005). A similar caveat is present with the high-affinity PtdIns(4,5)P_2-binding PDZ domains from syntenins. Domains from syntenin-1 detect plasma membrane PtdIns(4,5)P_2 (Zimmermann *et al.*, 2002), whereas the same regions of syntenin-2 reports the PtdIns(4,5)P_2-containing nuclear speckles (Mortier *et al.*, 2005); based on these results, it is clear that other interactions determine these probes' localisation.

Thus whereas much has been learnt through the expression of GFP-tagged probes for PIn, such an approach has serious drawbacks. In this thesis, an immunofluorescence approach was developed to detect PtdIns(4,5)P_2 in mast cells. This type of experiment was pursued since RPMCs are not amenable to transfection, and could not be maintained in culture (in the author's hands) for extended periods of time. It was thus not possible to use GFP-PH-PLCδ1 to follow PtdIns(4,5)P_2 dynamics during exocytosis. Immunofluorescence cannot be performed on live cells unless invasive microinjection techniques are employed. However, this technique presents a number of advantages over expression of GFP-tagged probes.

Firstly, since the antibody is applied to fixed cells, it passively reports the lipid distribution, and will therefore not impinge on PtdIns(4,5)P_2-dependent processes. Secondly, the antibodies are targeted solely by their target lipid, so do not have the potential bias observed with PIn-binding domains. They can therefore be used

to detect multiple pools of PtdIns(4,5)P_2, such as those at the plasma membrane and in the nucleus (chapter 4). Furthermore, recombinant PIn-binding proteins may be used in a similar manner to the antibodies to report PIn in fixed cells, as shown in chapter 4 and (Gillooly *et al.*, 2000). However, conditions were not found in which membrane and non-membranous, detergent-resistant pools of PIn can be determined simultaneously.

Use of fluorescence techniques presents the same problems of resolution as expression of GFP-tagged domains: apparent enrichment of the lipids is seen at regions of increased membrane density (figure 4.6) and (van Rheenen *et al.*, 2005)). However, such problems may be overcome by ultrastructural studies using immunogold labelling under the electron microscope (Gillooly *et al.*, 2000; Watt *et al.*, 2002; Watt *et al.*, 2004).

As reported with GFP-PH-PLCδ1 (Varnai and Balla, 1998), immunofluorescence was seen to accurately reflect the levels of PtdIns(4,5)P_2 in the cell. In addition, it presents certain advantages over biochemical methods, since the behaviour of individual cells as well as the population as a whole may be scrutinised. This led to the revelation that although the PtdIns(4,5)P_2 levels in a population of RPMCs drops by ~80% within 15 s of challenge with 48/80, this value actually reflects near total elimination of PtdIns(4,5)P_2 in ~80% of the cells (figure 4.14). Furthermore, using immunofluorescence it was possible to follow the different behaviours of plasma membrane and nuclear PtdIns(4,5)P_2 pools, which is very challenging using biochemical techniques. Quantitative analysis was eased by the use of automated software, although the technique should also prove amenable to high-throughput analysis using fluorescence-activated cell sorting (FACS) or computer-assisted automatic microscopy.

Therefore, despite drawbacks, quantitative immunofluorescence appears to be a powerful tool with which to study PIn dynamics in cells. It has been demonstrated to work with PtdIns(4,5)P_2 and PtdIns(3,4)P_2, providing proof-of-principle and strongly suggesting it may be applied for any PIn for which a specific probe is available. In this light, quantitative immunofluorescence presents a new technique that complements the current repertoire of tools, which includes expressed binding proteins and biochemical measurements.

6.2. Phosphoinositides involved in mast cell exocytosis

6.2.1. Synthesis of PPIn during permeabilisation and rundown

In chapter 3, experiments were conducted to determine whether synthesis of PPIn was required to maintain secretory competence in permeabilised RPMCs. Previous work had indicated that they were, since sequestering PPIn with neomycin mimicked the effect of depletion of ATP on the loss of secretory competence, and rundown was retarded by provision of PITP (Pinxteren *et al.*, 2001). From these studies, it appears that one or more PPIn forms part of a phosphorylation state required for exocytosis from RPMC. However, rundown also seems to occur in the presence of ATP due to a loss of the ability to couple stimulus with secretion, since it may be retarded by provision of the G-proteins that trigger exocytosis (O'Sullivan *et al.*, 1996; Brown *et al.*, 1998; Pinxteren *et al.*, 1998). Furthermore, it has been demonstrated that unlike exocytosis, the levels of PtdIns(4,5)P_2 and PtdIns4P do not rundown when RPMCs are permeabilised in the presence of 100 μM ATP for 10 minutes (Pinxteren *et al.*, 2001). Therefore, rundown does not occur due to the loss of these PPIn when ATP is provided, and may occur simply because of leakage of the machinery necessary to trigger exocytosis.

That said, levels of PtdIns(4,5)P_2 and PtdIns4P are seen to drop by > 50% when cells are permeabilised in the absence of ATP for 5 minutes (Pinxteren *et al.*, 2001), and the cells become refractory to stimulation under these conditions (Howell *et al.*, 1989). Therefore, synthesis of PPIn may indeed be required to maintain secretory competence in permeabilised mast cells. Further evidence for this concept was evident from the inhibition of exocytosis by neomycin and PH-βARK^{WAA}, agents that sequester PPIn (Pinxteren *et al.*, 1998; Pinxteren *et al.*, 2001). It was not possible to determine which PPIn these agents sequester in order to block exocytosis; indeed, it could be any of them in the case of PH-βARK^{WAA} (figure 3.3). However, it is almost certainly not PtdIns(4,5)P_2; this comes from the complementary observations that PH-PLCδ1 does not block exocytosis (figure 3.7) but effectively sequesters PtdIns(4,5)P_2 (figure 4.7), whereas PH-βARK^{WAA}

blocks exocytosis (figure 3.7) but does not sequester PtdIns(4,5)P_2 (figure 4.7). Another PIn is therefore implicated in exocytosis from permeabilised mast cells.

Recently, the LY294002- and wortmannin-resistant PI3K-C2α has been implicated in regulated exocytosis from endocrine cells (Maffucci *et al.*, 2003; Meunier *et al.*, 2005). However, the lack of inhibition by high concentrations of PI3K inhibitors or the PtdIns3P-sequestering 2xFYVE domain argue against a role for this enzyme in RPMC exocytosis. Indeed, the lack of effect of PI3K inhibitors or 3-PIn binding domains does not support a requirement for synthesis of this subclass of PPIn during RPMC exocytosis. However, whilst this thesis was in preparation, a novel wortmannin-resistant PtdIns(4,5)P_2 3-OH kinase activity was reported (Resnick *et al.*, 2005). Therefore, it is possible that this enzyme may synthesise a pool of PtdIns(3,4,5)P_3 required for exocytosis. However, this seems unlikely given that PtdIns(3,4,5)P_3-sequestering PH domains are without effect in permeabilised cells (figure 3.6), and that IPMK appears to synthesise PtdIns(3,4,5)P_3 in the nucleus (Resnick *et al.*, 2005).

If 3-PIn are not involved in maintaining secretory competence in permeabilised RPMCs, this leaves only the monophosphorylated isomers PtdIns4*P* and PtdIns5*P*. No tools were available to study the latter, so no conclusions can be drawn as to the role of PtdIns5*P* during RPMC exocytosis. Sequestration of PtdIns4*P* using PH-FAPP1 (Dowler *et al.*, 2000; Godi *et al.*, 2004) was able to retard rundown in preliminary experiments. This could occur by protecting PtdIns4*P* from degradation during rundown (and thus possibly increasing its levels), yet still permitting access to a high-affinity effector protein. In support for this concept, the effect of PITP in the presence of ATP on RPMCs was to elevate PtdIns4*P* levels, without altering levels of PtdIns(4,5)P_2 (Pinxteren *et al.*, 2001). Synthesis of PtdIns4*P* can therefore occur in mast cells, and is most likely mediated by the type II PI4K. This assertion is based on the observations that RPMCs contain type II activity (figure 3.8), that exocytosis in insensitive to concentrations of PI3K inhibitors that block the type III activity (Downing *et al.*, 1996) and that the effect of ATP is maximal at 50 μM, a concentration sufficient to drive type II but not type III PI4K (Howell *et al.*, 1989; Downing *et al.*, 1996; Pinxteren *et al.*, 2001). In this light, and with the caveats embedded in the use of

pharmacological approaches, it will be interesting to observe the effects of the type II PI4K inhibitors adenosine and Resveratrol on rundown (Barylko *et al.*, 2001; Minogue *et al.*, 2001; Naveen *et al.*, 2005).

If PtdIns4*P* synthesis is required for the maintenance of secretory competence in permeabilised RPMCs, does it act in its own right or as a substrate for synthesis of PtdIns(4,5)P_2? PtdIns4*P* has been implicated *per se* in the regulation of constitutive exocytosis at the Golgi complex in yeast (Hama *et al.*, 1999; Walch-Solimena and Novick, 1999; Audhya *et al.*, 2000) and mammalian cells (Wang *et al.*, 2003; Godi *et al.*, 2004). Furthermore, the PI4K activity required for neuronal and endocrine cell exocytosis is located on the secretory granules (Wiedemann *et al.*, 1996; Wiedemann *et al.*, 1998), whereas PtdIns(4,5)P_2 synthesis occurs at the plasma membrane (Holz *et al.*, 2000; Wenk *et al.*, 2001; Aikawa and Martin, 2003; Lawrence and Birnbaum, 2003). A similar PI4K activity is present in RPMC granules (Kurosawa and Parker, 1986). No evidence presents itself in the literature that this granule-associated PI4K is involved in the synthesis of plasma membrane PtdIns(4,5)P_2, so it may thus be possible that a pool of PtdIns4*P* exists on secretory vesicles and regulates exocytosis.

6.2.2. *A role for plasma membrane PtdIns(4,5)*P_2

In chapter 4, a plasma membrane pool of PtdIns(4,5)P_2 was identified which becomes transiently depleted during exocytosis. Thus PtdIns(4,5)P_2 was not observed at sites of membrane fusion (i.e. granule membranes) during compound exocytosis. This observation has implications in itself for the role of PtdIns(4,5)P_2 during this process, since its absence from internal membranes during compound exocytosis precludes a direct function in membrane fusion. In chapter 5, this depletion was ascribed to PLC activity, and this activity was required for exocytosis. Since PtdIns(4,5)P_2 depletion, but not recovery, was observed in permeabilised, degranulated RPMCs it was concluded that it is the depletion rather than re-synthesis of PtdIns(4,5)P_2 that is required for exocytosis. In this light, the failure of PH-PLCδ1 to block exocytosis can be explained by the fact that it was only applied at concentrations up to 10 μM, which are not sufficient to completely sequester PtdIns(4,5)P_2 (figure 4.8) and thus block PLC.

Experiments conducted in chapter 5 (figure 5.1) confirmed the role for PLCγ in calcium signalling during activation of mast cells (Penner, 1988; Kinet, 1999). However, PLC activity may not be required for generation of the calcium signal that triggers exocytosis in all types of mast cell (Melendez and Khaw, 2002), and indeed calcium may not be required at all under certain experimental conditions (Fernandez *et al.*, 1984; Penner, 1988; Churcher and Gomperts, 1990; Tatham and Gomperts, 1991; Koffer and Churcher, 1993).

However, experiments in permeabilised cells where the Ins(1,4,5)P_3/Ca^{2+} pathway is bypassed reveal other roles for PLC activity. Together, these results led to the conclusion that PtdIns(4,5)P_2 does not function at the moment of membrane fusion, but may be required for maintenance of a secretion-competent state (Martin, 2001). In support of this, depletion of PtdIns(4,5)P_2 with a bacterial PIn phosphatase was seen to accelerate rundown (figure 5.11).

6.2.3. Roles for other pools of PtdIns(4,5)P_2*?*

In chapter 4, two other pools of PtdIns(4,5)P_2 were identified in RPMCs: a granule membrane pool, which appears after exocytosis, and a nuclear pool, which increases dramatically after activation of cells with 48/80. These pools were not examined further, since they were not deemed as being important for the process of degranulation. This is because granule PtdIns(4,5)P_2 appears only after the completion of exocytosis, and the nuclear pool is increased, which would require ATP; exocytosis, on the other hand, can occur in the absence of ATP (Howell *et al.*, 1987). However, there may be non-exocytosis roles for these pools, which are speculated upon herein.

PtdIns(4,5)P_2 has been shown to regulate actin polymerisation in a variety of other cell types (Janmey and Lindberg, 2004). In activated RPMCs, polymerisation of actin to form centripetal filaments occurs (Norman *et al.*, 1994). One can speculate, therefore, that the granule membrane PtdIns(4,5)P_2 may direct this polymerisation. However, the granule membrane pool may simply be due to diffusion of PtdIns(4,5)P_2 through the plane of the fused membranes from the plasma membrane.

Nuclear PtdIns(4,5)P_2 and its metabolites have been implicated in several stages of gene expression, from chromatin remodelling to mRNA export (Hammond *et al.*, 2004). After activation, mast cells begin transcription and expression of various cytokines and chemokines, which are secreted in a sustained late response from the cell to mediate long-term inflammatory responses (Metcalfe *et al.*, 1997). It is tempting to speculate, therefore, that increases in nuclear PtdIns(4,5)P_2 reflect this activation of gene expression. In other words, it may simply reflect the nucleus "waking-up" in preparation for a sustained response to activation.

6.3. Functional outcomes of PtdIns(4,5)P_2 hydrolysis for exocytosis

6.3.1. Are DAG generation and PtdIns(4,5)P_2 depletion required for mast cell exocytosis?

As discussed in the introduction, an important function of PLC is the generation of DAG and its metabolites, as well as InsPP. However, a role for PLC-generated InsPP seems unlikely, for a number of reasons. Firstly, InsPP would be expected to leak rapidly from permeabilised cells through the ~30 nm SL-O lesions. Secondly, given that exocytosis may proceed in the absence of ATP (Howell *et al.*, 1987), phosphorylation of Ins(1,4,5)P_3 to higher InsPP is unlikely to occur, unless it transpires by (uncharacterised) ATP-independent phosphotransferases. Finally, neither Ins(1,4)P_2 nor Ins(1,4,5)P_3 were able to modify exocytosis at concentrations up to ~10^{-4} M, and inhibition of their degradation was also without effect. Together, there seems to be no evidence that InsPP may function during exocytosis from permeabilised cells triggered with Ca^{2+} and GTPγS. However, there is a role for Ins(1,4,5)P_3 in generating the calcium signal in intact, 48/80 triggered cells, and there may be other functions for Ins(1,4,5)P_3 or its metabolites under these conditions.

On the other hand, DAG and its analogues were seen to retard rundown from permeabilised cells, suggesting a role for DAG during exocytosis. However, the failure of DAG to restore exocytosis under conditions whereby PLC was inhibited demonstrated that DAG production is not sufficient for exocytosis to occur. Another function for PLC is the simple removal of PtdIns(4,5)P_2, as seen during

formation of phagosomes (Botelho *et al.*, 2000) and the modulation of ion channel activity (Suh and Hille, 2005). Removal of PtdIns(4,5)P_2 may also be mediated via inositol phosphatases, as seen with pathogenic strains of *Salmonella*, which utilise the SigD inositol phosphatase (Terebiznik *et al.*, 2002). However, this phosphatase was not sufficient to allow exocytosis to proceed when endogenous PLC was blocked. In fact, SigD accelerated rundown, suggesting roles for PtdIns(4,5)P_2 in maintaining secretory competence, possibly by acting as a substrate for PLC when activation occurs.

These observations lead to the hypothesis that the coincident removal of PtdIns(4,5)P_2 and generation of DAG by PLC may both be required for exocytosis to proceed. To test this possibility, the electroneutral complex between PtdIns(4,5)P_2 and neomycin (Gabev *et al.*, 1989) was employed to mimic removal of PtdIns(4,5)P_2 in permeabilised cells. If the hypothesis were correct, inhibition of exocytosis would be expected by the resulting inhibition of PLC (Sagawa *et al.*, 1983) and thus failure to generate DAG. However, this block would be lifted if DAG were applied independently of the endogenous PLC, via the cleavage of PtdIns by a bacterial PLC. The results from such an experiment showed a 2.5-fold increase of the IC_{50} of neomycin, which translates to a partial restoration of exocytosis by DAG. Similar results were obtained previously when 100 nM PMA was applied to neomycin-treated permeabilised RPMCs (Howell *et al.*, 1989).

Why should the restoration of exocytosis by DAG under conditions of PtdIns(4,5)P_2 sequestration be only partial? There could be a number of reasons for this experimental observation. Firstly, neomycin could displace an endogenous, soluble PtdIns(4,5)P_2-binding protein required for exocytosis, which is lost from the cell through the SL-O lesions (figure 5.13). A second possibility presents itself from the conclusions of chapter 3: that a PPIn, most likely PtdIns4*P* or PtdIns5*P*, is required for exocytosis in addition to PtdIns(4,5)P_2. Given that these monophosphorylated PIn isomers form lower affinity interactions with neomycin (Schacht, 1978) this would explain the increased concentration dependence of inhibition.

Together, the observations presented in chapter 5 confirm the importance of PtdIns(4,5)P_2 for regulated exocytosis. However, they suggest an unsuspected

dual mechanism of action for the lipid: it serves both as a membrane anchor for a protein required before and/or during exocytosis, and as a source of DAG required for membrane fusion (figure 5.13). This model remains speculative and requires further clarification. However, if the model is assumed to approximate the true situation for RPMC exocytosis, there are a number of candidate proteins that could perform the roles depicted in figure 5.13.

As discussed in the introduction, potential PtdIns(4,5)P_2-binding proteins required for exocytosis include Mints, CAPS, Rabphillin and synaptotagmin. Furthermore, the requisite syntaxins expressed in neurons and RPMCs contain a polybasic juxtamembrane regions that is capable of sequestering PtdIns(4,5)P_2 (S. McLaughlin, personal communication). Furthermore, SCAMP2, which has been shown to function during mast cell exocytosis (Guo *et al.*, 2002), also contains a polybasic motif capable of sequestering PtdIns(4,5)P_2 (Ellena *et al.*, 2004).

Several candidate DAG-regulated proteins that may be required for compound exocytosis have been previously characterised, including Munc13, chimaerins and Ras GRPs (Brose and Rosenmund, 2002). Although protein kinase C (PKC) is regulated by DAG (Parker and Murray-Rust, 2004), it seems that the catalytic activity of this enzyme is not required, for a number of reasons: firstly, the ATP-independence of exocytosis would not permit catalytic activity of PKC (Howell *et al.*, 1987). Secondly, RPMC exocytosis is insensitive to the PKC inhibitors H7, staurosporine and K252a, or to PKC inhibitor peptides (Howell *et al.*, 1989; Shefler *et al.*, 1998; Pinxteren *et al.*, 2001; Gloyna *et al.*, 2005). Given that RPMC exocytosis is blocked by the ether lipid DAG analogue AMG.C_{16}, another PKC inhibitor (Howell *et al.*, 1989; Pinxteren *et al.*, 2001), it will be interesting to verify whether this drug can antagonise other C1-domain containing proteins.

As discussed in the introduction, the cortical F-actin cytoskeleton breaks down during mast cell exocytosis; this could be facilitated by breakdown of PtdIns(4,5)P_2, as has been demonstrated during phagocytosis (Scott *et al.*, 2005). Indeed, partial depletion of the F-actin cytoskeleton with latrunculin or gelsolin increases mast cell exocytosis (Borovikov *et al.*, 1995; Martin-Verdeaux *et al.*, 2003; Sasaki *et al.*, 2005). In BMMCs, this treatment partially phenocopies the augmented degranulation in cells derived from mice genetically devoid of PI*P*K

Iα; these cells present reduced resting levels of PtdIns(4,5)P_2 and cortical F-actin (Sasaki *et al.*, 2005). From these observations, it was proposed that PtdIns(4,5)P_2 might regulate mast cell exocytosis in a negative manner, by maintaining an F-actin "barrier" at the plasma membrane (Sasaki *et al.*, 2005). Such a hypothesis would be consistent with the role of PLC reported in this thesis, and would account for one of the roles of PtdIns(4,5)P_2 during RPMC exocytosis.

However, such a "barrier" hypothesis for regulation of exocytosis by PtdIns(4,5)P_2 and F-actin appears over-simplified, based on two observations: firstly, complete removal of the F-actin cortex inhibits exocytosis from RPMCs (Pendleton and Koffer, 2001), and secondly, preventing actin depolymerisation with phalloidin does not inhibit exocytosis (Norman *et al.*, 1996; Sullivan *et al.*, 1999). Exocytosis could be facilitated by detachment of cortical F-actin from the plasma membrane, rather than by depolymerisation of actin. This might liberate a plasma membrane and F-actin-associated protein, which may then relocate to sites of membrane fusion. In this light, it is worth noting that SNAP-23, the Q-SNARE that relocates to granules from the plasma membrane, is associated with the actin cytoskeleton in resting RPMCs (Guo *et al.*, 1998).

6.3.2. Clues to PLC function from other cell types

PLC activity has been demonstrated during exocytosis from just about every cell type examined; examples include pancreatic exocrine cells (Hokin and Hokin, 1953; Hokin and Hokin, 1958), chromaffin cells (Whitaker, 1985), synapses after electrical stimulation (Bleasdale and Hawthorne, 1975; Pickard and Hawthorne, 1978) and RPMCs (Cockcroft and Gomperts, 1979). In fact, the only example where regulated exocytosis is observed in the absence of PLC activity is during barium-stimulated chromaffin cell secretion (Eberhard and Holz, 1987).

Inositol lipids were proposed to function during exocytosis based on the fact that depletion of the lipids with a bacterial PtdIns-specific PLC abolished ATP-dependent exocytosis (Eberhard *et al.*, 1990). However, in these studies the inhibition of exocytosis correlated well with the reduction in InsPP generation, a read-out of PLC activity. It could therefore be that depletion of inositol lipids prevented exocytosis in part by preventing PLC activity. Although Eberhard *et al*

found no effect of DAG or its analogues, such observations are consistent with those presented in this thesis, and can only lead to the conclusion that DAG generation is not sufficient to mediate the functional outcome of PLC activity during exocytosis.

Another report in broken PC12 cells found that a mammalian PLCδ could inhibit ATP-dependent exocytosis when applied before the stimulus, due to depletion of PtdIns(4,5)P_2 (Hay *et al.*, 1995). Once again this could simply produce a block of exocytosis by depleting substrate for PLC, so that activity is not available at the appropriate time, i.e. when the stimulus is provided.

Is there any evidence for a functional requirement for PLC activity in these model systems? Indeed, it was recently reported that chromaffin cell exocytosis is inhibited by the PLC inhibitor U73122, and by inhibitory antibodies against PLCγ1 and PLCβ3 (O'Connell *et al.*, 2003). There is also a well-established role for DAG in exocytosis from neuronal cells, since Munc13 is regulated by DAG and phorbol esters (Betz *et al.*, 1998); in fact, Munc13 activity is sufficient to account for the entire stimulatory effect of phorbol esters on SV exocytosis (Rhee *et al.*, 2002). Munc13 is indispensable for neuronal exocytosis, which is completely abolished in mice genetically devoid of Munc13-1 and Munc13-2 (Varoqueaux *et al.*, 2002). Further evidence comes from the studies on the cholinergic neuromuscular junction in the nematode worm *Caenorhabditis elegans*. In these cells, the heterotrimeric G protein G_q (EGL-30) was shown to activate PLCβ (EGL-8); in turn, DAG activates Unc13 and leads to elevated SV exocytosis and animal mobility. Indeed, phorbol esters are able to restore mobility in EGL-30 or EGL-8 mutant animals (Lackner *et al.*, 1999; Miller *et al.*, 1999). Furthermore, motility in these animals is negatively regulated by diacylglycerol kinase (DGK-1), which is believed to reduce DAG levels and so reduce Unc13 activation (Miller *et al.*, 1999; Nurrish *et al.*, 1999).

6.3.3. A model for PLC-regulation of exocytosis

In figure 6.1, a model is presented to explain the molecular requirements for PLC activity in the triggering of mast cell exocytosis. In particular, certain aspects pertaining to the interactions with Munc18 and Munc13 would be consistent with

current models of synaptic vesicle exocytosis (Galli and Haucke, 2004). However, the author is at pains to stress that, although consistent with the experimental evidence presented above, and supported by circumstantial evidence from other secretory systems, this model is entirely speculative. It is provided simply as a working hypothesis with which it may be possible to test the various events and interactions depicted within.

Activation via antigen receptor or polycations will lead to activation of PLCγ and PLCβ, respectively. The resulting Ins(1,4,5)P_3-stimulated calcium release (which may occur from the granules, (Quesada *et al.*, 2003)) may in turn activate PLCδ. The resulting PLC activity leads to consumption of plasma membrane PtdIns(4,5)P_2, with a number of functional ramifications.

Firstly, the cortical F-actin cytoskeleton becomes dissociated from the plasma membrane, liberating SNAP-23 from plasma membrane folds so that it may relocate to sites of membrane fusion at the cell surface. Secondly, loss of PtdIns(4,5)P_2 leads to dissociation of Mint from the syntaxin-4/Munc18 complex. Concurrently, the PLC-produced DAG recruits and activates Munc13, allowing dissociation of Munc18, opening of syntaxin-4 and the assembly of SNARE complexes at the plasma membrane; this expedites a round of cortical granule fusion. Subsequently, DAG and SNAP-23 relocate through the fused membranes to internal granule membranes, where they again allow formation of SNARE complexes after displacement of Munc18.

Although speculative, such a model would explain why depletion of PtdIns(4,5)P_2 does not lead to inhibition of ATP-independent, primed vesicle exocytosis (Eberhard *et al.*, 1990; Hay *et al.*, 1995). According to the above model, primed vesicles would already have been in the proximity of a round of PtdIns(4,5)P_2 hydrolysis, so the switch of Munc18 and Munc13 would already have occurred, and the SNARE complexes will already be assembled and awaiting the final Ca^{2+}-stimulus, which leads to membrane fusion (Chen *et al.*, 1999; Chen *et al.*, 2001).

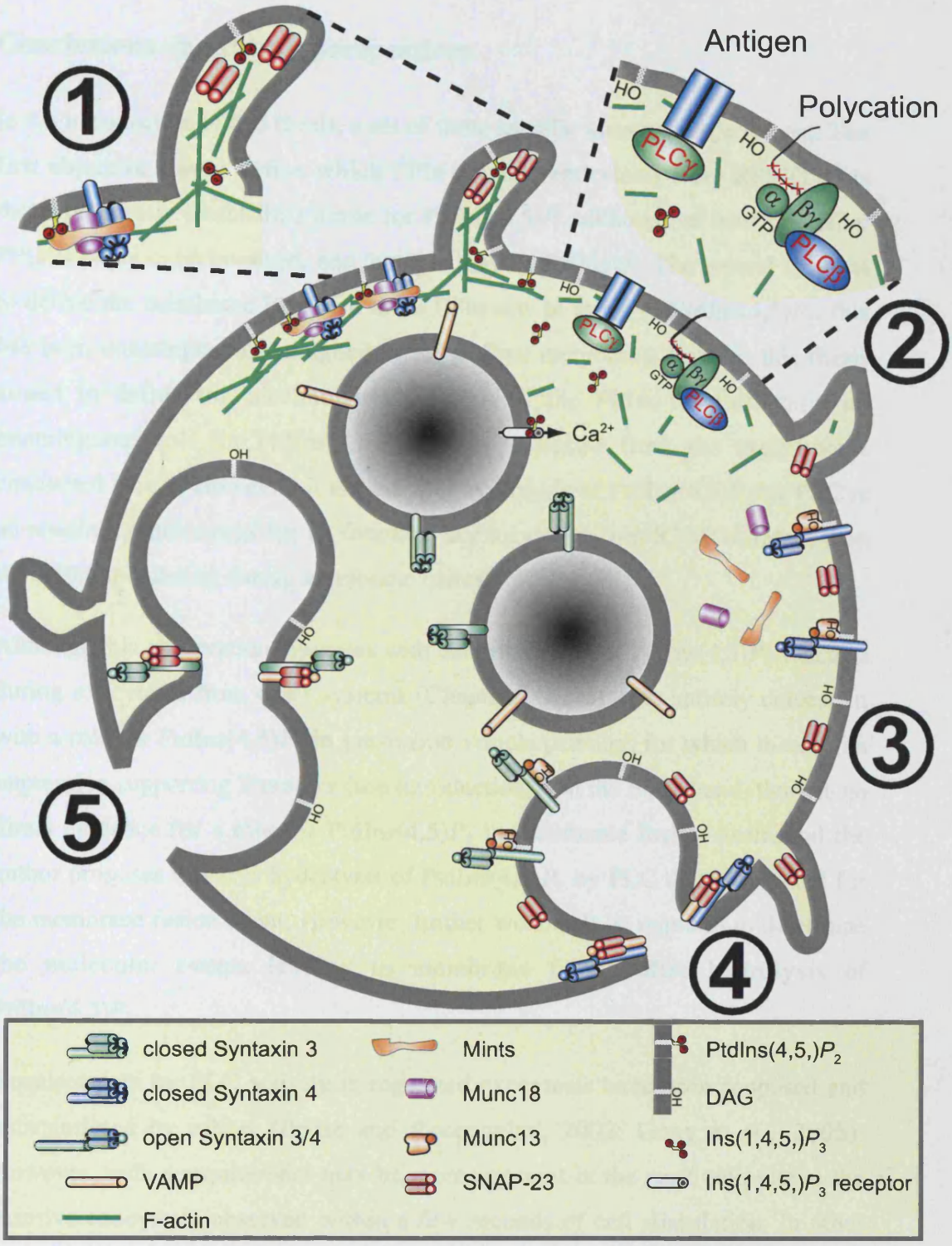

Figure 6.1: PLC activity during RPMC exocytosis. **(1) Priming:** PtdIns(4,5)P_2 localises F-actin and SNAP-23 at the plasma membrane, and is in a ternary complex with syntaxin 4, Munc18 and Mints. **(2) Activation:** PLC isoforms are recruited, and break down PtdIns(4,5)P_2 to Ins(1,4,5)P_3, which liberates Ca^{2+} from the granule stores. The actin cytoskeleton breaks down, releasing SNAP-23. **(3) SNARE preparation:** Mints is released from the ternary complex, and DAG activates Munc13, displacing Munc18 and opening syntaxin 4. **(4) Fusion:** SNARE complexes assemble at the plasma membrane; DAG and SNAP-23 diffuse onto the granule membrane. **(5) Compound exocytosis:** DAG activates Munc13 on granule membranes, which opens syntaxin 3. See text for details.

Conclusions and future perspectives

In the introduction to this thesis, a set of three specific aims were spelled out. The first objective was to define which PPIn regulate exocytosis from RPMC. This thesis has firmly established a role for PtdIns(4,5)P_2, although at least one other PPIn is likely to be involved, and that this may be PtdIns4*P*. The second aim was to define the membrane in which these PPIn act. In terms of PtdIns(4,5)P_2, this has been unambiguously assigned to the plasma membrane. Finally, this thesis aimed to define the mechanism of action of the PPIn; unfortunately, no unambiguous role for PtdIns(4,5)P_2 can be assigned from the experiments conducted herein. However, it is clear that hydrolysis of PtdIns(4,5)P_2 by PLC is an absolute requirement for its function in exocytosis from RPMCs, rather than the intact lipid acting during membrane fusion.

Although this observation disagrees with current models of PtdIns(4,5)P_2-function during exocytosis from other systems (Chapman, 2002), it is entirely consistent with a role for PtdIns(4,5)P_2 in pre-fusion vesicle priming, for which there is an impressive supporting literature (see introduction). On the other hand, there is no direct evidence for a role for PtdIns(4,5)P_2 in membrane fusion itself, and the author proposes that it is hydrolysis of PtdIns(4,5)P_2 by PLC that is required for the membrane fusion event. However, further work will be required to determine the molecular events leading to membrane fusion after hydrolysis of PtdIns(4,5)P_2.

Requirements for PLC activity in regulated exocytosis have been proposed and substantiated by others (Brose and Rosenmund, 2002; Gong *et al.*, 2005). However, such a requirement may be more apparent in the mast cell, due to the massive exocytosis observed within a few seconds of cell stimulation. In other cells, such as those in the nervous and endocrine systems, activity of PLC may be less apparent due to the restriction of PLC activity to small, polarised regions of secretory activity such as the synapse. Furthermore, since exocytosis can be sustained in these systems, break-down of plasma membrane PtdIns(4,5)P_2 would be less apparent due to concomitant synthesis of this lipid, which is required for priming of reserve vesicles and membrane recycling events. It is therefore because

of the unique biological properties of the mast cell that the importance of PLC in regulated exocytosis was so apparent.

Clearly, the work presented in this thesis is only the starting point for further clarification of the role of PtdIns(4,5)P_2 and its metabolites during regulated exocytosis. In particular, it will be interesting to define which PLC isoforms regulate exocytosis in mammalian cells, a question that may perhaps be answered through the use of genetic manipulation in mice. Furthermore, a more precise view of how PLC functions in the triggering of exocytosis is required. In particular, a more detailed understanding of the proteins associated with PtdIns(4,5)P_2 and its metabolites prior to and during exocytosis would aid in this endeavour. However, it is clear that exocytosis is regulated by a number of transient molecular contacts, and it will be of great value to elucidate how PtdIns(4,5)P_2 metabolism regulates these dynamic interactions at membranes.

Chapter 7: References

Abraham, S.N., and Malaviya, R. (1997). Mast cells in infection and immunity. Infect Immun *65*, 3501-3508.

Aikawa, Y., and Martin, T.F. (2003). ARF6 regulates a plasma membrane pool of phosphatidylinositol(4,5)bisphosphate required for regulated exocytosis. J Cell Biol *162*, 647-659.

Akin, C., and Metcalfe, D.D. (2004). Systemic mastocytosis. Annu Rev Med *55*, 419-432.

Ali, K., Bilancio, A., Thomas, M., Pearce, W., Gilfillan, A.M., Tkaczyk, C., Kuehn, N., Gray, A., Giddings, J., Peskett, E., Fox, R., Bruce, I., Walker, C., Sawyer, C., Okkenhaug, K., Finan, P., and Vanhaesebroeck, B. (2004). Essential role for the p110delta phosphoinositide 3-kinase in the allergic response. Nature *431*, 1007-1011.

Alvarez de Toledo, G., and Fernandez, J.M. (1990a). Compound versus multigranular exocytosis in peritoneal mast cells. J Gen Physiol *95*, 397-409.

Alvarez de Toledo, G., and Fernandez, J.M. (1990b). Patch-clamp measurements reveal multimodal distribution of granule sizes in rat mast cells. J Cell Biol *110*, 1033-1039.

An, S., and Zenisek, D. (2004). Regulation of exocytosis in neurons and neuroendocrine cells. Curr Opin Neurobiol *14*, 522-530.

Aoyagi, K., Sugaya, T., Umeda, M., Yamamoto, S., Terakawa, S., and Takahashi, M. (2005). The activation of exocytotic sites by the formation of phosphatidylinositol 4,5-bisphosphate microdomains at syntaxin clusters. J Biol Chem *280*, 17346-17352.

Arbuzova, A., Martushova, K., Hangyas-Mihalyne, G., Morris, A.J., Ozaki, S., Prestwich, G.D., and McLaughlin, S. (2000). Fluorescently labeled neomycin as a probe of phosphatidylinositol-4, 5-bisphosphate in membranes. Biochim Biophys Acta *1464*, 35-48.

Arcaro, A., and Wymann, M.P. (1993). Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-trisphosphate in neutrophil responses. Biochem J *296 (Pt 2)*, 297-301.

Aridor, M., Rajmilevich, G., Beaven, M.A., and Sagi-Eisenberg, R. (1993). Activation of exocytosis by the heterotrimeric G protein Gi3. Science *262*, 1569-1572.

Aridor, M., and Sagi-Eisenberg, R. (1990). Neomycin is a potent secretagogue of mast cells that directly activates a GTP-binding protein involved in exocytosis. J Cell Biol *111*, 2885-2891.

Aridor, M., Traub, L.M., and Sagi-Eisenberg, R. (1990). Exocytosis in mast cells by basic secretagogues: evidence for direct activation of GTP-binding proteins. J Cell Biol *111*, 909-917.

Audhya, A., Foti, M., and Emr, S.D. (2000). Distinct roles for the yeast phosphatidylinositol 4-kinases, Stt4p and Pik1p, in secretion, cell growth, and organelle membrane dynamics. Mol Biol Cell *11*, 2673-2689.

Auger, K.R., Serunian, L.A., Soltoff, S.P., Libby, P., and Cantley, L.C. (1989). PDGF-dependent tyrosine phosphorylation stimulates production of novel polyphosphoinositides in intact cells. Cell *57*, 167-175.

Augert, G., Blackmore, P.F., and Exton, J.H. (1989). Changes in the concentration and fatty acid composition of phosphoinositides induced by hormones in hepatocytes. J Biol Chem *264*, 2574-2580.

Bai, J., and Chapman, E.R. (2004). The C2 domains of synaptotagmin--partners in exocytosis. Trends Biochem Sci *29*, 143-151.

Bai, J., Tucker, W.C., and Chapman, E.R. (2004). PIP2 increases the speed of response of synaptotagmin and steers its membrane-penetration activity toward the plasma membrane. Nat Struct Mol Biol *11*, 36-44.

Baker, P.F., and Knight, D.E. (1978). Calcium-dependent exocytosis in bovine adrenal medullary cells with leaky plasma membranes. Nature *276*, 620-622.

Balla, A., Tuymetova, G., Barshishat, M., Geiszt, M., and Balla, T. (2002). Characterization of type II phosphatidylinositol 4-kinase isoforms reveals association of the enzymes with endosomal vesicular compartments. J Biol Chem *277*, 20041-20050.

Balla, A., Tuymetova, G., Tsiomenko, A., Varnai, P., and Balla, T. (2005). A plasma membrane pool of phosphatidylinositol 4-phosphate is generated by phosphatidylinositol 4-kinase type-III alpha: studies with the PH domains of the oxysterol binding protein and FAPP1. Mol Biol Cell *16*, 1282-1295.

Balla, T., Bondeva, T., and Varnai, P. (2000). How accurately can we image inositol lipids in living cells? Trends Pharmacol Sci *21*, 238-241.

Banfic, H., Downes, C.P., and Rittenhouse, S.E. (1998a). Biphasic activation of PKBalpha/Akt in platelets. Evidence for stimulation both by phosphatidylinositol 3,4-bisphosphate, produced via a novel pathway, and by phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem *273*, 11630-11637.

Banfic, H., Tang, X., Batty, I.H., Downes, C.P., Chen, C., and Rittenhouse, S.E. (1998b). A novel integrin-activated pathway forms PKB/Akt-stimulatory phosphatidylinositol 3,4-bisphosphate via phosphatidylinositol 3-phosphate in platelets. J Biol Chem *273*, 13-16.

Baram, D., Adachi, R., Medalia, O., Tuvim, M., Dickey, B.F., Mekori, Y.A., and Sagi-Eisenberg, R. (1999). Synaptotagmin II negatively regulates Ca2+-triggered exocytosis of lysosomes in mast cells. J Exp Med *189*, 1649-1658.

Baram, D., Linial, M., Mekori, Y.A., and Sagi-Eisenberg, R. (1998). Ca2+-dependent exocytosis in mast cells is stimulated by the Ca2+ sensor, synaptotagmin I. J Immunol *161*, 5120-5123.

Barylko, B., Gerber, S.H., Binns, D.D., Grichine, N., Khvotchev, M., Sudhof, T.C., and Albanesi, J.P. (2001). A novel family of phosphatidylinositol 4-kinases conserved from yeast to humans. J Biol Chem *276*, 7705-7708.

Befus, A.D., Mowat, C., Gilchrist, M., Hu, J., Solomon, S., and Bateman, A. (1999). Neutrophil defensins induce histamine secretion from mast cells: mechanisms of action. J Immunol *163*, 947-953.

Berridge, M.J. (1993). Cell signalling. A tale of two messengers. Nature *365*, 388-389.

Betz, A., Ashery, U., Rickmann, M., Augustin, I., Neher, E., Sudhof, T.C., Rettig, J., and Brose, N. (1998). Munc13-1 is a presynaptic phorbol ester receptor that enhances neurotransmitter release. Neuron *21*, 123-136.

Bhakdi, S., Tranum-Jensen, J., and Sziegoleit, A. (1985). Mechanism of membrane damage by streptolysin-O. Infect Immun *47*, 52-60.

Blank, U., Cyprien, B., Martin-Verdeaux, S., Paumet, F., Pombo, I., Rivera, J., Roa, M., and Varin-Blank, N. (2002). SNAREs and associated regulators in the control of exocytosis in the RBL-2H3 mast cell line. Mol Immunol *38*, 1341-1345.

Blank, U., and Rivera, J. (2004). The ins and outs of IgE-dependent mast-cell exocytosis. Trends Immunol *25*, 266-273.

Bleasdale, J.E., and Hawthorne, J.N. (1975). The effect of electrical stimulation on the turnover of phosphatidic acid in synaptosomes from guinea-pig brain. J Neurochem *24*, 373-379.

Bligh, E.G., and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Can J Biochem Physiol *37*, 911-917.

Blott, E.J., and Griffiths, G.M. (2002). Secretory lysosomes. Nat Rev Mol Cell Biol *3*, 122-131.

Bolland, S., Pearse, R.N., Kurosaki, T., and Ravetch, J.V. (1998). SHIP modulates immune receptor responses by regulating membrane association of Btk. Immunity *8*, 509-516.

Boronenkov, I.V., Loijens, J.C., Umeda, M., and Anderson, R.A. (1998). Phosphoinositide signaling pathways in nuclei are associated with nuclear speckles containing pre-mRNA processing factors. Mol Biol Cell *9*, 3547-3560.

Borovikov, Y.S., Norman, J.C., Price, L.S., Weeds, A., and Koffer, A. (1995). Secretion from permeabilised mast cells is enhanced by addition of gelsolin: contrasting effects of endogenous gelsolin. J Cell Sci *108*, 657-666.

Botelho, R.J., Teruel, M., Dierckman, R., Anderson, R., Wells, A., York, J.D., Meyer, T., and Grinstein, S. (2000). Localized biphasic changes in phosphatidylinositol-4,5-bisphosphate at sites of phagocytosis. J Cell Biol *151*, 1353-1368.

Bracher, A., Kadlec, J., Betz, H., and Weissenhorn, W. (2002). X-ray structure of a neuronal complexin-SNARE complex from squid. J Biol Chem *277*, 26517-26523.

Brose, N., and Rosenmund, C. (2002). Move over protein kinase C, you've got company: alternative cellular effectors of diacylglycerol and phorbol esters. J Cell Sci *115*, 4399-4411.

Brown, A.M., O_Sullivan, A.J., and Gomperts, B.D. (1998). Induction of exocytosis from permeabilized mast cells by the guanosine triphosphatases Rac and Cdc42. Mol Biol Cell *9*, 1053-1063.

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

Burgoyne, R.D., and Clague, M.J. (2003). Calcium and calmodulin in membrane fusion. Biochim Biophys Acta *1641*, 137-143.

Burgoyne, R.D., and Morgan, A. (2003). Secretory granule exocytosis. Physiol Rev *83*, 581-632.

Burwen, S.J., and Satir, B.H. (1977). Plasma membrane folds on the mast cell surface and their relationship to secretory activity. J Cell Biol *74*, 690-697.

Calleja, V., Ameer-Beg, S.M., Vojnovic, B., Woscholski, R., Downward, J., and Larijani, B. (2003). Monitoring conformational changes of proteins in cells by fluorescence lifetime imaging microscopy. Biochem J *372*, 33-40.

Chakrabarti, S., Kobayashi, K.S., Flavell, R.A., Marks, C.B., Miyake, K., Liston, D.R., Fowler, K.T., Gorelick, F.S., and Andrews, N.W. (2003). Impaired membrane resealing and autoimmune myositis in synaptotagmin VII-deficient mice. J Cell Biol *162*, 543-549.

Chapman, E.R. (2002). Synaptotagmin: a Ca(2+) sensor that triggers exocytosis? Nat Rev Mol Cell Biol *3*, 498-508.

Chen, Y.A., Scales, S.J., Patel, S.M., Doung, Y.C., and Scheller, R.H. (1999). SNARE complex formation is triggered by Ca2+ and drives membrane fusion. Cell *97*, 165-174.

Chen, Y.A., Scales, S.J., and Scheller, R.H. (2001). Sequential SNARE assembly underlies priming and triggering of exocytosis. Neuron *30*, 161-170.

Chen, Y.A., and Scheller, R.H. (2001). SNARE-mediated membrane fusion. Nat Rev Mol Cell Biol *2*, 98-106.

Chieregatti, E., and Meldolesi, J. (2005). Regulated exocytosis: new organelles for non-secretory purposes. Nat Rev Mol Cell Biol *6*, 181-187.

Choi, E.K., Park, H.J., Ma, J.S., Lee, H.C., Kang, H.C., Kim, B.G., and Kang, I.C. (2004). LY294002 inhibits monocyte chemoattractant protein-1 expression through a phosphatidylinositol 3-kinase-independent mechanism. FEBS Lett *559*, 141-144.

Churcher, Y., and Gomperts, B.D. (1990). ATP-dependent and ATP-independent pathways of exocytosis revealed by interchanging glutamate and chloride as the major anion in permeabilized mast cells. Cell Regul *1*, 337-346.

Cockcroft, S., and Gomperts, B.D. (1979). Evidence for a role of phosphatidylinositol turnover in stimulus-secretion coupling. Studies with rat peritoneal mast cells. Biochem J *178*, 681-687.

Cockcroft, S., Howell, T.W., and Gomperts, B.D. (1987). Two G-proteins act in series to control stimulus-secretion coupling in mast cells: use of neomycin to distinguish between G-proteins controlling polyphosphoinositide phosphodiesterase and exocytosis. J Cell Biol *105*, 2745-2750.

Cooke, F.T. (2002). Phosphatidylinositol 3,5-bisphosphate: metabolism and function. Arch Biochem Biophys *407*, 143-151.

Cooke, F.T., Dove, S.K., McEwen, R.K., Painter, G., Holmes, A.B., Hall, M.N., Michell, R.H., and Parker, P.J. (1998). The stress-activated phosphatidylinositol 3-phosphate 5-kinase Fab1p is essential for vacuole function in S. cerevisiae. Curr Biol *8*, 1219-1222.

Cullen, P.J., Cozier, G.E., Banting, G., and Mellor, H. (2001). Modular phosphoinositide-binding domains--their role in signalling and membrane trafficking. Curr Biol *11*, R882-893.

Currie, R.A., Walker, K.S., Gray, A., Deak, M., Casamayor, A., Downes, C.P., Cohen, P., Alessi, D.R., and Lucocq, J. (1999). Role of phosphatidylinositol 3,4,5-trisphosphate in regulating the activity and localization of 3-phosphoinositide-dependent protein kinase-1. Biochem J *337 (Pt 3)*, 575-583.

Czech, M.P. (2003). Dynamics of phosphoinositides in membrane retrieval and insertion. Annu Rev Physiol *65*, 791-815.

Damen, J.E., Liu, L., Rosten, P., Humphries, R.K., Jefferson, A.B., Majerus, P.W., and Krystal, G. (1996). The 145-kDa protein induced to associate with Shc by multiple cytokines is an inositol tetraphosphate and phosphatidylinositol 3,4,5-triphosphate 5-phosphatase. Proc Natl Acad Sci U S A *93*, 1689-1693.

Danilchik, M.V., Bedrick, S.D., Brown, E.E., and Ray, K. (2003). Furrow microtubules and localized exocytosis in cleaving Xenopus laevis embryos. J Cell Sci *116*, 273-283.

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

De Camilli, P. (1995). The eighth Datta Lecture. Molecular mechanisms in synaptic vesicle recycling. FEBS Lett *369*, 3-12.

Di Paolo, G., Moskowitz, H.S., Gipson, K., Wenk, M.R., Voronov, S., Obayashi, M., Flavell, R., Fitzsimonds, R.M., Ryan, T.A., and De Camilli, P. (2004). Impaired

PtdIns(4,5)P2 synthesis in nerve terminals produces defects in synaptic vesicle trafficking. Nature *431*, 415-422.

Ding, J., Vlahos, C.J., Liu, R., Brown, R.F., and Badwey, J.A. (1995). Antagonists of phosphatidylinositol 3-kinase block activation of several novel protein kinases in neutrophils. J Biol Chem *270*, 11684-11691.

DiNitto, J.P., Cronin, T.C., and Lambright, D.G. (2003). Membrane recognition and targeting by lipid-binding domains. Sci STKE *2003*, re16.

Domin, J., Pages, F., Volinia, S., Rittenhouse, S.E., Zvelebil, M.J., Stein, R.C., and Waterfield, M.D. (1997). Cloning of a human phosphoinositide 3-kinase with a C2 domain that displays reduced sensitivity to the inhibitor wortmannin. Biochem J *326*, 139-147.

Dove, S.K., Cooke, F.T., Douglas, M.R., Sayers, L.G., Parker, P.J., and Michell, R.H. (1997). Osmotic stress activates phosphatidylinositol-3,5-bisphosphate synthesis. Nature *390*, 187-192.

Dove, S.K., Piper, R.C., McEwen, R.K., Yu, J.W., King, M.C., Hughes, D.C., Thuring, J., Holmes, A.B., Cooke, F.T., Michell, R.H., Parker, P.J., and Lemmon, M.A. (2004). Svp1p defines a family of phosphatidylinositol 3,5-bisphosphate effectors. EMBO J *23*, 1922-1933.

Dowler, S., Currie, R.A., Campbell, D.G., Deak, M., Kular, G., Downes, C.P., and Alessi, D.R. (2000). Identification of pleckstrin-homology-domain-containing proteins with novel phosphoinositide-binding specificities. Biochem J *351*, 19-31.

Dowler, S., Currie, R.A., Downes, C.P., and Alessi, D.R. (1999). DAPP1: a dual adaptor for phosphotyrosine and 3-phosphoinositides. Biochem J *342 (Pt 1)*, 7-12.

Dowler, S., Kular, G., and Alessi, D.R. (2002). Protein lipid overlay assay. Sci STKE *2002*, PL6.

Downing, G.J., Kim, S., Nakanishi, S., Catt, K.J., and Balla, T. (1996). Characterization of a soluble adrenal phosphatidylinositol 4-kinase reveals wortmannin sensitivity of type III phosphatidylinositol kinases. Biochemistry *35*, 3587-3594.

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

Eberhard, D.A., Cooper, C.L., Low, M.G., and Holz, R.W. (1990). Evidence that the inositol phospholipids are necessary for exocytosis. Loss of inositol phospholipids

and inhibition of secretion in permeabilized cells caused by a bacterial phospholipase C and removal of ATP. Biochem J *268*, 15-25.

Eberhard, D.A., and Holz, R.W. (1987). Cholinergic stimulation of inositol phosphate formation in bovine adrenal chromaffin cells: distinct nicotinic and muscarinic mechanisms. J Neurochem *49*, 1634-1643.

Eisenhaber, B., Maurer-Stroh, S., Novatchkova, M., Schneider, G., and Eisenhaber, F. (2003). Enzymes and auxiliary factors for GPI lipid anchor biosynthesis and post-translational transfer to proteins. Bioessays *25*, 367-385.

El-Kholy, W., Macdonald, P.E., Lin, J.H., Wang, J., Fox, J.M., Light, P.E., Wang, Q., Tsushima, R.G., and Wheeler, M.B. (2003). The phosphatidylinositol 3-kinase inhibitor LY294002 potently blocks K(V) currents via a direct mechanism. Faseb J *17*, 720-722.

Ellena, J.F., Moulthrop, J., Wu, J., Rauch, M., Jaysinghne, S., Castle, J.D., and Cafiso, D.S. (2004). Membrane position of a basic aromatic peptide that sequesters phosphatidylinositol 4,5 bisphosphate determined by site-directed spin labeling and high-resolution NMR. Biophys J *87*, 3221-3233.

Endemann, G.C., Graziani, A., and Cantley, L.C. (1991). A monoclonal antibody distinguishes two types of phosphatidylinositol 4-kinase. Biochem J *273(Pt 1)*, 63-66.

Engisch, K.L., and Nowycky, M.C. (1998). Compensatory and excess retrieval: two types of endocytosis following single step depolarizations in bovine adrenal chromaffin cells. J Physiol *506 (Pt 3)*, 591-608.

Falasca, M., Logan, S.K., Lehto, V.P., Baccante, G., Lemmon, M.A., and Schlessinger, J. (1998). Activation of phospholipase C gamma by PI 3-kinase-induced PH domain-mediated membrane targeting. EMBO J *17*, 414-422.

Fernandez, J.M., Neher, E., and Gomperts, B.D. (1984). Capacitance measurements reveal stepwise fusion events in degranulating mast cells. Nature *312*, 453-455.

Fernandez-Alfonso, T., and Ryan, T.A. (2004). The kinetics of synaptic vesicle pool depletion at CNS synaptic terminals. Neuron *41*, 943-953.

Fernandez-Chacon, R., Alvarez de Toledo, G., Hammer, R.E., and Sudhof, T.C. (1999). Analysis of SCAMP1 function in secretory vesicle exocytosis by means of gene targeting in mice. J Biol Chem *274*, 32551-32554.

Ferry, X., Brehin, S., Kamel, R., and Landry, Y. (2002). G protein-dependent activation of mast cell by peptides and basic secretagogues. Peptides *23*, 1507-1515.

Ferry, X., Eichwald, V., Daeffler, L., and Landry, Y. (2001). Activation of betagamma Subunits of G(i2) and G(i3) Proteins by Basic Secretagogues Induces Exocytosis Through Phospholipase Cbeta and Arachidonate Release Through Phospholipase Cgamma in Mast Cells. J Immunol *167*, 4805-4813.

Fix, M., Melia, T.J., Jaiswal, J.K., Rappoport, J.Z., You, D., Sollner, T.H., Rothman, J.E., and Simon, S.M. (2004). Imaging single membrane fusion events mediated by SNARE proteins. Proc Natl Acad Sci U S A *101*, 7311-7316.

Flesch, F.M., Yu, J.W., Lemmon, M.A., and Burger, K.N. (2005). Membrane activity of the phospholipase C-delta1 pleckstrin homology (PH) domain. Biochem J *389*, 435-441.

Folch, J., Lees, M., and Sloane Stanley, G.H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem *226*, 497-509.

Ford, M.G., Mills, I.G., Peter, B.J., Vallis, Y., Praefcke, G.J., Evans, P.R., and McMahon, H.T. (2002). Curvature of clathrin-coated pits driven by epsin. Nature *419*, 361-366.

Ford, M.G., Pearse, B.M., Higgins, M.K., Vallis, Y., Owen, D.J., Gibson, A., Hopkins, C.R., Evans, P.R., and McMahon, H.T. (2001). Simultaneous binding of PtdIns(4,5)P2 and clathrin by AP180 in the nucleation of clathrin lattices on membranes. Science *291*, 1051-1055.

Friant, S., Pecheur, E.I., Eugster, A., Michel, F., Lefkir, Y., Nourrisson, D., and Letourneur, F. (2003). Ent3p Is a PtdIns(3,5)P2 effector required for protein sorting to the multivesicular body. Dev Cell *5*, 499-511.

Fruman, D.A., Meyers, R.E., and Cantley, L.C. (1998). Phosphoinositide kinases. Annu Rev Biochem *67*, 481-507.

Fujita-Yoshigaki, J. (1998). Divergence and convergence in regulated exocytosis: the characteristics of cAMP-dependent enzyme secretion of parotid salivary acinar cells. Cell Signal *10*, 371-375.

Fujita_Yoshigaki, J., Dohke, Y., Hara_Yokoyama, M., Kamata, Y., Kozaki, S., Furuyama, S., and Sugiya, H. (1996). Vesicle-associated membrane protein 2 is essential for cAMP-regulated exocytosis in rat parotid acinar cells. The inhibition

of cAMP-dependent amylase release by botulinum neurotoxin B. J Biol Chem *271*, 13130-13134.

Fukami, K., Furuhashi, K., Inagaki, M., Endo, T., Hatano, S., and Takenawa, T. (1992). Requirement of phosphatidylinositol 4,5-bisphosphate for alpha-actinin function. Nature *359*, 150-152.

Fukami, K., Matsuoka, K., Nakanishi, O., Yamakawa, A., Kawai, S., and Takenawa, T. (1988). Antibody to phosphatidylinositol 4,5-bisphosphate inhibits oncogene-induced mitogenesis. Proc Natl Acad Sci U S A *85*, 9057-9061.

Fukami, K., Sawada, N., Endo, T., and Takenawa, T. (1996). Identification of a phosphatidylinositol 4,5-bisphosphate-binding site in chicken skeletal muscle alpha-actinin. J Biol Chem *271*, 2646-2650.

Furuno, T., and Nakanishi, M. (2005). Live cell imaging to study signaling molecules in allergic reactions. Biol Pharm Bull *28*, 1551-1559.

Fushman, D., Najmabadi-Haske, T., Cahill, S., Zheng, J., LeVine, H., 3rd, and Cowburn, D. (1998). The solution structure and dynamics of the pleckstrin homology domain of G protein-coupled receptor kinase 2 (beta-adrenergic receptor kinase 1). A binding partner of Gbetagamma subunits. J Biol Chem *273*, 2835-2843.

Gabev, E., Kasianowicz, J., Abbott, T., and McLaughlin, S. (1989). Binding of neomycin to phosphatidylinositol 4,5-bisphosphate (PIP2). Biochim Biophys Acta *979*, 105-112.

Galli, T., and Haucke, V. (2004). Cycling of synaptic vesicles: how far? How fast! Sci STKE *2004*, re19.

Gambhir, A., Hangyas-Mihalyne, G., Zaitseva, I., Cafiso, D.S., Wang, J., Murray, D., Pentyala, S.N., Smith, S.O., and McLaughlin, S. (2004). Electrostatic sequestration of PIP2 on phospholipid membranes by basic/aromatic regions of proteins. Biophys J *86*, 2188-2207.

Gerhard, R., John, H., Aktories, K., and Just, I. (2003). Thiol-modifying phenylarsine oxide inhibits guanine nucleotide binding of Rho but not of Rac GTPases. Mol Pharmacol *63*, 1349-1355.

Ghalayini, A., and Eichberg, J. (1985). Purification of phosphatidylinositol synthetase from rat brain by CDP-diacylglycerol affinity chromatography and properties of the purified enzyme. J Neurochem *44*, 175-182.

Gillooly, D.J., Morrow, I.C., Lindsay, M., Gould, R., Bryant, N.J., Gaullier, J.M., Parton, R.G., and Stenmark, H. (2000). Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells. EMBO J *19*, 4577-4588.

Gillooly, D.J., Raiborg, C., and Stenmark, H. (2003). Phosphatidylinositol 3-phosphate is found in microdomains of early endosomes. Histochem Cell Biol *120*, 445-453.

Gloyna, W., Schmitz, F., and Seebeck, J. (2005). Inhibition of phospholipase C-independent exocytotic responses in rat peritoneal mast cells by U73122. Regul Pept *125*, 179-184.

Godi, A., Di Campli, A., Konstantakopoulos, A., Di Tullio, G., Alessi, D.R., Kular, G.S., Daniele, T., Marra, P., Lucocq, J.M., and De Matteis, M.A. (2004). FAPPs control Golgi-to-cell-surface membrane traffic by binding to ARF and PtdIns(4)P. Nat Cell Biol *6*, 393-404.

Goishi, K., Mizuno, K., Nakanishi, H., and Sasaki, T. (2004). Involvement of Rab27 in antigen-induced histamine release from rat basophilic leukemia 2H3 cells. Biochem Biophys Res Commun *324*, 294-301.

Gomperts, B.D. (1983). Involvement of guanine nucleotide-binding protein in the gating of Ca2+ by receptors. Nature *306*, 64-66.

Gomperts, B.D., Baldwin, J.M., and Micklem, K.J. (1983). Rat mast cells permeabilized with Sendai virus secrete histamine in response to Ca2+ buffered in the micromolar range. Biochem J *210*, 737-745.

Gomperts, B.D., and Tatham, P.E. (1992). Regulated exocytotic secretion from permeabilized cells. Methods Enzymol *219*, 178-189.

Gong, L.W., Di Paolo, G., Diaz, E., Cestra, G., Diaz, M.E., Lindau, M., De Camilli, P., and Toomre, D. (2005). Phosphatidylinositol phosphate kinase type I gamma regulates dynamics of large dense-core vesicle fusion. Proc Natl Acad Sci U S A *102*, 5204-5209.

Gozani, O., Karuman, P., Jones, D.R., Ivanov, D., Cha, J., Lugovskoy, A.A., Baird, C.L., Zhu, H., Field, S.J., Lessnick, S.L., Villasenor, J., Mehrotra, B., Chen, J., Rao, V.R., Brugge, J.S., Ferguson, C.G., Payrastre, B., Myszka, D.G., Cantley, L.C., Wagner, G., Divecha, N., Prestwich, G.D., and Yuan, J. (2003). The PHD finger of the chromatin-associated protein ING2 functions as a nuclear phosphoinositide receptor. Cell *114*, 99-111.

Gray, A., Van Der Kaay, J., and Downes, C.P. (1999). The pleckstrin homology domains of protein kinase B and GRP1 (general receptor for phosphoinositides-1) are sensitive and selective probes for the cellular detection of phosphatidylinositol 3,4-bisphosphate and/or phosphatidylinositol 3,4,5-trisphosphate in vivo. Biochem J *344 Pt 3*, 929-936.

Grishanin, R.N., Klenchin, V.A., Loyet, K.M., Kowalchyk, J.A., Ann, K., and Martin, T.F. (2002). Membrane association domains in Ca2+-dependent activator protein for secretion mediate plasma membrane and dense-core vesicle binding required for Ca2+-dependent exocytosis. J Biol Chem *277*, 22025-22034.

Grishanin, R.N., Kowalchyk, J.A., Klenchin, V.A., Ann, K., Earles, C.A., Chapman, E.R., Gerona, R.R., and Martin, T.F. (2004). CAPS acts at a prefusion step in dense-core vesicle exocytosis as a PIP2 binding protein. Neuron *43*, 551-562.

Gromada, J., Bark, C., Smidt, K., Efanov, A.M., Janson, J., Mandic, S.A., Webb, D.L., Zhang, W., Meister, B., Jeromin, A., and Berggren, P.O. (2005). Neuronal calcium sensor-1 potentiates glucose-dependent exocytosis in pancreatic beta cells through activation of phosphatidylinositol 4-kinase beta. Proc Natl Acad Sci U S A *102*, 10303-10308.

Gundelfinger, E.D., Kessels, M.M., and Qualmann, B. (2003). Temporal and spatial coordination of exocytosis and endocytosis. Nat Rev Mol Cell Biol *4*, 127-139.

Guo, J., Wenk, M.R., Pellegrini, L., Onofri, F., Benfenati, F., and De Camilli, P. (2003). Phosphatidylinositol 4-kinase type IIalpha is responsible for the phosphatidylinositol 4-kinase activity associated with synaptic vesicles. Proc Natl Acad Sci U S A *100*, 3995-4000.

Guo, S., Stolz, L.E., Lemrow, S.M., and York, J.D. (1999). SAC1-like domains of yeast SAC1, INP52, and INP53 and of human synaptojanin encode polyphosphoinositide phosphatases. J Biol Chem *274*, 12990-12995.

Guo, Z., Liu, L., Cafiso, D., and Castle, D. (2002). Perturbation of a very late step of regulated exocytosis by a secretory carrier membrane protein (SCAMP2)-derived peptide. J Biol Chem *277*, 35357-35363.

Guo, Z., Turner, C., and Castle, D. (1998). Relocation of the t-SNARE SNAP-23 from lamellipodia-like cell surface projections regulates compound exocytosis in mast cells. Cell *94*, 537-548.

Hafez, I., Stolpe, A., and Lindau, M. (2003). Compound exocytosis and cumulative fusion in eosinophils. J Biol Chem *278*, 44921-44928.

Halet, G. (2005). Imaging phosphoinositide dynamics using GFP-tagged protein domains. Biol Cell *97*, 501-518.

Halstead, J.R., Roefs, M., Ellson, C.D., D'Andrea, S., Chen, C., D'Santos, C.S., and Divecha, N. (2001). A novel pathway of cellular phosphatidylinositol(3,4,5)-trisphosphate synthesis is regulated by oxidative stress. Curr Biol *11*, 386-395.

Hama, H., Schnieders, E.A., Thorner, J., Takemoto, J.Y., and DeWald, D.B. (1999). Direct involvement of phosphatidylinositol 4-phosphate in secretion in the yeast Saccharomyces cerevisiae. J Biol Chem *274*, 34294-34300.

Hamada, K., Shimizu, T., Matsui, T., Tsukita, S., and Hakoshima, T. (2000). Structural basis of the membrane-targeting and unmasking mechanisms of the radixin FERM domain. EMBO J *19*, 4449-4462.

Hammond, G., Thomas, C.L., and Schiavo, G. (2004). Nuclear phosphoinositides and their functions. Curr Top Microbiol Immunol *282*, 177-206.

Harlan, J.E., Hajduk, P.J., Yoon, H.S., and Fesik, S.W. (1994). Pleckstrin homology domains bind to phosphatidylinositol-4,5-bisphosphate. Nature *371*, 168-170.

Hartmann, J., Scepek, S., Hafez, I., and Lindau, M. (2003). Differential regulation of exocytotic fusion and granule-granule fusion in eosinophils by Ca2+ and GTP analogs. J Biol Chem *278*, 44929-44934.

Harwood, J.L., and Hawthorne, J.N. (1969). Metabolism of the phosphoinositides in guinea-pig brain synaptosomes. J Neurochem *16*, 1377-1387.

Hay, J.C., Fisette, P.L., Jenkins, G.H., Fukami, K., Takenawa, T., Anderson, R.A., and Martin, T.F. (1995). ATP-dependent inositide phosphorylation required for Ca(2+)-activated secretion. Nature *374*, 173-177.

Hay, J.C., and Martin, T.F. (1992). Resolution of regulated secretion into sequential MgATP-dependent and calcium-dependent stages mediated by distinct cytosolic proteins. J Cell Biol *119*, 139-151.

Hay, J.C., and Martin, T.F. (1993). Phosphatidylinositol transfer protein required for ATP-dependent priming of Ca(2+)-activated secretion. Nature *366*, 572-575.

Hibi, T., Hirashima, N., and Nakanishi, M. (2000). Rat basophilic leukemia cells express syntaxin-3 and VAMP-7 in granule membranes. Biochem Biophys Res Commun *271*, 36-41.

Hide, I., Bennett, J.P., Pizzey, A., Boonen, G., Bar_Sagi, D., Gomperts, B.D., and Tatham, P.E. (1993). Degranulation of individual mast cells in response to Ca2+ and guanine nucleotides: an all-or-none event. J Cell Biol *123*, 585-593.

Hirose, K., Kadowaki, S., Tanabe, M., Takeshima, H., and Iino, M. (1999). Spatiotemporal dynamics of inositol 1,4,5-trisphosphate that underlies complex Ca2+ mobilization patterns. Science *284*, 1527-1530.

Hokin, L.E., and Hokin, M.R. (1955). Effects of acetylcholine on the turnover of phosphoryl units in individual phospholipids of pancreas slices and brain cortex slices. Biochim Biophys Acta *18*, 102-110.

Hokin, L.E., and Hokin, M.R. (1958). Phosphoinositides and protein secretion in pancreas slices. J Biol Chem *233*, 805-810.

Hokin, M.R., and Hokin, L.E. (1953). Enzyme secretion and the incorporation of P32 into phospholipides of pancreas slices. J Biol Chem *203*, 967-977.

Holz, R.W., Bittner, M.A., Peppers, S.C., Senter, R.A., and Eberhard, D.A. (1989). MgATP-independent and MgATP-dependent exocytosis. Evidence that MgATP primes adrenal chromaffin cells to undergo exocytosis. J Biol Chem *264*, 5412-5419.

Holz, R.W., Hlubek, M.D., Sorensen, S.D., Fisher, S.K., Balla, T., Ozaki, S., Prestwich, G.D., Stuenkel, E.L., and Bittner, M.A. (2000). A pleckstrin homology domain specific for phosphatidylinositol 4, 5-bisphosphate (PtdIns-4,5-P2) and fused to green fluorescent protein identifies plasma membrane PtdIns-4,5-P2 as being important in exocytosis. J Biol Chem *275*, 17878-17885.

Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A.J., Frohman, M.A., and Kanaho, Y. (1999). Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. Cell *99*, 521-532.

Howell, T.W., Cockcroft, S., and Gomperts, B.D. (1987). Essential synergy between Ca2+ and guanine nucleotides in exocytotic secretion from permeabilized rat mast cells. J Cell Biol *105*, 191-197.

Howell, T.W., and Gomperts, B.D. (1987). Rat mast cells permeabilised with streptolysin O secrete histamine in response to Ca2+ at concentrations buffered in the micromolar range. Biochim Biophys Acta *927*, 177-183.

Howell, T.W., Kramer, I.M., and Gomperts, B.D. (1989). Protein phosphorylation and the dependence on Ca2+ and GTP-gamma-S for exocytosis from permeabilised mast cells. Cell Signal *1*, 157-163.

Hu, C., Ahmed, M., Melia, T.J., Sollner, T.H., Mayer, T., and Rothman, J.E. (2003). Fusion of cells by flipped SNAREs. Science *300*, 1745-1749.

Hu, K., Carroll, J., Rickman, C., and Davletov, B. (2002). Action of complexin on SNARE complex. J Biol Chem *277*, 41652-41656.

Hughes, W.E., Woscholski, R., Cooke, F.T., Patrick, R.S., Dove, S.K., McDonald, N.Q., and Parker, P.J. (2000). SAC1 encodes a regulated lipid phosphoinositide phosphatase, defects in which can be suppressed by the homologous Inp52p and Inp53p phosphatases. J Biol Chem *275*, 801-808.

Irvine, R.F. (2002). Nuclear lipid signaling. Sci STKE *2002*, RE13.

Irvine, R.F., and Schell, M.J. (2001). Back in the water: the return of the inositol phosphates. Nat Rev Mol Cell Biol *2*, 327-338.

Itoh, T., Koshiba, S., Kigawa, T., Kikuchi, A., Yokoyama, S., and Takenawa, T. (2001). Role of the ENTH domain in phosphatidylinositol-4,5-bisphosphate binding and endocytosis. Science *291*, 1047-1051.

IUBMB. (1989). Numbering of atoms in myo-inositol. Recommendations 1988. Nomenclature Committee of the International Union of Biochemistry. Biochem J *258*, 1-2.

Jackson, T.R., Stephens, L.R., and Hawkins, P.T. (1992). Receptor specificity of growth factor-stimulated synthesis of 3-phosphorylated inositol lipids in Swiss 3T3 cells. J Biol Chem *267*, 16627-16636.

Jahn, R., Lang, T., and Sudhof, T.C. (2003). Membrane fusion. Cell *112*, 519-533.

Janmey, P.A., and Lindberg, U. (2004). Cytoskeletal regulation: rich in lipids. Nat Rev Mol Cell Biol *5*, 658-666.

Jefferson, A.B., and Majerus, P.W. (1995). Properties of type II inositol polyphosphate 5-phosphatase. J Biol Chem *270*, 9370-9377.

Jeffries, T.R., Dove, S.K., Michell, R.H., and Parker, P.J. (2004). PtdIns-specific MPR pathway association of a novel WD40 repeat protein, WIPI49. Mol Biol Cell *15*, 2652-2663.

Ju, S., Shaltiel, G., Shamir, A., Agam, G., and Greenberg, M.L. (2004). Human 1-D-myo-inositol-3-phosphate synthase is functional in yeast. J Biol Chem *279*, 21759-21765.

Kagayama, M., and Douglas, W.W. (1974). Electron microscope evidence of calcium-induced exocytosis in mast cells treated with 48-80 or the ionophores A-23187 and X-537A. J Cell Biol *62*, 519-526.

Kanai, F., Liu, H., Field, S.J., Akbary, H., Matsuo, T., Brown, G.E., Cantley, L.C., and Yaffe, M.B. (2001). The PX domains of p47phox and p40phox bind to lipid products of PI(3)K. *3*, 675-678.

Kapp-Barnea, Y., Melnikov, S., Shefler, I., Jeromin, A., and Sagi-Eisenberg, R. (2003). Neuronal calcium sensor-1 and phosphatidylinositol 4-kinase beta regulate IgE receptor-triggered exocytosis in cultured mast cells. J Immunol *171*, 5320-5327.

Kavran, J.M., Klein, D.E., Lee, A., Falasca, M., Isakoff, S.J., Skolnik, E.Y., and Lemmon, M.A. (1998). Specificity and promiscuity in phosphoinositide binding by pleckstrin homology domains. J Biol Chem *273*, 30497-30508.

Keller, P., and Simons, K. (1997). Post-Golgi biosynthetic trafficking. J Cell Sci *110 (Pt 24)*, 3001-3009.

Kent, C., Carman, G.M., Spence, M.W., and Dowhan, W. (1991). Regulation of eukaryotic phospholipid metabolism. Faseb J *5*, 2258-2266.

Kessler, S., and Kuhn, C. (1975). Scanning electron microscopy of mast cell degranulation. Lab Invest *32*, 71-77.

Kim, W.T., Chang, S., Daniell, L., Cremona, O., Di Paolo, G., and De Camilli, P. (2002). Delayed reentry of recycling vesicles into the fusion-competent synaptic vesicle pool in synaptojanin 1 knockout mice. Proc Natl Acad Sci U S A *99*, 17143-17148.

Kinet, J.P. (1999). The high-affinity IgE receptor (Fc epsilon RI): from physiology to pathology. Annu Rev Immunol *17*, 931-972.

Klarlund, J.K., Tsiaras, W., Holik, J.J., Chawla, A., and Czech, M.P. (2000). Distinct polyphosphoinositide binding selectivities for pleckstrin homology domains of GRP1-like proteins based on diglycine versus triglycine motifs. J Biol Chem *275*, 32816-32821.

Koffer, A. (1993). Calcium-induced secretion from permeabilized rat mast cells: requirements for guanine nucleotides. Biochim Biophys Acta *1176*, 231-239.

Koffer, A., and Churcher, Y. (1993). Calcium and GTP-gamma-S as single effectors of secretion from permeabilized rat mast cells: requirements for ATP. Biochim Biophys Acta *1176*, 222-230.

Koffer, A., and Gomperts, B.D. (1989). Soluble proteins as modulators of the exocytotic reaction of permeabilised rat mast cells. J Cell Sci *94*, 585-591.

Koffer, A., Williams, M., and Johansen, T. (2002). Vacuole formation in mast cells responding to osmotic stress and to F-actin disassembly. Cell Biol Int *26*, 885-892.

Kojima, T., Fukuda, M., Watanabe, Y., Hamazato, F., and Mikoshiba, K. (1997). Characterization of the pleckstrin homology domain of Btk as an inositol polyphosphate and phosphoinositide binding domain. Biochem Biophys Res Commun *236*, 333-339.

Krane, C.M., Fortner, C.N., Hand, A.R., McGraw, D.W., Lorenz, J.N., Wert, S.E., Towne, J.E., Paul, R.J., Whitsett, J.A., and Menon, A.G. (2001). Aquaporin 5-deficient mouse lungs are hyperresponsive to cholinergic stimulation. Proc Natl Acad Sci U S A *98*, 14114-14119.

Kurosawa, M., and Parker, C.W. (1986). A phosphatidylinositol kinase in rat mast cell granules. J Immunol *136*, 616-622.

Kweon, D.H., Kim, C.S., and Shin, Y.K. (2003). Regulation of neuronal SNARE assembly by the membrane. Nat Struct Biol *10*, 440-447.

Lackner, M.R., Nurrish, S.J., and Kaplan, J.M. (1999). Facilitation of synaptic transmission by EGL-30 Gqalpha and EGL-8 PLCbeta: DAG binding to UNC-13 is required to stimulate acetylcholine release. Neuron *24*, 335-346.

Laffargue, M., Calvez, R., Finan, P., Trifilieff, A., Barbier, M., Altruda, F., Hirsch, E., and Wymann, M.P. (2002). Phosphoinositide 3-kinase gamma is an essential amplifier of mast cell function. Immunity *16*, 441-451.

Lalli, G., Gschmeissner, S., and Schiavo, G. (2003). Myosin Va and microtubule-based motors are required for fast axonal retrograde transport of tetanus toxin in motor neurons. J Cell Sci *116*, 4639-4650.

Lamers, J.M., Dekkers, D.H., Mesaeli, N., Panagia, V., and van Heugten, H.A. (1993). Myocardial phosphoinositides do not share the same fatty acid profile. Biochem Biophys Res Commun *191*, 487-494.

Laux, T., Fukami, K., Thelen, M., Golub, T., Frey, D., and Caroni, P. (2000). GAP43, MARCKS, and CAP23 modulate PI(4,5)P(2) at plasmalemmal rafts, and regulate cell cortex actin dynamics through a common mechanism. J Cell Biol *149*, 1455-1472.

Lawrence, J.T., and Birnbaum, M.J. (2003). ADP-ribosylation factor 6 regulates insulin secretion through plasma membrane phosphatidylinositol 4,5-bisphosphate. Proc Natl Acad Sci U S A *100*, 13320-13325.

Lawson, D., Fewtrell, C., Gomperts, B., and Raff, M. (1975). Anti-immunoglobulin-induced histamine secretion by rat peritoneal mast cells studied by immunoferritin electron microscopy. J Exp Med *142*, 391-402.

Lawson, D., Fewtrell, C., and Raff, M.C. (1978). Localized mast cell degranulation induced by concanavalin A-sepharose beads. Implications for the Ca2+ hypothesis of stimulus-secretion coupling. J Cell Biol *79*, 394-400.

Lawson, D., Raff, M.C., Gomperts, B., Fewtrell, C., and Gilula, N.B. (1977). Molecular events during membrane fusion. A study of exocytosis in rat peritoneal mast cells. J Cell Biol *72*, 242-259.

Lemmon, M.A. (2003). Phosphoinositide recognition domains. Traffic *4*, 201-213.

Lemmon, M.A., and Ferguson, K.M. (2000). Signal-dependent membrane targeting by pleckstrin homology (PH) domains. Biochem J *350 Pt 1*, 1-18.

Lemmon, M.A., Ferguson, K.M., O'Brien, R., Sigler, P.B., and Schlessinger, J. (1995). Specific and high-affinity binding of inositol phosphates to an isolated pleckstrin homology domain. Proc Natl Acad Sci U S A *92*, 10472-10476.

Lillie, T.H., and Gomperts, B.D. (1992). Guanine nucleotide is essential and Ca2+ is a modulator in the exocytotic reaction of permeabilized rat mast cells. Biochem J *288 (Pt 1)*, 181-187.

Lippincott-Schwartz, J., Roberts, T.H., and Hirschberg, K. (2000). Secretory protein trafficking and organelle dynamics in living cells. Annu Rev Cell Dev Biol *16*, 557-589.

Liscovitch, M., Chalifa, V., Danin, M., and Eli, Y. (1991). Inhibition of neural phospholipase D activity by aminoglycoside antibiotics. Biochem J *279 (Pt 1)*, 319-321.

Liu, L., Guo, Z., Tieu, Q., Castle, A., and Castle, D. (2002). Role of Secretory Carrier Membrane Protein SCAMP2 in Granule Exocytosis. Mol Biol Cell *13*, 4266-4278.

Lowe, M. (2005). Structure and Function of the Lowe Syndrome Protein OCRL1. Traffic *6*, 711-719.

Loyet, K.M., Kowalchyk, J.A., Chaudhary, A., Chen, J., Prestwich, G.D., and Martin, T.F. (1998). Specific binding of phosphatidylinositol 4,5-bisphosphate to calcium-dependent activator protein for secretion (CAPS), a potential phosphoinositide effector protein for regulated exocytosis. J Biol Chem *273*, 8337-8343.

Lu, X., Zhang, F., McNew, J.A., and Shin, Y.K. (2005). Membrane fusion induced by neuronal SNAREs transits through hemifusion. J Biol Chem *280*, 30538-30541.

Lu, Y.B., Wu, M., and Zhou, H.L. (2004). Changes of phospholipase D activity of rat peritoneal mast cells in degranulation. Acta Pharmacol Sin *25*, 104-109.

Luo, B., Regier, D.S., Prescott, S.M., and Topham, M.K. (2004). Diacylglycerol kinases. Cell Signal *16*, 983-989.

Maeda, T., and Eisenberg, F., Jr. (1980). Purification, structure, and catalytic properties of L-myo-inositol-1-phosphate synthase from rat testis. J Biol Chem *255*, 8458-8464.

Maehama, T., and Dixon, J.E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem *273*, 13375-13378.

Maffucci, T., Brancaccio, A., Piccolo, E., Stein, R.C., and Falasca, M. (2003). Insulin induces phosphatidylinositol-3-phosphate formation through TC10 activation. EMBO J *22*, 4178-4189.

Maffucci, T., Cooke, F.T., Foster, F.M., Traer, C.J., Fry, M.J., and Falasca, M. (2005). Class II phosphoinositide 3-kinase defines a novel signaling pathway in cell migration. J Cell Biol *169*, 789-799.

Mahal, L.K., Sequeira, S.M., Gureasko, J.M., and Sollner, T.H. (2002). Calcium-independent stimulation of membrane fusion and SNAREpin formation by synaptotagmin I. J Cell Biol *158*, 273-282.

Marche, P., Koutouzov, S., and Girard, A. (1983). Impairment of membrane phosphoinositide metabolism by aminoglycoside antibiotics: streptomycin,

amikacin, kanamycin, dibekacin, gentamicin and neomycin. J Pharmacol Exp Ther *227*, 415-420.

Marche, P., Olier, B., Girard, A., Fillastre, J.P., and Morin, J.P. (1987). Aminoglycoside-induced alterations of phosphoinositide metabolism. Kidney Int *31*, 59-64.

Marcus, S.L., Wenk, M.R., Steele-Mortimer, O., and Finlay, B.B. (2001). A synaptojanin-homologous region of Salmonella typhimurium SigD is essential for inositol phosphatase activity and Akt activation. FEBS Lett *494*, 201-207.

Marone, G., Triggiani, M., and de Paulis, A. (2005). Mast cells and basophils: friends as well as foes in bronchial asthma? Trends Immunol *26*, 25-31.

Marquardt, D.L., Alongi, J.L., and Walker, L.L. (1996). The phosphatidylinositol 3-kinase inhibitor wortmannin blocks mast cell exocytosis but not IL-6 production. J Immunol *156*, 1942-1945.

Marshall, A.J., Krahn, A.K., Ma, K., Duronio, V., and Hou, S. (2002). TAPP1 and TAPP2 are targets of phosphatidylinositol 3-kinase signaling in B cells: sustained plasma membrane recruitment triggered by the B-cell antigen receptor. Mol Cell Biol *22*, 5479-5491.

Martin, T.F. (1997). Phosphoinositides as spatial regulators of membrane traffic. Curr Opin Neurobiol *7*, 331-338.

Martin, T.F. (2001). PI(4,5)P(2) regulation of surface membrane traffic. Curr Opin Cell Biol *13*, 493-499.

Martin-Verdeaux, S., Pombo, I., Iannascoli, B., Roa, M., Varin-Blank, N., Rivera, J., and Blank, U. (2003). Evidence of a role for Munc18-2 and microtubules in mast cell granule exocytosis. J Cell Sci *116*, 325-334.

Masuda, E.S., Luo, Y., Young, C., Shen, M., Rossi, A.B., Huang, B.C., Yu, S., Bennett, M.K., Payan, D.G., and Scheller, R.H. (2000). Rab37 is a novel mast cell specific GTPase localized to secretory granules. FEBS Lett *470*, 61-64.

Matsuda, M., Paterson, H.F., Rodriguez, R., Fensome, A.C., Ellis, M.V., Swann, K., and Katan, M. (2001). Real time fluorescence imaging of PLC gamma translocation and its interaction with the epidermal growth factor receptor. J Cell Biol *153*, 599-612.

Matuoka, K., Fukami, K., Nakanishi, O., Kawai, S., and Takenawa, T. (1988). Mitogenesis in response to PDGF and bombesin abolished by microinjection of antibody to PIP2. Science *239*, 640-643.

Mayer, A. (2001). What drives membrane fusion in eukaryotes? Trends Biochem Sci *26*, 717-723.

McBride, H.M., Rybin, V., Murphy, C., Giner, A., Teasdale, R., and Zerial, M. (1999). Oligomeric complexes link Rab5 effectors with NSF and drive membrane fusion via interactions between EEA1 and syntaxin 13. Cell *98*, 377-386.

McEwen, R.K., Dove, S.K., Cooke, F.T., Painter, G.F., Holmes, A.B., Shisheva, A., Ohya, Y., Parker, P.J., and Michell, R.H. (1999). Complementation analysis in PtdInsP kinase-deficient yeast mutants demonstrates that Schizosaccharomyces pombe and murine Fab1p homologues are phosphatidylinositol 3-phosphate 5-kinases. J Biol Chem *274*, 33905-33912.

McLaughlin, S., Wang, J., Gambhir, A., and Murray, D. (2002). PIP(2) and proteins: interactions, organization, and information flow. Annu Rev Biophys Biomol Struct *31*, 151-175.

McNew, J.A., Parlati, F., Fukuda, R., Johnston, R.J., Paz, K., Paumet, F., Sollner, T.H., and Rothman, J.E. (2000a). Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. Nature *407*, 153-159.

McNew, J.A., Weber, T., Engelman, D.M., Sollner, T.H., and Rothman, J.E. (1999). The length of the flexible SNAREpin juxtamembrane region is a critical determinant of SNARE-dependent fusion. Mol Cell *4*, 415-421.

McNew, J.A., Weber, T., Parlati, F., Johnston, R.J., Melia, T.J., Sollner, T.H., and Rothman, J.E. (2000b). Close is not enough: SNARE-dependent membrane fusion requires an active mechanism that transduces force to membrane anchors. J Cell Biol *150*, 105-117.

McPherson, P.S., Garcia, E.P., Slepnev, V.I., David, C., Zhang, X., Grabs, D., Sossin, W.S., Bauerfeind, R., Nemoto, Y., and De Camilli, P. (1996). A presynaptic inositol-5-phosphatase. Nature *379*, 353-357.

Mehrotra, B., Myszka, D.G., and Prestwich, G.D. (2000). Binding kinetics and ligand specificity for the interactions of the C2B domain of synaptogmin II with inositol polyphosphates and phosphoinositides. Biochemistry *39*, 9679-9686.

Melendez, A.J., and Khaw, A.K. (2002). Dichotomy of Ca2+ signals triggered by different phospholipid pathways in antigen stimulation of human mast cells. J Biol Chem *277*, 17255-17262.

Metcalfe, D.D., Baram, D., and Mekori, Y.A. (1997). Mast cells. Physiol Rev *77*, 1033-1079.

Meunier, F.A., Osborne, S.L., Hammond, G.R., Cooke, F.T., Parker, P.J., Domin, J., and Schiavo, G. (2005). PI3-Kinase C2{alpha} Is Essential for ATP-dependent Priming of Neurosecretory Granule Exocytosis. Mol Biol Cell.

Meyers, R.E., and Cantley, L.C. (1998). Phosphatidylinositol 4-kinases. Assays and product analysis. Methods Mol Biol *105*, 99-108.

Micheva, K.D., Buchanan, J., Holz, R.W., and Smith, S.J. (2003). Retrograde regulation of synaptic vesicle endocytosis and recycling. Nat Neurosci *6*, 925-932.

Micheva, K.D., Holz, R.W., and Smith, S.J. (2001). Regulation of presynaptic phosphatidylinositol 4,5-biphosphate by neuronal activity. J Cell Biol *154*, 355-368.

Miller, K.G., Emerson, M.D., and Rand, J.B. (1999). Goalpha and diacylglycerol kinase negatively regulate the Gqalpha pathway in C. elegans. Neuron *24*, 323-333.

Milosevic, I., Sorensen, J.B., Lang, T., Krauss, M., Nagy, G., Haucke, V., Jahn, R., and Neher, E. (2005). Plasmalemmal phosphatidylinositol-4,5-bisphosphate level regulates the releasable vesicle pool size in chromaffin cells. J Neurosci *25*, 2557-2565.

Minogue, S., Anderson, J.S., Waugh, M.G., dos Santos, M., Corless, S., Cramer, R., and Hsuan, J.J. (2001). Cloning of a human type II phosphatidylinositol 4-kinase reveals a novel lipid kinase family. J Biol Chem *276*, 16635-16640.

Misra, S., and Hurley, J.H. (1999). Crystal structure of a phosphatidylinositol 3-phosphate-specific membrane-targeting motif, the FYVE domain of Vps27p. Cell *97*, 657-666.

Mitra, P., Zhang, Y., Rameh, L.E., Ivshina, M.P., McCollum, D., Nunnari, J.J., Hendricks, G.M., Kerr, M.L., Field, S.J., Cantley, L.C., and Ross, A.H. (2004). A novel phosphatidylinositol(3,4,5)P3 pathway in fission yeast. J Cell Biol *166*, 205-211.

Mori, S., Saino, T., and Satoh, Y. (2000). Effect of low temperatures on compound 48/80-induced intracellular Ca2+ changes and exocytosis of rat peritoneal mast cells. Arch Histol Cytol *63*, 261-270.

Morris, J.B., Hinchliffe, K.A., Ciruela, A., Letcher, A.J., and Irvine, R.F. (2000). Thrombin stimulation of platelets causes an increase in phosphatidylinositol 5-phosphate revealed by mass assay. FEBS Lett *475*, 57-60.

Mortier, E., Wuytens, G., Leenaerts, I., Hannes, F., Heung, M.Y., Degeest, G., David, G., and Zimmermann, P. (2005). Nuclear speckles and nucleoli targeting by PIP2-PDZ domain interactions. EMBO J *24*, 2556-2565.

Mozdy, A.D., and Shaw, J.M. (2003). A fuzzy mitochondrial fusion apparatus comes into focus. Nat Rev Mol Cell Biol *4*, 468-478.

Nahorski, S.R., Young, K.W., John Challiss, R.A., and Nash, M.S. (2003). Visualizing phosphoinositide signalling in single neurons gets a green light. Trends Neurosci *26*, 444-452.

Naveen, B., Shankar, B.S., and Subrahmanyam, G. (2005). FcvarepsilonRI cross-linking activates a type II phosphatidylinositol 4-kinase in RBL 2H3 cells. Mol Immunol *42*, 1541-1549.

Neale, E.A., Bowers, L.M., Jia, M., Bateman, K.E., and Williamson, L.C. (1999). Botulinum neurotoxin A blocks synaptic vesicle exocytosis but not endocytosis at the nerve terminal. J Cell Biol *147*, 1249-1260.

Neco, P., Gil, A., Del Mar Frances, M., Viniegra, S., and Gutierrez, L.M. (2002). The role of myosin in vesicle transport during bovine chromaffin cell secretion. Biochem J *368*, 405-413.

Neeft, M., Wieffer, M., de Jong, A.S., Negroiu, G., Metz, C.H., van Loon, A., Griffith, J., Krijgsveld, J., Wulffraat, N., Koch, H., Heck, A.J., Brose, N., Kleijmeer, M., and van der Sluijs, P. (2005). Munc13-4 is an effector of rab27a and controls secretion of lysosomes in hematopoietic cells. Mol Biol Cell *16*, 731-741.

Nemeth, A., and Rohlich, P. (1982). Early membrane retrieval following exocytosis in rat mast cells. Eur J Cell Biol *28*, 39-46.

Nemoto, Y., Kearns, B.G., Wenk, M.R., Chen, H., Mori, K., Alb, J.G., Jr., De Camilli, P., and Bankaitis, V.A. (2000). Functional characterization of a mammalian Sac1 and mutants exhibiting substrate-specific defects in phosphoinositide phosphatase activity. J Biol Chem *275*, 34293-34305.

Nishida, K., Yamasaki, S., Ito, Y., Kabu, K., Hattori, K., Tezuka, T., Nishizumi, H., Kitamura, D., Goitsuka, R., Geha, R.S., Yamamoto, T., Yagi, T., and Hirano, T. (2005). Fc{epsilon}RI-mediated mast cell degranulation requires calcium-

independent microtubule-dependent translocation of granules to the plasma membrane. J Cell Biol *170*, 115-126.

Norman, J.C., Price, L.S., Ridley, A.J., Hall, A., and Koffer, A. (1994). Actin filament organization in activated mast cells is regulated by heterotrimeric and small GTP-binding proteins. J Cell Biol *126*, 1005-1015.

Norman, J.C., Price, L.S., Ridley, A.J., and Koffer, A. (1996). The small GTP-binding proteins, Rac and Rho, regulate cytoskeletal organization and exocytosis in mast cells by parallel pathways. Mol Biol Cell *7*, 1429-1442.

Norris, F.A., Atkins, R.C., and Majerus, P.W. (1997). The cDNA cloning and characterization of inositol polyphosphate 4-phosphatase type II. Evidence for conserved alternative splicing in the 4-phosphatase family. J Biol Chem *272*, 23859-23864.

Nurrish, S., Segalat, L., and Kaplan, J.M. (1999). Serotonin inhibition of synaptic transmission: Galpha(0) decreases the abundance of UNC-13 at release sites. Neuron *24*, 231-242.

O'Connell, G.C., Douglas, S.A., and Bunn, S.J. (2003). The involvement of specific phospholipase C isozymes in catecholamine release from digitonin permeabilized bovine adrenal medullary chromaffin cells. Neurosci Lett *342*, 1-4.

O'Sullivan, A.J., Brown, A.M., Freeman, H.N., and Gomperts, B.D. (1996). Purification and identification of FOAD-II, a cytosolic protein that regulates secretion in streptolysin-O permeabilized mast cells, as a rac/rhoGDI complex. Mol Biol Cell *7*, 397-408.

Ochocka, A.M., and Pawelczyk, T. (2003). Isozymes delta of phosphoinositide-specific phospholipase C and their role in signal transduction in the cell. Acta Biochim Pol *50*, 1097-1110.

Ohara-Imaizumi, M., Nakamichi, Y., Tanaka, T., Katsuta, H., Ishida, H., and Nagamatsu, S. (2002). Monitoring of exocytosis and endocytosis of insulin secretory granules in the pancreatic beta-cell line MIN6 using pH-sensitive green fluorescent protein (pHluorin) and confocal laser microscopy. Biochem J *363*, 73-80.

Okamoto, M., and Sudhof, T.C. (1997). Mints, Munc18-interacting proteins in synaptic vesicle exocytosis. J Biol Chem *272*, 31459-31464.

Olsen, H.L., Hoy, M., Zhang, W., Bertorello, A.M., Bokvist, K., Capito, K., Efanov, A.M., Meister, B., Thams, P., Yang, S.N., Rorsman, P., Berggren, P.O., and

Gromada, J. (2003). Phosphatidylinositol 4-kinase serves as a metabolic sensor and regulates priming of secretory granules in pancreatic beta cells. Proc Natl Acad Sci U S A *100*, 5187-5192.

Osborne, S.L., Thomas, C.L., Gschmeissner, S., and Schiavo, G. (2001). Nuclear PtdIns(4,5)P(2) assembles in a mitotically regulated particle involved in pre-mRNA splicing. J Cell Sci *114*, 2501-2511.

Osipchuk, Y., and Cahalan, M. (1992). Cell-to-cell spread of calcium signals mediated by ATP receptors in mast cells. Nature *359*, 241-244.

Pagliarini, D.J., Worby, C.A., and Dixon, J.E. (2004). A PTEN-like phosphatase with a novel substrate specificity. J Biol Chem *279*, 38590-38596.

Palmer, M., Harris, R., Freytag, C., Kehoe, M., Tranum-Jensen, J., and Bhakdi, S. (1998). Assembly mechanism of the oligomeric streptolysin O pore: the early membrane lesion is lined by a free edge of the lipid membrane and is extended gradually during oligomerization. EMBO J *17*, 1598-1605.

Papayannopoulos, V., Co, C., Prehoda, K.E., Snapper, S., Taunton, J., and Lim, W.A. (2005). A polybasic motif allows N-WASP to act as a sensor of PIP(2) density. Mol Cell *17*, 181-191.

Parker, P.J., and Murray-Rust, J. (2004). PKC at a glance. J Cell Sci *117*, 131-132.

Parsons, T.D., Coorssen, J.R., Horstmann, H., and Almers, W. (1995). Docked granules, the exocytic burst, and the need for ATP hydrolysis in endocrine cells. Neuron *15*, 1085-1096.

Paumet, F., Le Mao, J., Martin, S., Galli, T., David, B., Blank, U., and Roa, M. (2000). Soluble NSF attachment protein receptors (SNAREs) in RBL-2H3 mast cells: functional role of syntaxin 4 in exocytosis and identification of a vesicle-associated membrane protein 8-containing secretory compartment. J Immunol *164*, 5850-5857.

Pendaries, C., Tronchere, H., Plantavid, M., and Payrastre, B. (2003). Phosphoinositide signaling disorders in human diseases. FEBS Lett *546*, 25-31.

Pendleton, A., and Koffer, A. (2001). Effects of latrunculin reveal requirements for the actin cytoskeleton during secretion from mast cells. Cell Motil Cytoskeleton *48*, 37-51.

Penner, R. (1988). Multiple signaling pathways control stimulus-secretion coupling in rat peritoneal mast cells. Proc Natl Acad Sci U S A *85*, 9856-9860.

Peters, C., Bayer, M.J., Buhler, S., Andersen, J.S., Mann, M., and Mayer, A. (2001). Trans-complex formation by proteolipid channels in the terminal phase of membrane fusion. Nature *409*, 581-588.

Pickard, M.R., and Hawthorne, J.N. (1978). The labelling of nerve ending phospholipids in guinea-pig brain in vivo and the effect of electrical stimulation on phosphatidylinositol metabolism in prelabelled synaptosomes. J Neurochem *30*, 145-155.

Pinxteren, J.A., Gomperts, B.D., Rogers, D., Phillips, S.E., Tatham, P.E., and Thomas, G.M. (2001). Phosphatidylinositol transfer proteins and protein kinase C make separate but non-interacting contributions to the phosphorylation state necessary for secretory competence in rat mast cells. Biochem J *356*, 287-296.

Pinxteren, J.A., O_Sullivan, A.J., Larbi, K.Y., Tatham, P.E., and Gomperts, B.D. (2000). Thirty years of stimulus-secretion coupling: from Ca(2+) toGTP in the regulation of exocytosis. Biochimie *82*, 385-393.

Pinxteren, J.A., O_Sullivan, A.J., Tatham, P.E., and Gomperts, B.D. (1998). Regulation of exocytosis from rat peritoneal mast cells by G protein beta gamma-subunits. EMBO J *17*, 6210-6218.

Powis, G., Seewald, M.J., Gratas, C., Melder, D., Riebow, J., and Modest, E.J. (1992). Selective inhibition of phosphatidylinositol phospholipase C by cytotoxic ether lipid analogues. Cancer Res *52*, 2835-2840.

Price, L.S., Norman, J.C., Ridley, A.J., and Koffer, A. (1995). The small GTPases Rac and Rho as regulators of secretion in mast cells. Curr Biol *5*, 68-73.

Quesada, I., Chin, W.C., and Verdugo, P. (2003). ATP-independent luminal oscillations and release of Ca2+ and H+ from mast cell secretory granules: implications for signal transduction. Biophys J *85*, 963-970.

Raab, W.P. (1968). Mast cell depleting properties of neomycin. J Invest Dermatol *50*, 138-140.

Raffaniello, R.D., and Raufman, J.P. (1993). Guanine nucleotides activate multiple signaling pathways in permeabilized gastric chief cells. Evidence for GTP gamma S-induced calcium-independent pepsinogen secretion. J Biol Chem *268*, 8491-8496.

Rajkumar, T., Gooden, C.S., Lemoine, N.R., Gullick, W.J., and Goden, C.S. (1993). Expression of the c-erbB-3 protein in gastrointestinal tract tumours determined by monoclonal antibody RTJ1. J Pathol *170*, 271-278.

Rameh, L.E., Tolias, K.F., Duckworth, B.C., and Cantley, L.C. (1997). A new pathway for synthesis of phosphatidylinositol-4,5-bisphosphate. Nature *390*, 192-196.

Reddy, A., Caler, E.V., and Andrews, N.W. (2001). Plasma membrane repair is mediated by Ca(2+)-regulated exocytosis of lysosomes. Cell *106*, 157-169.

Reim, K., Mansour, M., Varoqueaux, F., McMahon, H.T., Sudhof, T.C., Brose, N., and Rosenmund, C. (2001). Complexins regulate a late step in Ca2+-dependent neurotransmitter release. Cell *104*, 71-81.

Resnick, A.C., Snowman, A.M., Kang, B.N., Hurt, K.J., Snyder, S.H., and Saiardi, A. (2005). Inositol polyphosphate multikinase is a nuclear PI3-kinase with transcriptional regulatory activity. Proc Natl Acad Sci U S A *102*, 12783-12788.

Rettig, J., and Neher, E. (2002). Emerging roles of presynaptic proteins in Ca++-triggered exocytosis. Science *298*, 781-785.

Rhee, J.S., Betz, A., Pyott, S., Reim, K., Varoqueaux, F., Augustin, I., Hesse, D., Sudhof, T.C., Takahashi, M., Rosenmund, C., and Brose, N. (2002). Beta phorbol ester- and diacylglycerol-induced augmentation of transmitter release is mediated by Munc13s and not by PKCs. Cell *108*, 121-133.

Rhee, S.G. (2001). Regulation of phosphoinositide-specific phospholipase C. Annu Rev Biochem *70*, 281-312.

Rhee, S.G., Suh, P.G., Ryu, S.H., and Lee, S.Y. (1989). Studies of inositol phospholipid-specific phospholipase C. Science *244*, 546-550.

Richmond, J.E., and Broadie, K.S. (2002). The synaptic vesicle cycle: exocytosis and endocytosis in Drosophila and C. elegans. Curr Opin Neurobiol *12*, 499-507.

Richmond, J.E., Weimer, R.M., and Jorgensen, E.M. (2001). An open form of syntaxin bypasses the requirement for UNC-13 in vesicle priming. Nature *412*, 338-341.

Rigoni, M., Schiavo, G., Weston, A.E., Caccin, P., Allegrini, F., Pennuto, M., Valtorta, F., Montecucco, C., and Rossetto, O. (2004). Snake presynaptic neurotoxins with phospholipase A2 activity induce punctate swellings of neurites and exocytosis of synaptic vesicles. J Cell Sci *117*, 3561-3570.

Rizzoli, S.O., and Betz, W.J. (2004). The structural organization of the readily releasable pool of synaptic vesicles. Science *303*, 2037-2039.

Roberts, H.F., Clarke, J.H., Letcher, A.J., Irvine, R.F., and Hinchliffe, K.A. (2005). Effects of lipid kinase expression and cellular stimuli on phosphatidylinositol 5-phosphate levels in mammalian cell lines. FEBS Lett *579*, 2868-2872.

Rohlich, P., Anderson, P., and Uvnas, B. (1971). Electron microscope observations on compounds 48-80-induced degranulation in rat mast cells. Evidence for sequential exocytosis of storage granules. J Cell Biol *51*, 465-483.

Rosenmund, C., Rettig, J., and Brose, N. (2003). Molecular mechanisms of active zone function. Curr Opin Neurobiol *13*, 509-519.

Roth, M.G. (2004). Phosphoinositides in constitutive membrane traffic. Physiol Rev *84*, 699-730.

Roy, A., and Levine, T.P. (2004). Multiple pools of phosphatidylinositol 4-phosphate detected using the pleckstrin homology domain of Osh2p. J Biol Chem *279*, 44683-44689.

Sagawa, N., Bleasdale, J.E., and Di Renzo, G.C. (1983). The effects of polyamines and aminoglycosides on phosphatidylinositol-specific phospholipase C from human amnion. Biochim Biophys Acta *752*, 153-161.

Saito, H., Ishizaka, K., and Ishizaka, T. (1989). Effect of nonhydrolyzable guanosine phosphate on IgE-mediated activation of phospholipase C and histamine release from rodent mast cells. J Immunol *143*, 250-258.

Salinas, E., Ventura, J., Cordova, L.E., and Luis Quintanar, J. (2004). Presence of SNAP-25 in rat mast cells. Immunol Lett *95*, 105-108.

Sankaran, V.G., Klein, D.E., Sachdeva, M.M., and Lemmon, M.A. (2001). High-affinity binding of a FYVE domain to phosphatidylinositol 3-phosphate requires intact phospholipid but not FYVE domain oligomerization. Biochemistry *40*, 8581-8587.

Sankaranarayanan, S., and Ryan, T.A. (2000). Real-time measurements of vesicle-SNARE recycling in synapses of the central nervous system. Nat Cell Biol *2*, 197-204.

Santagata, S., Boggon, T.J., Baird, C.L., Gomez, C.A., Zhao, J., Shan, W.S., Myszka, D.G., and Shapiro, L. (2001). G-protein signaling through tubby proteins. Science *292*, 2041-2050.

Sasaki, J., Sasaki, T., Yamazaki, M., Matsuoka, K., Taya, C., Shitara, H., Takasuga, S., Nishio, M., Mizuno, K., Wada, T., Miyazaki, H., Watanabe, H., Iizuka, R., Kubo,

S., Murata, S., Chiba, T., Maehama, T., Hamada, K., Kishimoto, H., Frohman, M.A., Tanaka, K., Penninger, J.M., Yonekawa, H., Suzuki, A., and Kanaho, Y. (2005). Regulation of anaphylactic responses by phosphatidylinositol phosphate kinase type I {alpha}. J Exp Med *201*, 859-870.

Sassa, T., Harada, S., Ogawa, H., Rand, J.B., Maruyama, I.N., and Hosono, R. (1999). Regulation of the UNC-18-Caenorhabditis elegans syntaxin complex by UNC-13. J Neurosci *19*, 4772-4777.

Saunders, C.M., Larman, M.G., Parrington, J., Cox, L.J., Royse, J., Blayney, L.M., Swann, K., and Lai, F.A. (2002). PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development. Development *129*, 3533-3544.

Sbrissa, D., Ikonomov, O.C., and Shisheva, A. (1999). PIKfyve, a mammalian ortholog of yeast Fab1p lipid kinase, synthesizes 5-phosphoinositides. Effect of insulin. J Biol Chem *274*, 21589-21597.

Schacht, J. (1978). Purification of polyphosphoinositides by chromatography on immobilized neomycin. J Lipid Res *19*, 1063-1067.

Schacht, J., and Agranoff, B.W. (1972). Effects of acetylcholine on labeling of phosphatidate and phosphoinositides by (32 P) orthophosphate in nerve ending fractions of guinea pig cortex. J Biol Chem *247*, 771-777.

Schaletzky, J., Dove, S.K., Short, B., Lorenzo, O., Clague, M.J., and Barr, F.A. (2003). Phosphatidylinositol-5-phosphate activation and conserved substrate specificity of the myotubularin phosphatidylinositol 3-phosphatases. Curr Biol *13*, 504-509.

Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B.R., and Montecucco, C. (1992). Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. Nature *359*, 832-835.

Schiavo, G., Gu, Q.M., Prestwich, G.D., Sollner, T.H., and Rothman, J.E. (1996). Calcium-dependent switching of the specificity of phosphoinositide binding to synaptotagmin. Proc Natl Acad Sci U S A *93*, 13327-13332.

Schiavo, G., Matteoli, M., and Montecucco, C. (2000). Neurotoxins affecting neuroexocytosis. Physiol Rev *80*, 717-766.

Schu, P.V., Takegawa, K., Fry, M.J., Stack, J.H., Waterfield, M.D., and Emr, S.D. (1993). Phosphatidylinositol 3-kinase encoded by yeast VPS34 gene essential for protein sorting. Science *260*, 88-91.

Scott, C.C., Dobson, W., Botelho, R.J., Coady-Osberg, N., Chavrier, P., Knecht, D.A., Heath, C., Stahl, P., and Grinstein, S. (2005). Phosphatidylinositol-4,5-bisphosphate hydrolysis directs actin remodeling during phagocytosis. J Cell Biol *169*, 139-149.

Sekiya, K., Danbara, H., Yase, K., and Futaesaku, Y. (1996). Electron microscopic evaluation of a two-step theory of pore formation by streptolysin O. J Bacteriol *178*, 6998-7002.

Shefler, I., Taube, Z., Medalia, O., and Sagi-Eisenberg, R. (1998). Basic secretagogues activate protein tyrosine phosphorylation and release of arachidonic acid in mast cells via a novel protein kinase C and phosphatidylinositol 3-kinase-dependent mechanism. Eur J Immunol *28*, 3468-3478.

Shin, H.W., Hayashi, M., Christoforidis, S., Lacas-Gervais, S., Hoepfner, S., Wenk, M.R., Modregger, J., Uttenweiler-Joseph, S., Wilm, M., Nystuen, A., Frankel, W.N., Solimena, M., De Camilli, P., and Zerial, M. (2005). An enzymatic cascade of Rab5 effectors regulates phosphoinositide turnover in the endocytic pathway. J Cell Biol *170*, 607-618.

Shin, O.H., Rhee, J.S., Tang, J., Sugita, S., Rosenmund, C., and Sudhof, T.C. (2003). Sr2+ binding to the Ca2+ binding site of the synaptotagmin 1 C2B domain triggers fast exocytosis without stimulating SNARE interactions. Neuron *37*, 99-108.

Siraganian, R.P. (2003). Mast cell signal transduction from the high-affinity IgE receptor. Curr Opin Immunol *15*, 639-646.

Smith, C., and Neher, E. (1997). Multiple forms of endocytosis in bovine adrenal chromaffin cells. J Cell Biol *139*, 885-894.

Speidel, D., Bruederle, C.E., Enk, C., Voets, T., Varoqueaux, F., Reim, K., Becherer, U., Fornai, F., Ruggieri, S., Holighaus, Y., Weihe, E., Bruns, D., Brose, N., and Rettig, J. (2005). CAPS1 regulates catecholamine loading of large dense-core vesicles. Neuron *46*, 75-88.

Stephens, L.R., Hughes, K.T., and Irvine, R.F. (1991). Pathway of phosphatidylinositol(3,4,5)-trisphosphate synthesis in activated neutrophils. Nature *351*, 33-39.

Stocker, H., Andjelkovic, M., Oldham, S., Laffargue, M., Wymann, M.P., Hemmings, B.A., and Hafen, E. (2002). Living with lethal PIP3 levels: viability of flies

lacking PTEN restored by a PH domain mutation in Akt/PKB. Science *295*, 2088-2091.

Sudhof, T.C. (2002). Synaptotagmins: why so many? J Biol Chem *277*, 7629-7632.

Suh, B.C., and Hille, B. (2005). Regulation of ion channels by phosphatidylinositol 4,5-bisphosphate. Curr Opin Neurobiol *15*, 370-378.

Sullivan, R., Price, L.S., and Koffer, A. (1999). Rho controls cortical F-actin disassembly in addition to, but independently of, secretion in mast cells. J Biol Chem *274*, 38140-38146.

Szule, J.A., and Coorssen, J.R. (2003). Revisiting the role of SNAREs in exocytosis and membrane fusion. Biochim Biophys Acta *1641*, 121-135.

Szule, J.A., Jarvis, S.E., Hibbert, J.E., Spafford, J.D., Braun, J.E., Zamponi, G.W., Wessel, G.M., and Coorssen, J.R. (2003). Calcium-triggered membrane fusion proceeds independently of specific presynaptic proteins. J Biol Chem *278*, 24251-24254.

Tadokoro, S., Nakanishi, M., and Hirashima, N. (2005). Complexin II facilitates exocytotic release in mast cells by enhancing Ca2+ sensitivity of the fusion process. J Cell Sci *118*, 2239-2246.

Takahashi, N., Kadowaki, T., Yazaki, Y., Miyashita, Y., and Kasai, H. (1997). Multiple exocytotic pathways in pancreatic beta cells. J Cell Biol *138*, 55-64.

Takenawa, T., and Itoh, T. (2001). Phosphoinositides, key molecules for regulation of actin cytoskeletal organization and membrane traffic from the plasma membrane. Biochim Biophys Acta *1533*, 190-206.

Tatham, P.E., and Gomperts, B.D. (1991). Rat mast cells degranulate in response to microinjection of guanine nucleotide. J Cell Sci *98 (Pt 2)*, 217-224.

Taylor, G.S., Maehama, T., and Dixon, J.E. (2000). Inaugural article: myotubularin, a protein tyrosine phosphatase mutated in myotubular myopathy, dephosphorylates the lipid second messenger, phosphatidylinositol 3-phosphate. Proc Natl Acad Sci U S A *97*, 8910-8915.

Terebiznik, M.R., Vieira, O.V., Marcus, S.L., Slade, A., Yip, C.M., Trimble, W.S., Meyer, T., Finlay, B.B., and Grinstein, S. (2002). Elimination of host cell PtdIns(4,5)P(2) by bacterial SigD promotes membrane fission during invasion by Salmonella. Nat Cell Biol *4*, 766-773.

Theoharides, T.C., and Cochrane, D.E. (2004). Critical role of mast cells in inflammatory diseases and the effect of acute stress. J Neuroimmunol *146*, 1-12.

Thomas, C.C., Dowler, S., Deak, M., Alessi, D.R., and van Aalten, D.M. (2001). Crystal structure of the phosphatidylinositol 3,4-bisphosphate-binding pleckstrin homology (PH) domain of tandem PH-domain-containing protein 1 (TAPP1): molecular basis of lipid specificity. Biochem J *358*, 287-294.

Thomas, C.L., Steel, J., Prestwich, G.D., and Schiavo, G. (1999). Generation of phosphatidylinositol-specific antibodies and their characterization. Biochem Soc Trans *27*, 648-652.

Thomas, G.M., Cunningham, E., Fensome, A., Ball, A., Totty, N.F., Truong, O., Hsuan, J.J., and Cockcroft, S. (1993). An essential role for phosphatidylinositol transfer protein in phospholipase C-mediated inositol lipid signaling. Cell *74*, 919-928.

Thomas, G.M., and Pinxteren, J.A. (2000). Phosphatidylinositol transfer proteins: one big happy family or strangers with the same name? Mol Cell Biol Res Commun. *4*, 1-9.

Thompson, A.K., Mostafapour, S.P., Denlinger, L.C., Bleasdale, J.E., and Fisher, S.K. (1991). The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. A role for Gp in receptor compartmentation. J Biol Chem *266*, 23856-23862.

Tolias, K.F., Rameh, L.E., Ishihara, H., Shibasaki, Y., Chen, J., Prestwich, G.D., Cantley, L.C., and Carpenter, C.L. (1998). Type I phosphatidylinositol-4-phosphate 5-kinases synthesize the novel lipids phosphatidylinositol 3,5-bisphosphate and phosphatidylinositol 5-phosphate. J Biol Chem *273*, 18040-18046.

Tooze, S.A., Martens, G.J., and Huttner, W.B. (2001). Secretory granule biogenesis: rafting to the SNARE. Trends Cell Biol *11*, 116-122.

Torrealba, F., and Carrasco, M.A. (2004). A review on electron microscopy and neurotransmitter systems. Brain Res Brain Res Rev *47*, 5-17.

Touhara, K., Koch, W.J., Hawes, B.E., and Lefkowitz, R.J. (1995). Mutational analysis of the pleckstrin homology domain of the beta-adrenergic receptor kinase. Differential effects on G beta gamma and phosphatidylinositol 4,5-bisphosphate binding. J Biol Chem *270*, 17000-17005.

Tucker, W.C., Weber, T., and Chapman, E.R. (2004). Reconstitution of Ca2+-regulated membrane fusion by synaptotagmin and SNAREs. Science *304*, 435-438.

van der Wal, J., Habets, R., Varnai, P., Balla, T., and Jalink, K. (2001). Monitoring agonist-induced phospholipase C activation in live cells by fluorescence resonance energy transfer. J Biol Chem *276*, 15337-15344.

van Rheenen, J., Achame, E.M., Janssen, H., Calafat, J., and Jalink, K. (2005). PIP2 signaling in lipid domains: a critical re-evaluation. EMBO J *24*, 1664-1673.

Vanhaesebroeck, B., Leevers, S.J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P.C., Woscholski, R., Parker, P.J., and Waterfield, M.D. (2001). Synthesis and function of 3-phosphorylated inositol lipids. Annu Rev Biochem *70*, 535-602.

Varlamov, O., Volchuk, A., Rahimian, V., Doege, C.A., Paumet, F., Eng, W.S., Arango, N., Parlati, F., Ravazzola, M., Orci, L., Sollner, T.H., and Rothman, J.E. (2004). i-SNAREs: inhibitory SNAREs that fine-tune the specificity of membrane fusion. J Cell Biol *164*, 79-88.

Varnai, P., and Balla, T. (1998). Visualization of phosphoinositides that bind pleckstrin homology domains: calcium- and agonist-induced dynamic changes and relationship to myo-[3H]inositol-labeled phosphoinositide pools. J Cell Biol *143*, 501-510.

Varoqueaux, F., Sigler, A., Rhee, J.S., Brose, N., Enk, C., Reim, K., and Rosenmund, C. (2002). Total arrest of spontaneous and evoked synaptic transmission but normal synaptogenesis in the absence of Munc13-mediated vesicle priming. Proc Natl Acad Sci U S A *99*, 9037-9042.

Vlahos, C.J., Matter, W.F., Hui, K.Y., and Brown, R.F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J Biol Chem *269*, 5241-5248.

Volinia, S., Dhand, R., Vanhaesebroeck, B., MacDougall, L.K., Stein, R., Zvelebil, M.J., Domin, J., Panaretou, C., and Waterfield, M.D. (1995). A human phosphatidylinositol 3-kinase complex related to the yeast Vps34p-Vps15p protein sorting system. EMBO J *14*, 3339-3348.

Walch-Solimena, C., and Novick, P. (1999). The yeast phosphatidylinositol-4-OH kinase pik1 regulates secretion at the Golgi. Nat Cell Biol *1*, 523-525.

Walent, J.H., Porter, B.W., and Martin, T.F. (1992). A novel 145 kd brain cytosolic protein reconstitutes Ca(2+)-regulated secretion in permeable neuroendocrine cells. Cell *70*, 765-775.

Walker, D.M., Urbe, S., Dove, S.K., Tenza, D., Raposo, G., and Clague, M.J. (2001). Characterization of MTMR3. an inositol lipid 3-phosphatase with novel substrate specificity. Curr Biol *11*, 1600-1605.

Wang, D., Feng, J., Wen, R., Marine, J.C., Sangster, M.Y., Parganas, E., Hoffmeyer, A., Jackson, C.W., Cleveland, J.L., Murray, P.J., and Ihle, J.N. (2000). Phospholipase Cgamma2 is essential in the functions of B cell and several Fc receptors. Immunity *13*, 25-35.

Wang, J., Gambhir, A., Hangyas-Mihalyne, G., Murray, D., Golebiewska, U., and McLaughlin, S. (2002). Lateral sequestration of phosphatidylinositol 4,5-bisphosphate by the basic effector domain of myristoylated alanine-rich C kinase substrate is due to nonspecific electrostatic interactions. J Biol Chem *277*, 34401-34412.

Wang, L., Li, G., and Sugita, S. (2005). A central kinase domain of type I phosphatidylinositol phosphate kinases is sufficient to prime exocytosis: isoform specificity and its underlying mechanism. J Biol Chem *280*, 16522-16527.

Wang, Y.J., Li, W.H., Wang, J., Xu, K., Dong, P., Luo, X., and Yin, H.L. (2004). Critical role of PIP5KI{gamma}87 in InsP3-mediated Ca(2+) signaling. J Cell Biol *167*, 1005-1010.

Wang, Y.J., Wang, J., Sun, H.Q., Martinez, M., Sun, Y.X., Macia, E., Kirchhausen, T., Albanesi, J.P., Roth, M.G., and Yin, H.L. (2003). Phosphatidylinositol 4 phosphate regulates targeting of clathrin adaptor AP-1 complexes to the Golgi. Cell *114*, 299-310.

Watson, R.T., Kanzaki, M., and Pessin, J.E. (2004). Regulated membrane trafficking of the insulin-responsive glucose transporter 4 in adipocytes. Endocr Rev *25*, 177-204.

Watt, S.A., Kimber, W.A., Fleming, I.N., Leslie, N.R., Downes, C.P., and Lucocq, J.M. (2004). Detection of novel intracellular agonist responsive pools of phosphatidylinositol 3,4-bisphosphate using the TAPP1 pleckstrin homology domain in immunoelectron microscopy. Biochem J *377*, 653-663.

Watt, S.A., Kular, G., Fleming, I.N., Downes, C.P., and Lucocq, J.M. (2002). Subcellular localization of phosphatidylinositol 4,5-bisphosphate using the pleckstrin homology domain of phospholipase C delta1. Biochem J *363*, 657-666.

Way, G., Morrice, N., Smythe, C., and O'Sullivan, A.J. (2002). Purification and identification of secernin, a novel cytosolic protein that regulates exocytosis in mast cells. Mol Biol Cell *13*, 3344-3354.

Way, G., O_luanaigh, N., and Cockcroft, S. (2000). Activation of exocytosis by cross-linking of the IgE receptor is dependent on ADP-ribosylation factor 1-regulated phospholipase D in RBL-2H3 mast cells: evidence that the mechanism of activation is via regulation of phosphatidylinositol 4,5-bisphosphate synthesis. Biochem J *346 Pt 1*, 63-70.

Weber, T., Zemelman, B.V., McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T.H., and Rothman, J.E. (1998). SNAREpins: minimal machinery for membrane fusion. Cell *92*, 759-772.

Welch, H.C., Coadwell, W.J., Ellson, C.D., Ferguson, G.J., Andrews, S.R., Erdjument-Bromage, H., Tempst, P., Hawkins, P.T., and Stephens, L.R. (2002). P-Rex1, a PtdIns(3,4,5)P3- and Gbetagamma-regulated guanine-nucleotide exchange factor for Rac. Cell *108*, 809-821.

Wen, R., Jou, S.T., Chen, Y., Hoffmeyer, A., and Wang, D. (2002). Phospholipase C gamma 2 is essential for specific functions of Fc epsilon R and Fc gamma R. J Immunol *169*, 6743-6752.

Wenk, M.R., Lucast, L., Di Paolo, G., Romanelli, A.J., Suchy, S.F., Nussbaum, R.L., Cline, G.W., Shulman, G.I., McMurray, W., and De Camilli, P. (2003). Phosphoinositide profiling in complex lipid mixtures using electrospray ionization mass spectrometry. Nat Biotechnol *21*, 813-817.

Wenk, M.R., Pellegrini, L., Klenchin, V.A., Di Paolo, G., Chang, S., Daniell, L., Arioka, M., Martin, T.F., and De Camilli, P. (2001). PIP kinase Igamma is the major PI(4,5)P(2) synthesizing enzyme at the synapse. Neuron *32*, 79-88.

Whitaker, M. (1985). Polyphosphoinositide hydrolysis is associated with exocytosis in adrenal medullary cells. FEBS Lett *189*, 137-140.

Wiedemann, C., Schafer, T., and Burger, M.M. (1996). Chromaffin granule-associated phosphatidylinositol 4-kinase activity is required for stimulated secretion. EMBO J *15*, 2094-2101.

Wiedemann, C., Schafer, T., Burger, M.M., and Sihra, T.S. (1998). An essential role for a small synaptic vesicle-associated phosphatidylinositol 4-kinase in neurotransmitter release. J Neurosci *18*, 5594-5602.

Wishart, M.J., and Dixon, J.E. (2002). PTEN and myotubularin phosphatases: from 3-phosphoinositide dephosphorylation to disease. Trends Cell Biol *12*, 579-585.

Xu, C., Watras, J., and Loew, L.M. (2003). Kinetic analysis of receptor-activated phosphoinositide turnover. J Cell Biol *161*, 779-791.

Xu, Y., Hortsman, H., Seet, L., Wong, S.H., and Hong, W. (2001). SNX3 regulates endosomal function through its PX-domain-mediated interaction with PtdIns(3)P. Nat Cell Biol *3*, 658-666.

Xu, Y., Zhang, F., Su, Z., McNew, J.A., and Shin, Y.K. (2005). Hemifusion in SNARE-mediated membrane fusion. Nat Struct Mol Biol *12*, 417-422.

Yokogawa, T., Nagata, S., Nishio, Y., Tsutsumi, T., Ihara, S., Shirai, R., Morita, K., Umeda, M., Shirai, Y., Saitoh, N., and Fukui, Y. (2000). Evidence that 3'-phosphorylated polyphosphoinositides are generated at the nuclear surface: use of immunostaining technique with monoclonal antibodies specific for PI 3,4-P(2). FEBS Lett *473*, 222-226.

York, J.D., Odom, A.R., Murphy, R., Ives, E.B., and Wente, S.R. (1999). A phospholipase C-dependent inositol polyphosphate kinase pathway required for efficient messenger RNA export. Science *285*, 96-100.

Yu, J.W., and Lemmon, M.A. (2001). All phox homology (PX) domains from Saccharomyces cerevisiae specifically recognize phosphatidylinositol 3-phosphate. J Biol Chem *276*, 44179-44184.

Yu, J.W., Mendrola, J.M., Audhya, A., Singh, S., Keleti, D., DeWald, D.B., Murray, D., Emr, S.D., and Lemmon, M.A. (2004). Genome-wide analysis of membrane targeting by S. cerevisiae pleckstrin homology domains. Mol Cell *13*, 677-688.

Yule, D.I., and Williams, J.A. (1992). U73122 inhibits Ca2+ oscillations in response to cholecystokinin and carbachol but not to JMV-180 in rat pancreatic acinar cells. J Biol Chem *267*, 13830-13835.

Zhang, J., Banfic, H., Straforini, F., Tosi, L., Volinia, S., and Rittenhouse, S.E. (1998). A type II phosphoinositide 3-kinase is stimulated via activated integrin in platelets. A source of phosphatidylinositol 3-phosphate. J Biol Chem *273*, 14081-14084.

Zhang, X., Jefferson, A.B., Auethavekiat, V., and Majerus, P.W. (1995). The protein deficient in Lowe syndrome is a phosphatidylinositol-4,5-bisphosphate 5-phosphatase. Proc Natl Acad Sci U S A *92*, 4853-4856.

Zhang, X., Loijens, J.C., Boronenkov, I.V., Parker, G.J., Norris, F.A., Chen, J., Thum, O., Prestwich, G.D., Majerus, P.W., and Anderson, R.A. (1997). Phosphatidylinositol-4-phosphate 5-kinase isozymes catalyze the synthesis of 3-phosphate-containing phosphatidylinositol signaling molecules. J Biol Chem *272*, 17756-17761.

Zheng, Q., McFadden, S.C., and Bobich, J.A. (2004). Phosphatidylinositol 4,5-bisphosphate promotes both [3H]-noradrenaline and [14C]-glutamate exocytosis from nerve endings. Neurochem Int *44*, 243-250.

Zhu, D., Tan, K.S., Zhang, X., Sun, A.Y., Sun, G.Y., and Lee, J.C. (2005). Hydrogen peroxide alters membrane and cytoskeleton properties and increases intercellular connections in astrocytes. J Cell Sci *118*, 3695-3703.

Zimmermann, P., Meerschaert, K., Reekmans, G., Leenaerts, I., Small, J.V., Vandekerckhove, J., David, G., and Gettemans, J. (2002). PIP(2)-PDZ domain binding controls the association of syntenin with the plasma membrane. Mol Cell *9*, 1215-1225.