Elimination of plasma membrane phosphatidylinositol (4,5)-bisphosphate is required for exocytosis from mast cells

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Summary

The inositol lipid phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P_2] is involved in a myriad of cellular processes, including the regulation of exocytosis and endocytosis. In this paper, we address the role of PtdIns(4,5)P_2 in compound exocytosis from rat peritoneal mast cells. This process involves granule-plasma membrane fusion as well as homotypic granule membrane fusion and occurs without any immediate compensatory endocytosis. Using a novel quantitative immunofluorescence technique, we report that plasma membrane PtdIns(4,5)P_2 becomes transiently depleted upon activation of exocytosis, and is not detected on the membranes of fusing granules. Depletion is caused by phospholipase C activity, and is mandatory for exocytosis. Although phospholipase C is required for Ca^{2+} release from internal stores, the majority of the requirement for PtdIns(4,5)P_2 hydrolysis occurs downstream of Ca^{2+} signalling – as shown in permeabilised cells, where the inositol (1,4,5)-trisphosphate–Ca^{2+} pathway is bypassed. Neither generation of the PtdIns(4,5)P_2 metabolite, diacylglycerol (DAG) or simple removal and/or sequestration of PtdIns(4,5)P_2 are sufficient for exocytosis to occur. However, treatment of permeabilised cells with DAG induces a small potentiation of exocytosis, indicating that it may be required. We propose that a cycle of PtdIns(4,5)P_2 synthesis and breakdown is crucial for exocytosis to occur in mast cells, and may have a more general role in all professional secretory cells.

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Introduction

Phosphoinositides are a family of phospholipids containing *myo*-inositol as their headgroup. Despite a relatively low abundance in biological membranes, phosphoinositides regulate a myriad of cellular processes owing to their high rate of metabolic turnover. One such lipid, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], is required for many aspects of cellular physiology, including multiple stages of membrane traffic (Czech, 2003; Hammond et al., 2004; Janmey and Lindberg, 2004). In particular, PtdIns(4,5)P_2 is implicated in the fusion of secretory vesicles with the plasma membrane in response to a specific stimulus (Martin, 2001), a process known as regulated exocytosis.

Studies in neuroendocrine cells revealed that a process of ATP-dependent priming is required before secretory vesicles are competent to undergo regulated exocytosis (Hay and Martin, 1992; Holz et al., 1989). Early work demonstrated that ATP is required, at least in part, to generate phosphoinositides and that depletion of phosphoinositides prevented priming (Eberhard et al., 1990). Subsequent work has revealed requirements for enzymes that generate PtdIns(4,5)P_2 during ATP-dependent priming (Hay et al., 1995; Hay and Martin, 1993; Wiedemann et al., 1996), indicating a specific requirement for this lipid. Recent genetic approaches, whereby cellular PtdIns(4,5)P_2 levels had been manipulated in endocrine cells showed corresponding changes in the number of primed vesicles (Gong et al., 2005; Milosevic et al., 2005; Olsen et al., 2003). Furthermore, use of the monogamous PtdIns(4,5)P_2-binding pleckstrin homology (PH) domain from phospholipase C δ1 (PLCδ1) fused to green fluorescent protein (GFP) has identified plasma membrane pools of PtdIns(4,5)P_2 as being important in the regulation of exocytosis in neuroendocrine cells (Aikawa and Martin, 2003; Holz et al., 2000), hippocampal neurons (Micheva et al., 2001) and pancreatic beta cells (Lawrence and Birnbaum, 2003).

How does plasma membrane PtdIns(4,5)P_2 mediate vesicle priming? Several proteins required for regulated exocytosis bind PtdIns(4,5)P_2 in vitro, including Mints (Okamoto and Sudhof, 1997), Rabphilin 3A (Chung et al., 1998), synaptotagmins (Bai and Chapman, 2004) and CAPS

Journal of Cell Science

Journal of Cell Science

(Grishanin et al., 2004). Indeed, it has even been suggested that the interaction between PtdIns(4,5)P_2 and synaptotagmin I plays a direct role in the membrane fusion process (Bai et al., 2004). However, exactly how PtdIns(4,5)P_2 regulates exocytosis is still far from clear. Furthermore, exocytosis from neurons and endocrine cells is accompanied by compensatory endocytosis (Gundelfinger et al., 2003), a process itself reliant on PtdIns(4,5)P_2 (Wenk and De Camilli, 2004). Therefore, it can be difficult to resolve precisely at which stage in the exo-endocytic cycle PtdIns(4,5)P_2 acts; for example, neurons from mice deficient in phosphatidylinositol 4-phosphate 5-kinase Iγ (PIPK Iγ) bear defects at several stages of the synaptic vesicle cycle, with a predominant impairment in vesicle retrieval after exocytosis (Di Paolo et al., 2004).

Upon activation, mast cells release pro-inflammatory mediators in an acute, rapid and massive exocytosis from around a thousand pre-formed granules, a process termed degranulation. This involves compound exocytosis, whereby only an outer cohort of granules undergo heterotypic fusion at the plasma membrane; the remaining granules fuse with neighbouring-fused granules (pseudo-heterotypic fusion), in a wave that propagates from the surface into the cell (Alvarez de Toledo and Fernandez, 1990). As a result, a series of large intracellular cavities communicating with the extracellular milieu and containing the granule cores are formed. Notably, mast cell exocytosis proceeds without significant compensatory endocytosis during or immediately following degranulation (Fernandez et al., 1984). Like neuroendocrine cells, mast cells require an ATP-dependent priming reaction prior to exocytosis (Howell et al., 1989), which again involves the generation of phosphoinositides (Pinxteren et al., 2001). However, mast cells from PIPK Iα-deficient mice display reduced PtdIns(4,5)P_2 levels yet, intriguingly, augmented degranulation (Sasaki et al., 2005). Therefore, it seems likely that PtdIns(4,5)P_2 plays a key – yet poorly defined – role in mast cell exocytosis.

In this paper, we have employed a novel quantitative immunofluorescence technique to follow PtdIns(4,5)P_2 dynamics during compound exocytosis from rat peritoneal mast cells (RPMCs). We report that PtdIns(4,5)P_2 is present at the plasma membrane and absent from the granule membranes; during exocytosis, PtdIns(4,5)P_2 is eliminated from the plasma membrane and remains absent from membranes of fusing granules. This depletion is mediated by phosphatidylinositol-specific phospholipase C (PLC), the activity of which is required for exocytosis beyond its established role in Ca^{2+} signalling.

Results

PtdIns(4,5)P_2 is present at the plasma membrane of resting mast cells

To investigate a potential role for PtdIns(4,5)P_2 in exocytosis from mast cells, we began by establishing the subcellular localisation of this lipid. RPMCs are not amenable to transfection with GFP-tagged probes. Therefore, we used the specific monoclonal antibody 2C11 against PtdIns(4,5)P_2 (Osborne et al., 2001; Thomas et al., 1999), opting for a staining protocol performed at 4°C to preserve membrane localisation of the lipid (Watt et al., 2002). Discontinuous thread-like structures labelled by the 2C11 antibody were observed at the cell surface (Fig. 1A), which are reminiscent

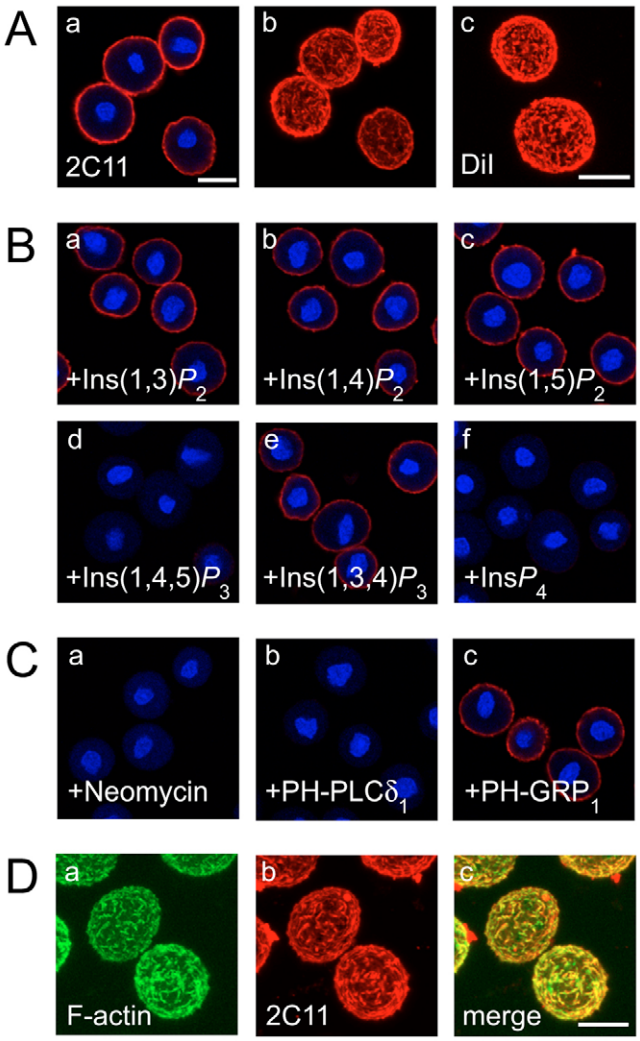

Fig. 1. PtdIns(4,5)P_2 is enriched at the plasma membrane of resting RPMCs. (A) The monoclonal anti-PtdIns(4,5)P_2 antibody 2C11 stains the plasma membrane. RPMCs were fixed and stained at 4°C with (a,b) 2C11 (red) and Draq5™ (blue) or (c) CM-DiI as described in Materials and Methods; (a) shows an equatorial confocal section, (b,c) are projections of confocal sections taken at 1 μm intervals throughout the cell. (B) 2C11 binds PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3. 2C11 was pre-incubated with 1000-fold molar excess of the indicated inositol phosphate before staining as in (A); equatorial confocal sections are shown. (C) 2C11 specifically detects PtdIns(4,5)P_2 on the plasma membrane of RPMCs. Cells were preincubated with 50 μM of the indicated PH domain before staining (b,c), or with 1 mM neomycin during staining with 2C11 (a); equatorial confocal sections are shown. (D) PtdIns(4,5)P_2 colocalises with the cortical actin cytoskeleton. Cells were co-stained with 2C11 (red) and Alexa Fluor 488-phalloidin (green) as described in Materials and Methods. A projection of confocal sections taken at 1 μm intervals throughout the cell is shown. (A-C) Micrographs are at the same magnification, except (Ac), which is at the same magnification as (D). Bars, 10 μm.

of the plasma membrane folds seen in mast cells when viewed by scanning electron microscopy (Burwen and Satir, 1977). Since a similar pattern was observed with the generic plasma membrane dye, CM-DiI (Fig. 1A), the staining is consistent with a homogenous distribution of PtdIns(4,5)P_2 within the

Journal of Cell Science

plasma membrane, and we could not detect any evidence for local enrichment of PtdIns(4,5)P_2 at this level of resolution.

We performed two independent sets of controls to demonstrate that 2C11 was detecting PtdIns(4,5)P_2 in RPMCs. First, the antibody was pre-incubated with a molar excess of various inositol phosphates before applying the antibody to cells. Under these conditions, both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 competed with the cellular antigen (Fig. 1B), indicating that this could be PtdIns(4,5)P_2 or PtdIns(3,4,5)P_3. However, it is unlikely that PtdIns(3,4,5)P_3 is giving rise to the staining observed, because this molecule is not detectable in resting RPMCs whereas PtdIns(4,5)P_2 is abundant (G.R.V.H. and S.K.D., unpublished observations). To formally exclude this possibility, we performed a second set of control experiments: cells were pre-incubated with agents that would specifically bind endogenous phosphoinositide, therefore preventing their labelling with the antibody. Binding of 2C11 was effectively prevented by incubation of the cells with neomycin, an aminoglycoside antibiotic that binds with high affinity to several phosphoinositides, including PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 (Fig. 1B) (Schacht, 1978) (S.K.D., unpublished observations). In parallel, cells were pre-incubated with GST-tagged probes of exquisite specificity: the PH domain from PLCδ1, which binds PtdIns(4,5)P_2 (Lemmon et al., 1995), and the 2G splice-variant of the PH domain from GRP1, which is specific for PtdIns(3,4,5)P_3 (Klarlund et al., 2000). Only the PH domain from PLCδ1 effectively competed with 2C11 (Fig. 1C), demonstrating that the antibody is indeed detecting endogenous PtdIns(4,5)P_2.

Resting RPMCs contain a cortical actin cytoskeleton (Norman et al., 1996), which has been linked to plasma membrane PtdIns(4,5)P_2 in a number of cell types (Janmey and Lindberg, 2004). Indeed, when mast cells were counter-stained for F-actin with fluorescent phalloidin, we observed an excellent colocalisation with PtdIns(4,5)P_2 (Fig. 1D). Thus, resting RPMCs appear to contain a pool of PtdIns(4,5)P_2 at the plasma membrane, juxtaposed to the cortical F-actin network. Importantly, we did not observe any staining of internal granule membranes (Fig. 1A-C).

PtdIns(4,5)P_2 dynamics during exocytosis

Mast cell exocytosis involves both granule-plasma membrane and granule-granule membrane fusion. Therefore, if PtdIns(4,5)P_2 were required for membrane fusion, we would expect either relocation of plasma membrane PtdIns(4,5)P_2 to granule membranes or synthesis of PtdIns(4,5)P_2 at the these sites during exocytosis. To test these hypotheses, we stimulated cells for various times with the polycationic agonist, compound 48/80 (48/80). Cells were then fixed and stained with both 2C11 and fluorescent concanavalin A, a membrane impermeable reagent that binds to granule cores with high affinity. Only granule cores that have been exposed to the extracellular milieu are labelled by concanavalin A, making this reagent an effective tool to monitor exocytosis (Norman et al., 1996).

Surprisingly, cells stimulated for short periods when degranulation was well underway showed almost complete elimination of plasma membrane PtdIns(4,5)P_2, which remained absent from the membranes of fusing granules (Fig. 2A). Only after a further 2.5 minutes, well after the completion of exocytosis (Penner, 1988), was PtdIns(4,5)P_2 seen to return at the plasma- as well as granule-membranes at levels comparable with resting cells (Fig. 2A,B). Analysis of single-

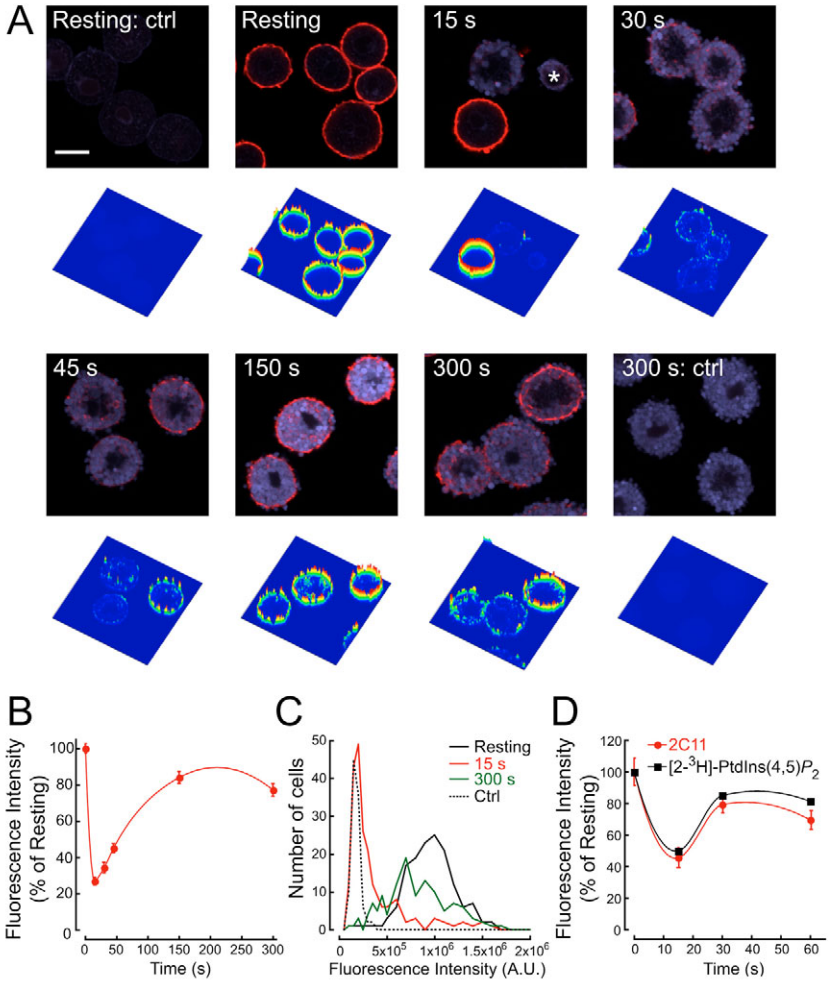

Fig. 2. Plasma membrane PtdIns(4,5)P_2 is transiently depleted during degranulation. (A) Cells were fixed with ice-cold 3% glutaraldehyde and stained either at rest or after stimulation for the indicated times with compound 48/80. First and third row of panels show merged images of 2C11 (red) and Alexa Fluor 647-concanavalin A (blue). Second and fourth row of panels show fluorescence intensity profiles for 2C11. Bar, 10 μm. Ctrl indicates that 2C11 has been omitted during staining. *, contaminating neutrophil. (B,C) 2C11 fluorescence was quantified as described in Materials and Methods. (B,C) Mean fluorescence (± s.e.m.) normalised to the resting level (B), distribution of fluorescence at the indicated time points (C); n>100 cells per time point. (D) RPMCs were loaded with unlabelled or [2-^{3}H]inositol, and stimulated for the indicated time period with compound 48/80. Cells were either fixed and stained with 2C11 and quantified as in B (2C11), or extracted and analysed for PtdIns(4,5)P_2 content by HPLC ([2-^{3}H]-PtdIns(4,5)P_2).

cell fluorescence intensity revealed that after 15 seconds of stimulation with 48/80, the majority of cells had undergone complete elimination of PtdIns(4,5)P_2, because the intensity was comparable with that of cells stained with only secondary antibody (Fig. 2C). Thus the ~80% drop in fluorescence intensity observed after 15 seconds (Fig. 2B) represents elimination of PtdIns(4,5)P_2 in ~80% of the cells (Fig. 2C); the remaining ~20% overlap with the fluorescence observed in resting cells (Fig. 2C), because these cells had yet to begin exocytosis (e.g. Fig. 2A, 15 seconds, lower cell).

Is the 2C11 antibody an accurate tool with which to study PtdIns(4,5)P_2 dynamics? In principle, PtdIns(4,5)P_2 binding to an endogenous effector might produce a decrease in staining. Although this seems unlikely given the harsh fixation procedure used in these experiments, we directly measured PtdIns(4,5)P_2 by metabolic labelling of mast cells with [2-^{3}H]-inositol. RPMCs were left at rest or stimulated with 48/80 for up to 60 seconds; cells were then either fixed and stained, or lysed in acid and lipids were extracted and analysed by HPLC. Once again, we saw evidence of transient depletion of plasma membrane PtdIns(4,5)P_2 (Fig. 2D and supplementary material Fig. S2). The relative decrease in fluorescence intensity was less marked in cells labelled with inositol for 19 hours (compare Fig. 2B and 2D). However, this was owing to decreased fluorescence intensity in the resting population of labelled RPMCs (compare Fig. 2C with supplementary material Fig. S2B), rather than an incomplete elimination of PtdIns(4,5)P_2 in stimulated cells. Furthermore, the labelled RPMCs displayed a more rapid recovery of PtdIns(4,5)P_2 after labelling in vitro for 19 hours. However, despite these differences, the levels of [2-^{3}H]-PtdIns(4,5)P_2 were in excellent agreement with the fluorescence data (Fig. 2D).

Biochemical estimates of PtdIns(4,5)P_2 levels tended to be slightly higher than fluorescence estimates after stimulation (Fig. 2D). We reasoned that this might be due to the existence of a second, minor pool of PtdIns(4,5)P_2 not detected after saponin permeabilisation (see Materials and Methods). Many cells contain a detergent-resistant nuclear pool of phosphoinositides (Hammond et al., 2004). Indeed, after extraction of membranes with Triton X-100, we observed a punctate staining of RPMC nuclei (supplementary material Fig. S1A), consistent with previous reports using 2C11 in other cells (Osborne et al., 2001). This staining was observed to increase dramatically after stimulation of cells with 48/80 (supplementary material Fig. S1A,B). Although direct comparison of membrane and nuclear PtdIns(4,5)P_2 pools was not possible owing to the different staining protocols, the nuclear pool contained approximately 1% of the fluorescence compared with the membrane pool, accounting for the discrepancy with biochemical measurements (Fig. 2D). Therefore, we conclude that after the onset of exocytosis, plasma membrane PtdIns(4,5)P_2 becomes depleted from mast cells, while a minor nuclear pool increases.

PLC mediates the depletion of plasma membrane PtdIns(4,5)P_2

A clue to the cause of PtdIns(4,5)P_2 depletion during exocytosis was given by experiments akin to those illustrated in Fig. 2D. When the total phosphoinositide levels are plotted as a function of time after stimulation, a steady decline to approximately 85% of control after 60 seconds is observed (data not shown). This depletion was predominately from PtdIns, and indicates the action of a phospholipase.

To test whether PLC caused the depletion of plasma membrane PtdIns(4,5)P_2, we used two inhibitors of PLC: the thiol-reactive U73-122 (Horowitz et al., 2005) and Et-18-OMe (Powis et al., 1992). U73-122 inhibited exocytosis with an apparent IC_{50} of ~2 μM, whereas the inactive analogue U73-343 had an IC_{50} approximately tenfold higher (Fig. 3A). Et-18-OMe inhibited exocytosis with an IC_{50} of ~27 μM (Fig. 3B), whereas the vehicle control had no discernible effect. Both inhibitors caused release of β-hexosaminidase (β-hex) from unstimulated cells at higher concentrations, indicating cell lysis (data not shown). Pre-incubation of cells with 5 μM U73-122 or 40 μM Et-18-OMe prevented exocytosis and blocked the

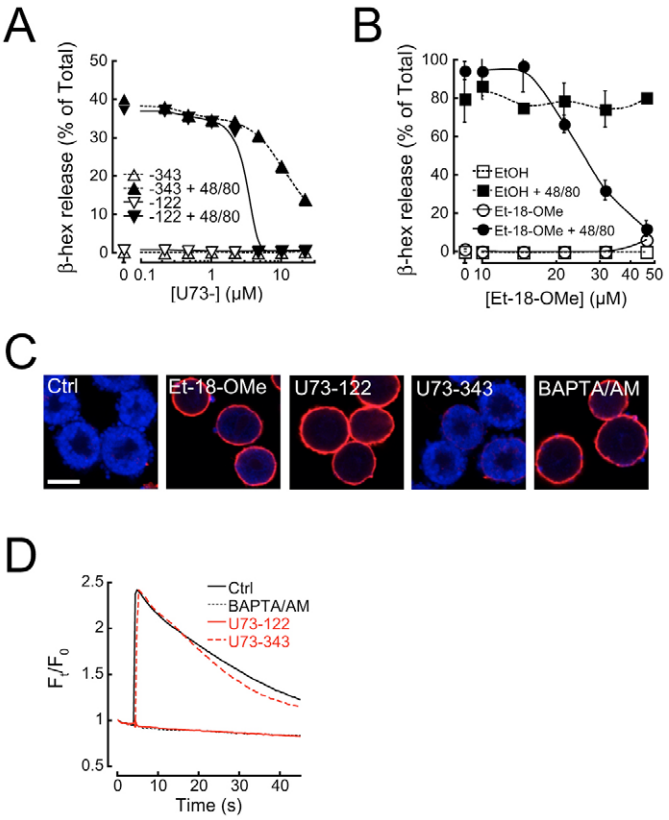

Fig. 3. Phospholipase C causes the depletion of plasma membrane PtdIns(4,5)P_2, and the release of Ca^{2+} from intracellular stores. (A,B) PLC inhibitors prevent mast cell degranulation. RPMCs were pre-incubated with U73-122 and/or U73-343 for 5 minutes (A), or Et-18-OMe (or EtOH, as vehicle control) for 20 minutes (B), at the indicated concentration before stimulation with 48/80 at 25°C. After 10 minutes, the medium was assayed for released β-hexosaminidase (β-hex) activity. Data are means of triplicates ± s.e.m. (C) PLC inhibitors block depletion of PtdIns(4,5)P_2. Mast cells were untreated (ctrl) or incubated with 5 μM BAPTA/AM or as described in A and B. Subsequently, degranulation was evoked with 48/80 for 30 seconds. Cells were fixed and stained with 2C11 (red) and Alexa Fluor 647-concanavalin A (blue). Bar, 10 μm. (D) PLC inhibitors block Ca^{2+} signalling. RPMCs were loaded for 20 minutes with Fluo3/AM, pre-incubated with the indicated compound as in A-C or for 20 minutes with 5 μM BAPTA/AM, and activated with 48/80. Normalised fluorescence intensity traces are shown from a single representative cell for each condition.

depletion of PtdIns(4,5)P_2, whereas no effect of 5 μM U73-343 was observed (Fig. 3C). Thus, PLC activity seems to cause the reduction in plasma membrane PtdIns(4,5)P_2, and is required for exocytosis.

Bone marrow derived mast cells require PLCγ2 for Ca^{2+} release from intracellular stores as well as for exocytosis (Wen et al., 2002), suggesting a similar function in RPMCs. To test this possibility, mast cells were loaded with the Ca^{2+} indicator Fluo3/AM and stimulated with 48/80. In agreement with previous studies (Penner, 1988), we observed a rapid increase in the intracellular Ca^{2+} concentration in control cells after activation, which declines slowly (Fig. 3D). When cells were treated with 5 μM U73-122 this change in cytosolic Ca^{2+} levels was completely abolished (Fig. 3D), whereas U73-343 was without effect. Thus, it appears that PLC is required for the Ca^{2+} release from intracellular stores, consistent with the previous observation that injection of neomycin, which also inhibits PLC, blocks Ca^{2+} release from intracellular stores in RPMCs (Penner, 1988).

Is Ca^{2+} release from internal stores strictly required for exocytosis to occur? This seems to be the case, because loading cells with the cell-permeant Ca^{2+} chelator BAPTA/AM inhibits exocytosis. However, depletion of plasma membrane PtdIns(4,5)P_2 was also inhibited (Fig. 3C). This was most probably due to the reduction of basal Ca^{2+} levels and thus inhibition of PLC, which is a Ca^{2+}-dependent enzyme (Rhee et al., 1989).

PLC activity in permeabilised mast cells

To investigate whether hydrolysis of bulk PtdIns(4,5)P_2 was required for mast cell exocytosis, we utilised cell permeabilisation with streptolysin-O (SL-O). Permeabilised RPMCs undergo exocytosis in the presence of Ca^{2+} buffered in the μM range, although provision of GTP or its non-hydrolysable analogue GTPγS is absolutely required (Howell et al., 1987). Since Ca^{2+} is buffered with EGTA, Ca^{2+} release from stores will not lead to an elevation in Ca^{2+} concentration, thus bypassing Ins(1,4,5)P_3-mediated Ca^{2+} release.

When RPMCs are permeabilised for 3 minutes before fixation in the presence of 100 nM free Ca^{2+} (pCa 7) and 100 μM MgATP, PtdIns(4,5)P_2 displays a distribution similar to that observed in intact, resting cells and no exocytosis is detected (Fig. 4A). However, if cells are permeabilised in the presence of MgATP, 10 μM GTPγS and 10 μM free Ca^{2+} (pCa 5) a stochastic activation of the cells is observed (Hide et al., 1993): the majority of cells appeared to have degranulated after 3 minutes, although a minority have not yet begun exocytosis. The proportion of mast cells undergoing exocytosis under these conditions increases with time, although for individual cells exocytosis may take a matter of seconds (G.R.V.H., unpublished observations). Strikingly, in cells that are yet to begin exocytosis, PtdIns(4,5)P_2 remains on the plasma membrane and may even be increased (Fig. 4A). On the contrary, cells that undergo exocytosis show almost complete elimination of PtdIns(4,5)P_2. No recovery of PtdIns(4,5)P_2 is observed in any of the cells within 3 minutes (Fig. 4A). In ten experiments, the proportion of degranulated cells varied widely from 44% to 100%, with a mean ± s.d. of 79%±20%.

What causes the depletion of PtdIns(4,5)P_2 from permeabilised cells? Under these conditions, PLC, phosphoinositide 3-kinases and inositol phosphatases, all of

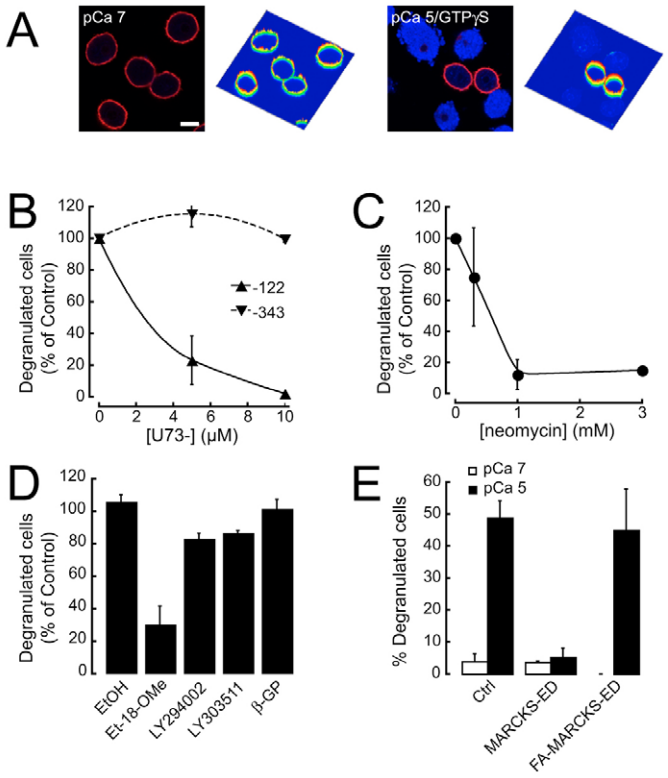

Fig. 4. PLC causes depletion of PtdIns(4,5)P_2 from permeabilised mast cells. (A) RPMCs were permeabilised with SL-O in the presence of 100 μM MgATP and 3 mM Ca:EGTA at pCa 7, or 100 μM MgATP, 10 μM GTPγS and 3 mM Ca:EGTA at pCa 5 for 3 minutes at 30°C. Cells were then fixed and stained with 2C11 (red) and Alexa Fluor 647-concanavalin A (blue). Bar, 10 μm. (B-D) PLC inhibitors prevent PtdIns(4,5)P_2 depletion and block degranulation of permeabilised cells. Mast cells 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. (E) RPMCs were permeabilised in the presence of 100 μM MgATP and 300 μM Ca:EGTA at pCa 8 at 30°C. After 2 minutes, the indicated peptide was added to 100 μM. After a further 5 minutes, cells were activated by the addition of the indicated buffers at the same concentrations as (A). 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.

which can deplete plasma membrane PtdIns(4,5)P_2, may be activated. Treatment of cells with U73-122 during permeabilisation blocked exocytosis and the depletion of PtdIns(4,5)P_2, whereas U73-343 was without effect (Fig. 4B, Fig. 5A). At 40 μM, Et-18-OMe did not have such dramatic effects in permeabilised cells, but still decreased the proportion of degranulating cells by approximately 70% relative to untreated controls (Fig. 4D). Neomycin at 1-3 mM (Fig. 4C) also blocked exocytosis, although results were extremely variable at 300 μM (Fig. 4C). However, in some cells 1-3 mM neomycin appeared to cause a reduction in the extent of PtdIns(4,5)P_2 staining despite a lack of degranulation (Fig. 5A). This is possibly caused by the interaction of neomycin

Journal of Cell Science

with PtdIns(4,5)P_2, which can effectively inhibit 2C11 labelling at these concentrations (Fig. 1B).

We also used the myristoylated alanine-rich C-kinase substrate basic effector domain (MARCKS-ED) because this peptide was shown to laterally sequester PtdIns(4,5)P_2 in the membrane, inhibiting PLC but sparing other interactions such as with the PH domain from PLCδ1 (Gambhir et al., 2004). Equilibration of 100 μM peptide into permeabilised RPMCs abolished exocytosis (Fig. 4E) as well as breakdown of PtdIns(4,5)P_2 (Fig. 5B). By contrast, a mutant peptide in which four Phe residues are mutated to Ala (FA-MARCKS-ED) was shown to display a weaker interaction with PtdIns(4,5)P_2 at the membrane and, consequently, a far less potent inhibition of PLC (Gambhir et al., 2004); this peptide affected neither exocytosis (Fig. 4E) or plasma membrane PtdIns(4,5)P_2 depletion (Fig. 5B). Together, these results are therefore consistent with a role for PLC-mediated PtdIns(4,5)P_2 breakdown in exocytosis.

By contrast, use of β-glycerophosphate (β-GP, 5 mM) as a generic phosphatase inhibitor did not effect exocytosis or depletion of PtdIns(4,5)P_2 (Fig. 4D, Fig. 5A). At 100 μM, the phosphoinositide 3-kinase inhibitor LY294002 produced a 20% reduction in the number of degranulating cells (Fig. 4D). However, its inactive analogue LY303511 (Vlahos et al., 1994) produced an identical effect (Fig. 4D), allowing us to conclude that the inhibition was not due to impairment of phosphoinositide 3-kinases.

RPMCs not undergoing exocytosis were replete with plasma membrane PtdIns(4,5)P_2, whereas cells that showed signs of degranulation showed almost complete elimination of PtdIns(4,5)P_2 (Fig. 4A, Fig. 5). We occasionally observed cells that had not degranulated yet still lost their PtdIns(4,5)P_2 staining, but these were always a minority (< 5%) for most of the conditions shown in Figs 4 and 5. The presence of U73-122 increased this proportion to approximately 7% at 5 μM and as high as 12% at 10 μM. The lack of PtdIns(4,5)P_2 immunoreactivity might be due to the action of phosphatases in the absence of PLC-mediated PtdIns(4,5)P_2 hydrolysis. However, we never observed cells with signs of compound exocytosis that retained PtdIns(4,5)P_2 under any experimental condition tested. Therefore, the depletion of plasma membrane PtdIns(4,5)P_2 observed from permeabilised cells is indispensable for exocytosis to occur and appears to be mediated by PLC.

We also tested the effect of U73-122 and Et-18-OMe on the release of β-hex from permeabilised RPMCs in suspension and found both inhibited, with apparent IC_{50}s of 2 and 20 μM, respectively (supplementary material Fig. S3). Once again, U73-343 had only a minor effect, and Et-18-OMe became lytic at higher concentrations. Our results with U73-122 and U73-343 are consistent with those reported independently by another group (Gloyna et al., 2005) in SL-O permeabilised mast cells.

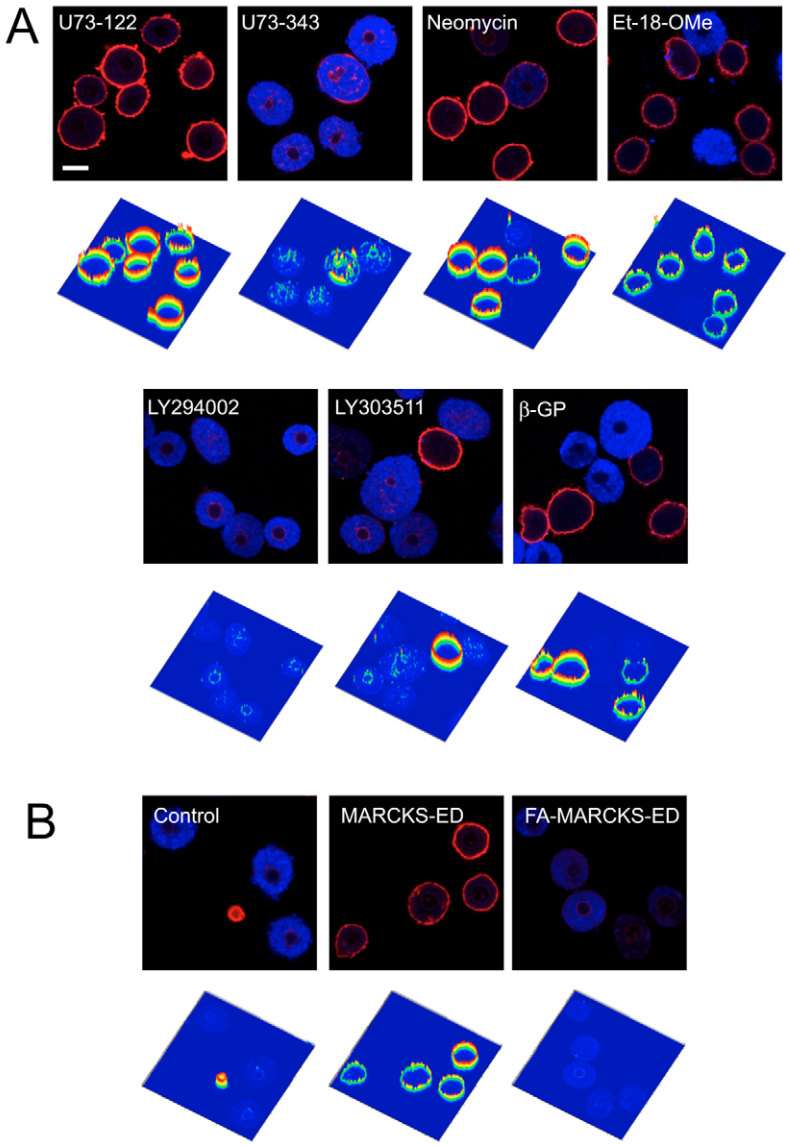

Fig. 5. (A) Mast cells were permeabilised with SL-O in the presence of 100 μM MgATP, 10 μM GTPγS and 3 mM Ca:EGTA at pCa 5 for 3 minutes at 30°C as in Fig. 4A, in the presence of 10 μM U73-122 or U73-343, 3 mM neomycin, 40 μM Et-18-OMe, 100 μM LY294002, 100 μM LY303511 or 5 mM β-GP. (B) Cells were permeabilised in the presence of 300 μM Ca:EGTA at pCa 8; after two minutes, the indicated peptide was added to 100 μM. After a further 5 minutes at 30°C, Ca:EGTA at pCa 5 and GTPγS were added to the same final concentrations as (A), and the cells incubated for 5 minutes. Cells were then fixed and stained with 2C11 (red) and Alexa Fluor 647-concanavalin A (blue). Top panels show merged images of 2C11 and concanavalin A; bottom panels show the fluorescence intensity profile for 2C11. Bar, 10 μm.

Downstream of PLC activation

How does hydrolysis of PtdIns(4,5)P_2 by PLC control exocytosis? Although in a permeabilised cell system with buffered Ca^{2+} we can rule out a role for Ins(1,4,5)P_3 in mediating Ca^{2+} release, there might be other functions for this inositol phosphate. However, Ins(1,4,5)P_3 and Ins(1,4)P_2 at concentrations of up to 100 μM failed to modify exocytosis from permeabilised cells in the presence or absence of PLC inhibitors (data not shown). Furthermore, a degradation

Journal of Cell Science

product of Ins(1,4,5)P_3 is probably not involved because mast cell exocytosis is insensitive to mM concentrations of LiCl, (Cockcroft et al., 1987), which block the dephosphorylation of Ins(1,4)P_2. Finally, Ins(1,4,5)P_3 can be phosphorylated by an InsP_3 3-kinase and enter into the metabolic pathway of inositol polyphosphates. These molecules have many functions including regulation at multiple steps of membrane traffic (Irvine and Schell, 2001). However, because mast cell exocytosis proceeds when the cells are depleted of ATP (Howell et al., 1987), InsP_3 3-kinase activity is probably not required for exocytosis. This leaves us with two non-mutually exclusive possibilities. First, the other product of PLC, 1,2-diacyl-*sn*-glycerol (DAG) may be required. Second, elimination of plasma membrane PtdIns(4,5)P_2 may be needed for exocytosis, as has been shown for phagosome formation (Scott et al., 2005), inactivation of certain ion channels (Suh and Hille, 2005) and for *Salmonella* invasion (Terebiznik et al., 2002).

To test whether removal of plasma membrane PtdIns(4,5)P_2 was sufficient to allow exocytosis to proceed, we utilised the bacterial inositol phosphatase SigD, which hydrolyses PtdIns(4,5)P_2 (Marcus et al., 2001; Terebiznik et al., 2002).

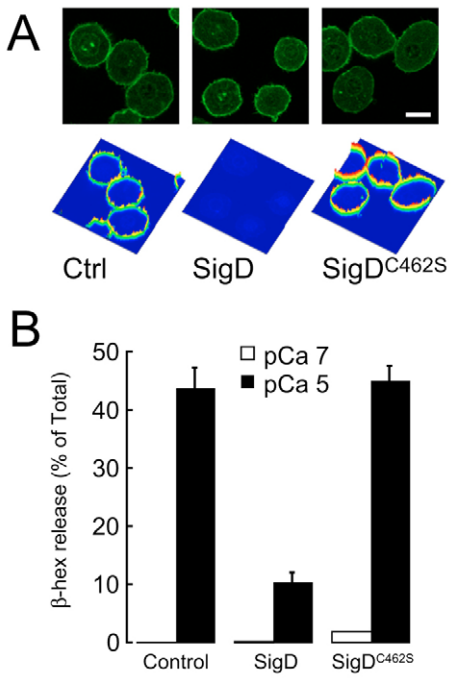

Fig. 6. SigD depletes plasma membrane PtdIns(4,5)P_2 and inhibits mast cell degranulation. (A) RPMCs were permeabilised with SL-O in the presence of 100 μM MgATP and 300 μM Ca:EGTA at pCa 8 in the absence (Ctrl) or presence of 100 μg/ml of SigD wild-type or the inactive SigD^{C462S} mutant. After 10 minutes at 30°C, cells were fixed and stained with 2C11 and Alexa Fluor 488-phalloidin. Top panels show phalloidin staining, bottom panels show the fluorescence intensity profile for 2C11. Bar, 10 μm. (B) Mast cells in suspension were treated as in A. After 10 minutes at 30°C, cells were kept inactive by addition of 3 mM Ca:EGTA at pCa 7, or stimulated with 3 mM Ca:EGTA at pCa 5 and 100 μM GTPγS (SigD^{C462S}) as indicated. After a further 10 minutes, cells were quenched with ice-cold 5 mM EGTA, centrifuged and supernatants assayed for β-hexosaminidase (β-hex) activity.

Equilibration of 100 μg/ml recombinant SigD into mast cells permeabilised with SL-O causes the complete disappearance of plasma membrane PtdIns(4,5)P_2 (Fig. 6A); however, when cells are stimulated under these conditions exocytosis is inhibited (Fig. 6B). This depends on the catalytic activity of SigD, because a point mutation in its active site (C462S) (Marcus et al., 2001; Terebiznik et al., 2002) renders the enzyme incapable of cleaving plasma membrane PtdIns(4,5)P_2 (Fig. 6A) and does not affect exocytosis (Fig. 6B). Therefore, simple elimination of PtdIns(4,5)P_2 is not sufficient for exocytosis to occur.

To test whether DAG is required for exocytosis, we utilised a bacterial, PtdIns-specific PLC (PtdIns-PLC) that hydrolyses PtdIns as opposed to PtdIns(4,5)P_2. This enzyme reproducibly promoted exocytosis after equilibration into permeabilised cells (Fig. 7A), an effect which was not due to loss of plasma membrane PtdIns(4,5)P_2 (supplementary material Fig. S4) and not observed with heat-inactivated enzyme (Fig. 7A,B). This effect was consistent with that observed with the DAG analogues phorbol 12-myristate 13-acetate (PMA, but not the inactive 4-α-PMA), 12,13-dibutyrate (PDBu) and 1-oleoyl 2-acetyl-*sn*-glycerol (OAG) (Fig. 7C) as reported previously (Howell et al., 1989). However, PtdIns-PLC did not restore exocytosis when endogenous PLC was blocked with Et-18-OMe (Fig. 7A), indicating that DAG generation per se is not sufficient for exocytosis.

This left the possibility that concomitant removal of plasma membrane PtdIns(4,5)P_2 and generation of DAG are required for exocytosis. We attempted to test this hypothesis by simultaneous equilibration of SigD and PtdIns-PLC into permeabilised cells. However, the independent effects of the two enzymes were simply additive, suggesting that they acted on separate processes (Fig. 7D). The catalytic product of SigD is probably a PtdIns*P* isomer, which may exhibit some of the function(s) of PtdIns(4,5)P_2. Therefore, we used neomycin because this aminoglycoside forms an electroneutral complex with PtdIns(4,5)P_2, disrupting interactions with this lipid and preventing activity of PLC (Gabev et al., 1989). When PtdIns-PLC was equilibrated into permeabilised cells for 5 minutes, the concentration of neomycin required to inhibit exocytosis was increased (Fig. 7E), indicating a partial restoration of exocytosis by DAG. These data are consistent with a previous report using PMA (Howell et al., 1989), and suggest that generation of DAG might indeed be an important factor in the triggering of exocytosis, with PtdIns(4,5)P_2 hydrolysis also being required.

Discussion

Immunofluorescence as a quantitative tool to monitor PtdIns(4,5)P_2 dynamics

Our early experiments with the anti-PtdIns(4,5)P_2 antibody 2C11 yielded a punctate cytoplasmic staining, which was poorly colocalised with membrane markers (not shown). While this work was under way, Watt and colleagues demonstrated that PtdIns(4,5)P_2 is shed from membranes of cryo-sections at room temperature; this problem was overcome by performing all post-sectioning steps at temperatures at 4°C or below (Watt et al., 2002). We applied the principle of staining at reduced temperatures to our immunofluorescence protocols. This method yielded excellent membrane retention of PtdIns(4,5)P_2 and, in combination with quantitative analysis of fluorescence

intensity, we observed a transient depletion of plasma membrane PtdIns(4,5)P_2 during exocytosis.

Our fluorescence measurements yielded excellent agreement with biochemical estimates of PtdIns(4,5)P_2 levels (Fig. 2). However, both methods suffer the drawback of taking measurements from cells post mortem. In general, immunofluorescence techniques are not applicable to live cell imaging, unless invasive microinjection is employed. Many studies have followed phosphoinositide dynamics in live cells using specific phosphoinositide-binding domains fused to GFP (Halet, 2005), and measurements of fluorescence under such conditions can also produce similar results to biochemical estimates (Varnai and Balla, 1998). However, such phosphoinositide-binding modules are almost certainly targeted by protein determinants acting in conjunction with phosphoinositides, thus might only label a sub-pool of phosphoinositides and interfere with phosphoinositide-dependent processes (Raucher et al., 2000; Varnai et al., 2002). Moreover, immunofluorescence relies on antibodies raised against the pure lipid, thus can detect multiple pools of PtdIns(4,5)P_2 in cells – a cumbersome task with biochemical methods. It is also amenable to high throughput analysis, and has the advantage over biochemical methods of detecting the behaviour of individual cells in a population, as well as the population mean (Fig. 2). In conclusion, we believe quantitative immunofluorescence of phosphoinositides adds a powerful new tool to the repertoire of techniques available to study phosphoinositide dynamics in cells.

PLC-mediated hydrolysis is required for mast cell exocytosis

In this study, we report a complete elimination of plasma membrane PtdIns(4,5)P_2 at the onset of exocytosis; notably, PtdIns(4,5)P_2 also remained undetectable on the membranes of fusing granules. This observation has important implications how PtdIns(4,5)P_2 might regulate exocytosis, because as the bulk lipid is destroyed, it cannot function directly in the membrane fusion process. Using a combination of biochemical and pharmacological approaches, we have defined the pathway of PtdIns(4,5)P_2 depletion as PLC-mediated. PLC appeared necessary to initiate the Ca^{2+} signal at the onset of exocytosis (Fig. 4D) yet, conversely, Ca^{2+} appears necessary for PLC activity (Fig. 4C). Further experiments, where the Ins(1,4,5)P_3-Ca^{2+} pathway was bypassed with Ca^{2+} buffers in permeabilised cells, revealed additional roles for PLC-mediated PtdIns(4,5)P_2 depletion, beyond its role in Ca^{2+} signalling.

Our results contrast with an earlier study conducted in RPMCs that excluded additional roles for PLC in RPMC exocytosis (Cockcroft et al., 1987). This study relied solely on the use of neomycin, showing inhibition of PLC activity at lower concentrations than those that inhibit exocytosis. Our results demonstrate a good agreement between concentrations of several PLC inhibitors, including neomycin, that block exocytosis and PLC activity. We have also found that neomycin may interfere with the extraction of Ins(1,4,5)P_3 under the conditions used in the former study (data not shown), precluding accurate determination of PLC activity. Furthermore, a small undetectable hydrolysis may have persisted in these experiments, leading to the generation of sufficient DAG under conditions where bulk PtdIns(4,5)P_2 was sequestered by neomycin.

What could be the role of PLC-mediated PtdIns(4,5)P_2

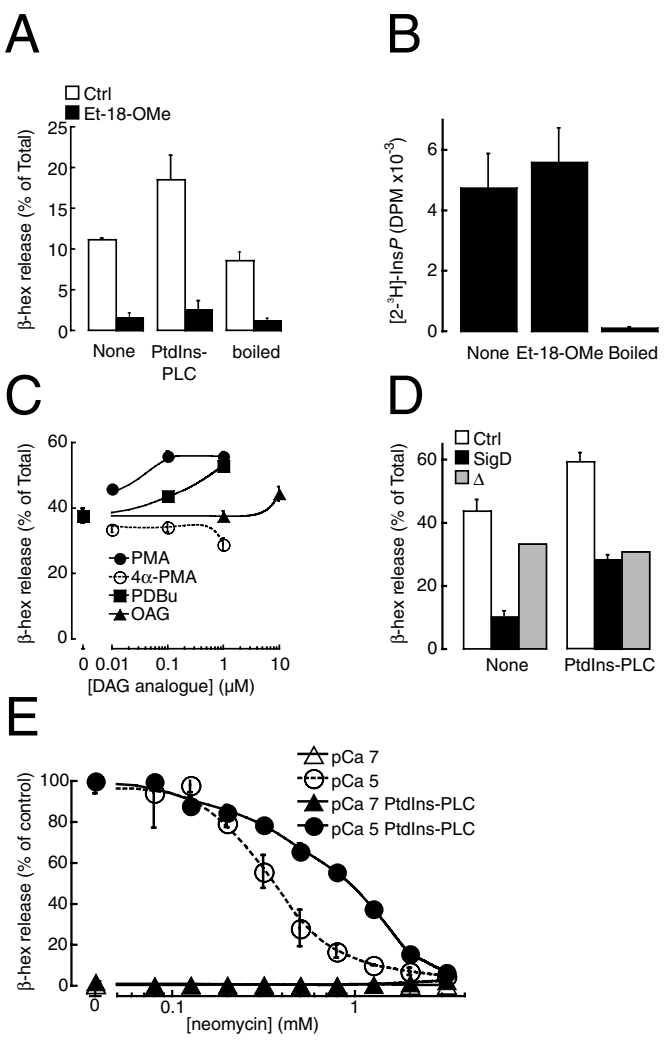

Fig. 7. DAG partially restores exocytosis from permeabilised mast cells. RPMCs in suspension were permeabilised for the indicated time at pCa 8 and then stimulated as described in Fig. 6B. Unstimulated cells (pCa 7) released ⩽ 2% of their total β-hexosaminidase (β-hex) activity. (A) Cells were permeabilised for 10 minutes in the absence (Ctrl) or presence of 0.3 U/ml PtdIns-PLC, with or without 20 μM Et-18-OMe as indicated. (B) In vitro activity of PtdIns-PLC against [2-^{3}H]-PtdIns in the absence or presence of 20 μM Et-18-OMe. Boiled PtdIns-PLC refers to 0.3 U/ml of the enzyme inactivated at 110°C for 15 minutes. (C) Indicated DAG analogues were applied for 10 minutes before stimulation. (D) Combined effect of PtdIns-PLC and SigD. Mast cells were permeabilised in the presence of 0.3 U/ml PtdIns-PLC and 100 μg/ml SigD for 10 minutes as indicated; Δ refers to the difference in β-hex release between incubations with and without SigD. (E) Effect of PtdIns-PLC on the IC_{50} of neomycin. Cells were permeabilised in the presence of the indicated concentration of neomycin with or without 0.3 U/ml PtdIns-PLC for 5 minutes before stimulation. Data are normalised to the levels of secretion observed without neomycin, which were 92±4% of the total cellular β-hex activity in the presence of PtdIns-PLC, and 72±4% in its absence. Data are means ± s.e.m. (n=3) for stimulated cells (for inactive cells at pCa 7, n=1).

Journal of Cell Science

depletion during exocytosis? PLC depletes PtdIns(4,5)P_2 from incipient phagosomes, causing disassembly of the surrounding F-actin (Scott et al., 2005). Furthermore, SigD eliminates plasma membrane PtdIns(4,5)P_2, causing a reduction in membrane cytoskeletal rigidity to facilitate invasion by *Salmonella typhimurium* (Terebiznik et al., 2002). Mast cells contain an F-actin cortex that breaks down at the onset of degranulation (Nishida et al., 2005; Norman et al., 1996). Disruption of this cortex is seen to enhance degranulation (Borovikov et al., 1995; Martin-Verdeaux et al., 2003), and the augmented degranulation by PIPK Iα-deficient mast cells is partially mimicked by depletion of the F-actin cortex in wild-type cells (Sasaki et al., 2005). Notably, although SigD eliminated plasma membrane PtdIns(4,5)P_2, it failed to reduce the actin cortex in RPMCs (Fig. 5A). Therefore, it seems possible that PtdIns(4,5)P_2 breakdown is associated with the disassembly of the cortical actin cytoskeleton. However, stabilisation of this cortex with phalloidin is not seen to affect exocytosis (Norman et al., 1996), so simple removal of an F-actin 'barrier' seems unlikely. Alternatively, removal of plasma membrane PtdIns(4,5)P_2 might lead to the dissociation of an F-actin-associated factor. Notably, the SNARE protein SNAP-23, which relocates from plasma to granule membranes for compound exocytosis, is associated with F-actin inside the membrane folds of resting RPMCs (Guo et al., 1998).

The enhancement of exocytosis from RPMCs by DAG and its analogues, along with its sparing effect on the inhibition by neomycin (Fig. 7) lead us to suspect that PLC-mediated generation of this lipid is also be important for exocytosis, even if it is not sufficient. However, it is unlikely to act through DAG-kinase or protein kinase C (PKC), because mast cell exocytosis does not require ATP (Howell et al., 1987), and is insensitive to PKC inhibitors or PKC pseudosubstrate peptides (Gloyna et al., 2005; Howell et al., 1989; Pinxteren et al., 2001; Shefler et al., 1998). Instead, it might act through another C1-domain-containing protein (Brose and Rosenmund, 2002) such as Munc13, which is the sole target of DAG for neuroexocytosis (Rhee et al., 2002).

A general role for PLC in exocytosis?

We have demonstrated a crucial role for bulk hydrolysis of plasma membrane PtdIns(4,5)P_2 in mast cell exocytosis. Might such a mechanism operate during exocytosis in other cell types? Several lines of evidence lead us to suspect so. First, a late activation of PLC is entirely consistent with a role of PtdIns(4,5)P_2 in priming of exocytosis. Second, there is an obligate role for DAG generation during exocytosis in neurons to facilitate activation of Munc13 (Brose and Rosenmund, 2002). Third, activation of exocytosis stimulates PLC in a number of cell types, including adrenal chromaffin cells (Whitaker, 1985) and RPMCs (Cockcroft and Gomperts, 1979). Finally, U73-122 and isoform-specific PLC antibodies were found to inhibit chromaffin-cell exocytosis (O'Connell et al., 2003). However, PtdIns(4,5)P_2 depletion might not be apparent in neural and endocrine cells, because sustained synthesis of PtdIns(4,5)P_2 would be required to permit multiple rounds of vesicle priming and compensatory endocytosis that accompany exocytosis.

Conclusion

We have used a novel quantitative immunofluorescence technique to follow PtdIns(4,5)P_2 dynamics during exocytosis from mast cells. We have established that elimination of plasma membrane PtdIns(4,5)P_2 by PLC is required for exocytosis, independent of the Ins(1,4,5)-P_3–Ca^{2+} pathway. Further work will be required to understand the molecular basis behind this pathway, but it most likely involves both consumption of PtdIns(4,5)P_2 and concomitant production of DAG. We propose that such a cycle underpins the regulation of priming and exocytosis in the majority of professional secretory cells.

Materials and Methods

Materials and recombinant proteins

Et-18-OMe, U73-122 and U73-343 were from Calbiochem, neomycin from Alexis Biochemicals and *B. cereus* PtdIns-specific phospholipase C from Sigma. Radionuclides were from Amersham Biosciences. All other reagents of analytical quality or higher were obtained from standard commercial sources.

GST-tagged PH-PLCδ_1 was a kind gift of M. Katan (Institute of Cancer Research, London, UK); GST-tagged PH-GRP$_1$ was generated as described (Klarlund et al., 2000) and cloned into a modified pGEX-4T3 vector. His_6-SigD wild-type and C462S (Marcus et al., 2001) were kind gifts of B. Finlay (Michael Smith Laboratories, University of British Columbia, Vancouver, Canada). Plasmids were transformed into BL21(DE3) and grown to an OD_{600} 0.8-1.4; protein expression was induced with 400 μM isopropyl β-D-1-thiogalactopyranoside for 3 hours at 37°C (PH-PLCδ_1 or PH-GRP$_1$), or 100 μM for 16 hours at 18°C (SigD). Bacteria were harvested, washed twice in PBS containing 0.05% Tween-20, and lysed in a French press upon resuspension in breaking buffer [PBS with EDTA-free protease inhibitor tablets (Roche), 2 mM EDTA, 4 μg/ml pepstatin, 0.1% 2-mercaptoethanol and either 0.05% Tween-20 (for PH-PLCδ_1 and PH-GRP$_1$) or 1% Triton X-100 and 10 mM imidazole (SigD)]. Lysates were then cleared of insoluble material by centrifugation at 27,000 ***g*** for 15 minutes followed by 160,000 ***g*** for 20 minutes. Supernatants were then purified on glutathione-agarose or Ni-agarose and eluted with 2.5 mM reduced glutathione or 250 mM imidazole, respectively. Finally, proteins were dialysed into intracellular buffer (IB: 20 mM PIPES-NaOH, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1 mg/ml BSA, pH 6.8) without BSA, flash-frozen in liquid nitrogen and stored at –80°C.

Stimulation of cells and secretion assay

RPMCs were purified from male retired-breeder Sprague Dawley rats (Charles River) as described previously (Gomperts and Tatham, 1992). Briefly, 50 ml of peritoneal washings (in 0.9% NaCl, 1 mg/ml BSA) were pelleted, resuspended to 8.5 ml in extracellular buffer (EB: 20 mM HEPES-NaOH, 137 mM NaCl, 2.7 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5.6 mM glucose, 1 mg/ml BSA, pH 7.2) and filtered through a nylon mesh. Mast cells were then purified from the suspension by centrifugation through a 1.5 ml Percoll cushion at 1.113 g/ml. Purified RPMCs were washed once and seeded on glass eight-well multi-test slides (MP Biomedicals) or plastic 96-well plates (Corning) for 30 minutes at room temperature.

When left intact, cells were pre-incubated as described in figure legends before stimulating with 10 μg/ml compound 48/80 (Sigma) in EB for the stated time periods. For permeabilisation experiments, adherent cells were rinsed twice with 5 mM EGTA in IB to remove extracellular Ca^{2+}. Subsequently, they were chilled on ice and incubated for 8 minutes in IB containing 1.6 IU/ml streptolysin-O (SL-O: iTest plus Ltd). Excess SL-O was then removed by rinsing once with ice-cold IB, and ice-cold stimulation buffers were added. These consisted of IB containing 0.3-3 mM Ca:EGTA and 100 μM MgATP; GTPγS (Lithium salt solution, Roche Molecular Biochemicals) was included at 10 or 100 μM, as were compounds or recombinant protein as described in the figure legends. Permeabilisation was then initiated by warming the slides to 30°C.

For secretion assays on cells in suspension, experiments were performed exactly as described previously (Pinxteren et al., 2001), using a fluorometric assay for secreted β-hexosaminidase (Gomperts and Tatham, 1992).

Immunofluorescence staining

At the end of the stimulation period, mast cells on multi-test slides were transferred to ice and quenched with ice-cold fixative (either 3% glutaraldehyde or 0.2% glutaraldehyde with 4% paraformaldehyde in PBS); they were then left to fix for 3 hours at 4°C. All subsequent steps were performed either on ice or at 4°C, with care taken not to allow slides to warm above 4°C. After fixation, the cells were rinsed thrice with 50 mM NH_4Cl in PBS, stained for 5 minutes with 200 μg/ml Alexa Fluor 647-concanavalin A (Molecular Probes) and rinsed twice in PBS. Cells were then permeabilised and blocked for 4 hours with 0.5% saponin, 5% normal goat serum (NGS, Gibco RBL) and 50 mM NH_4Cl in sodium glutamate buffer (NaGB: 20 mM PIPES-NaOH, 137 mM sodium glutamate, 2 mM $MgCl_2$, 1 mg/ml BSA, pH 6.8). For competition by PH domains, 50 μM of

Journal of Cell Science

the relevant recombinant proteins were included. This solution was then removed and replaced with antibody solution (0.1% saponin and 5% NGS in NaGB), containing 16 μg/ml anti-PtdIns(4,5)P_2 antibody 2C11 (Osborne et al., 2001; Thomas et al., 1999). For competition experiments, 16 μg/ml 2C11 was pre-incubated with a 10,000-fold molar excess (approximately 200 μM, assuming 10 PtdIns(4,5)P_2-binding sites per molecule of 2C11 IgM) of inositol polyphosphates (Cell Signals Inc.). Antibody-inositol polyphosphate complexes were allowed to form for 30 minutes at room temperature, before chilling the mixture on ice. Cells were incubated with 2C11 overnight, and then washed twice for 10 minutes with NaGB, before adding 10 μg/ml Alexa Fluor 555-anti mouse IgM with or without 5 U/ml Alexa Fluor 488-phalloidin or 5 μM BODIPY-ceramide:BSA (Molecular Probes) in antibody solution for 4 hours. Cells were then washed four times for 10 minutes with NaGB; DAPI (Roche) or Draq5™ (Alexis Biochemicals) at 1:2000 were included in the second wash when used. Cells were next post-fixed for 10 minutes on ice, then 5 minutes at room temperature. Finally, cells were rinsed four times with 50 mM NH_4Cl in PBS and mounted in Mowiol4-88. For nuclear PtdIns(4,5)P_2 staining, the above protocol was modified by fixing cells with 4% paraformaldehyde, replacing NaGB with PBS, and using blocking- and antibody-solutions which contain 5% NGS, 0.2% Triton X-100. All stages were performed in the cold except for the blocking step, which was performed at room temperature.

When staining the plasma membrane, cells were incubated with 10 μM CM-DiI_{18} (Molecular Probes) for 10 minutes, rinsed twice in EB and then fixed for 3 hours with 0.2% glutaraldehyde, 4% paraformaldehyde in PBS at 4°C. Cells were rinsed and mounted as above.

Confocal microscopy and PtdIns(4,5)P_2 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 63× 1.4 NA PlanApochromat oil-immersion lens. Image intensity profiles were created with the Zeiss LSM 3.2 software.

For quantitative image analysis, 3D-image stacks comprising four (nuclei) or six (whole cell) 4 μm sections were acquired using a 40× 1.3 NA PlanApochromat oil-immersion lens and 4× averaging. Image stacks were saved in the original Zeiss format, with 2C11 (Alexa Fluor 555, Alexa Fluor 543 nm excitation) in the red channel. Laser-power- and detector-gain and -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 using a custom written journal in MetaMorph 6.3 image analysis software (Molecular Devices). 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 for 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-^{3}H]inositol labelling and HPLC

RPMCs prepared from four Sprague Dawley rats (≥500 g) were washed in Medium 199 (Gibco RBL) supplemented with 1 mg/ml BSA, 60 μg/ml penicillin and 100 μg/ml streptomycin and then seeded in four 35 mm dishes (2 ml/dish) in the presence of 25 μCi/ml [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 lysing cells at the indicated times with 0.5 ml 1 M HCl, supplemented with 5 mM tetrabutyl ammonium hydrogen sulphate. Lipids were then extracted as described (Jackson et al., 1992); extracts were dried under a stream of nitrogen, deacylated and analysed by HPLC (Dove et al., 1997).

Ca^{2+} imaging

Cells were seeded in the presence of 2 μM Fluo3/AM (Molecular Probes) for 30 minutes, before incubating in the presence of the stated compounds. Cells were then stimulated with 48/80 whilst images were acquired at approximately three frames/second with a 100× 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 milliseconds. 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).

In vitro assay of PtdIns-PLC

PtdIns-PLC (0.3 U/ml) was assayed in 20 μl of IB (without BSA) with or without Et-18-OMe. Heat-inactivated PtdIns-PLC was treated at 110°C for 15 minutes. 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 minutes 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. Then, 50 μl of aqueous phase was assayed for released [2-^{3}H]-Ins*P* by scintillation counting.

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