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

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Mast cells permeabilized by streptolysin O undergo exocytosis when stimulated with Ca^{2+} and guanosine 5'-[γ -thio]triphosphate but become progressively refractory to this stimulus if it is delayed. This run-down of responsiveness occurs over a period of 20–30 min, during which the cells leak soluble and tethered proteins. We show here that withdrawal of ATP during the process of run-down is strongly inhibitory but that as little as 25 μM ATP can extend responsiveness significantly; this effect is maximal at 50 μM . When phosphatidylinositol transfer proteins (PITPs) are provided to cells at the time of permeabilization, run-down is retarded. We conclude that in the presence of ATP they convey substrates for phosphorylation that are essential for exocytosis and thus interact with the regulatory machinery. Furthermore, we show that PITP α and PITP β have additive effects in this mechanism, suggesting that they are not functionally redundant. Alternatively, secretion from run-down cells can be

inhibited by the aminoglycoside antibiotic neomycin, which is understood to bind to phosphoinositide headgroups, and by a PH (pleckstrin homology) domain polypeptide that binds phosphoinositides. The apparent displacement of neomycin by exogenous PITPs suggests that these proteins screen essential lipids. Secretion from run-down cells is also inhibited by 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol (AMG-C₁₆), an inhibitor of protein kinase C. The lack of synergy between neomycin and AMG-C₁₆ suggests that protein kinase C independently provides a second essential component through protein phosphorylation and that there are two independent phosphorylation pathways necessary for secretion competence.

Key words: exocytosis, GTP-binding protein, neomycin, pleckstrin homology domain, streptolysin O.

INTRODUCTION

Mast cells permeabilized by streptolysin O (SLO) undergo exocytosis when stimulated with Ca^{2+} and guanosine 5'-[γ -thio]triphosphate (GTP[S]). For cells stimulated at the time of permeabilization (prompt stimulation), nearly all of the secretory products (measured as hexosaminidase) can be released. There is no requirement for ATP and therefore the reactions regulating exocytosis do not involve the phosphorylation of either proteins or lipids (inositides) [1]. However, for cells stimulated to secrete after a delay of some minutes after permeabilization (the run-down procedure), the presence of ATP becomes mandatory [2]. This indicates that particular phosphorylation states of cellular components are required for secretion to occur.

To elucidate the nature of the phosphorylation state that determines competence to undergo secretion, we have examined the effect of neomycin on cells stimulated at various times after permeabilization. This aminoglycoside antibiotic binds to the headgroup of PtdIns(4,5) P_2 (and other phosphoinositides), thereby preventing the access of phospholipase C (PLC) [3] and possibly other proteins as well. In spite of this, secretion from promptly stimulated cells is rather insensitive to the presence of neomycin [4]. At concentrations of neomycin that prevent phosphoinositide hydrolysis by PLC, only a partial decrease in secretion (approx. 15%) was recorded. Here we report that, after permeabilization, the secretory reaction becomes progressively

more sensitive to inhibition by neomycin and that this can be reversed by providing, from the outset, phosphatidylinositol transfer proteins (PITPs) or PtdIns(4,5) P_2 in soluble form, D-(+)-*sn*-1,2-di-*O*-octanoyl PtdIns(4,5) P_2 (3-*O*-phospho-linked) [diC₈-PtdIns(4,5) P_2]. Even after substantial run-down, the requirement for ATP can be saturated at 50 μM , which is sufficient to allow the operation of phosphoinositide 3-kinases (PI-3Ks) and phosphoinositide 4-kinases (PI-4Ks) [5,6] and also some isoforms of protein kinase C (PKC) [7]. Further experiments in which we applied neomycin, 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol (AMG-C₁₆) (an inhibitor of PKC) and PITPs indicate that the phosphorylation state required for secretory competence in mast cells is composed of two distinct components. These act independently and without synergy.

EXPERIMENTAL

Materials

SLO was from Abbott Laboratories (Dartford, Kent, U.K.; now Corgenix, Peterborough, Cambs., U.K.). AMG-C₁₆ was from Bachem AG (Bubendorf, Switzerland). Neomycin sulphate and dithiothreitol were from Alexis (Läufelfingen, Switzerland). diC₈-PtdIns(4,5) P_2 was from Echelon (Salt Lake City, UT, U.S.A.). Secondary antibodies were from Bio-Rad. Protein concentrations were determined with bicinchoninic acid assay

Abbreviations used: AMG-C₁₆, 1-*O*-hexadecyl-2-*O*-methyl-*rac*-glycerol; $\beta\text{ARK}_1\text{-Cp}$, β -adrenergic receptor kinase C-terminal peptide; diC₈-PtdIns(4,5) P_2 , D-(+)-*sn*-1,2-di-*O*-octanoyl PtdIns(4,5) P_2 (3-*O*-phospho-linked); GTP[S], guanosine 5'-[γ -thio]triphosphate; IEF, isoelectric focusing; PH, pleckstrin homology; PI-3K, phosphoinositide 3-kinase; PI-4K, phosphoinositide 4-kinase; PITP, phosphatidylinositol transfer protein; PKC, protein kinase C; PLC, phospholipase C; PtdCho, phosphatidylcholine; SLO, streptolysin O.

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kits from Pierce (Chester, Cheshire, U.K.). All other chemicals were of the highest quality from standard commercial sources.

All chromatographic columns and equipment were obtained from Pharmacia (Uppsala, Sweden).

Phosphorescence imaging plates (Fuji), phosphorescence imaging apparatus (FujiBAS1000) and software (Tina) were from Raytek (Sheffield, U.K.).

Sec14p^{WT}, Sec14p^{K66,239A} proteins and antibodies against Sec14p were provided by the laboratory of V. Bankaitis (Birmingham, AL, U.S.A.). Antibodies against PITP α and PITP β were provided by B. A. Hamilton (Cambridge, MA, U.S.A.).

The plasmid encoding the WAA mutant (two alanine residues inserted after Trp-643) of the C-terminal peptide of β -adrenergic receptor kinase (β ARK₁-Cp) was provided by R. Lefkowitz (Durham, NC, U.S.A.).

Mast cells

Cells were isolated by peritoneal lavage of male Sprague–Dawley rats (more than 300 g) and mast cells were purified (more than 98 %) by centrifugation through a Percoll cushion as described previously [8].

Calcium buffers

Calcium/EGTA buffers were prepared by mixing solutions, made up at identical concentrations adjusted to pH 6.8, of EGTA and end-point-titrated Ca/EGTA, according to a computer program, as previously described [8].

Secretion measurements

Cells, suspended in an iso-osmotic salts buffer solution [137 mM NaCl/2.47 mM KCl/1 mM MgCl₂/20 mM Pipes (pH 6.8)] supplemented with BSA (1 mg/ml) were incubated with metabolic inhibitors [2-deoxyglucose (0.6 mM) and antimycin A (10 μ M)] for 5 min at 30 °C, then cooled on ice and added to SLO (final concentration 1.6 i.u./ml) in the same buffer. After 5 min, unbound SLO was removed by dilution and centrifugation [9]. Run-down was initiated by transferring the cells to prewarmed (30 °C to cause permeabilization) iso-osmotic buffer supplemented with Ca/EGTA (0.3 mM to regulate to pCa 8) and MgATP²⁻ (100 μ M) together with the proteins being tested contained in the wells of a 96-well microtitre plate. The cell concentration was approx. 3×10^4 per well. After allowing predetermined times for run-down to occur, the cells were stimulated to secrete by adding prewarmed solutions containing Ca/EGTA buffer (3 mM final concentration) formulated to regulate to pCa 5 (or pCa 7 as control) and GTP[S] to a final concentration of 100 μ M. After 10 min to permit secretion to proceed to completion, the incubations were quenched by the addition of an equal volume of ice-cold iso-osmotic buffer supplemented with 5 mM EGTA. The cells were sedimented by centrifugation and the supernatants were sampled for the measurement of secreted hexosaminidase as described previously [10].

Secretion is expressed as the percentage of total cellular hexosaminidase released, calibrated by reference to appropriate reagent blanks and the total cell content released by 0.2 % (w/v) Triton X-100. All determinations were in quadruplicate unless stated otherwise and all experiments were repeated at least three times.

Analysis of polyphosphoinositides by TLC

Mast cells (10^7) were plated out in a 25 cm² flask and were incubated at 37 °C with 3.7 MBq of [2-³H]myo-inositol for 12 h in 10 ml of Medium 199, supplemented with penicillin and

streptomycin (both at 100 μ g/ml). Cells were washed by dilution and centrifugation in buffered salts solution. They were then permeabilized by treatment with SLO and left to run down as described above and under the conditions specified in the Results section. Phospholipids were extracted and prepared as described previously [11] and then separated by TLC. The radioactivity of the spots was determined by phosphorescence imaging.

Recombinant proteins

Recombinant mouse PITP α in pET3d expression vector was a gift from K. W. A. Wirtz (University of Utrecht, Utrecht, The Netherlands). A clone of mouse PITP β was obtained by PCR amplification from a rat cDNA library by using sense primer 5'-TAAATCCATGGTGCTGATTAAGGAATTCC-3' and antisense primer 5'-AAAGGATCCTAGGCATCAGCAGCCGACGTG-3'. To aid cloning, *Nco*I and *Bam*HI restriction sites (underlined) were included in the sense and antisense primers respectively. The PCR product was subcloned into pET3d vector (Novagen) between the *Nco*I and *Bam*HI sites by standard methods. Each strand of the PITP β insert in the final construct was sequenced (MWG-Biotech, Ebersberg, Germany) to confirm the identity of the product. *Escherichia coli* BL21(DE3) pLysS cells were transformed with either PITP α or PITP β plasmids and protein expression was induced by the addition of 1 mM isopropyl- β -D-thiogalactoside to exponentially growing cultures. After 4 h, cells were collected by centrifugation and resuspended in buffer comprising 50 mM Tris, pH 8.0, and 25 % (w/v) sucrose (20 ml per litre of culture). After being frozen and thawed, the suspensions were treated for 20 min at room temperature with 1 mg/ml lysozyme. A solution of 50 mM Tris/HCl (pH 8.0)/100 mM MgCl₂/0.2 % (w/v) Triton X-100 was then added (8 ml per litre of original culture) and the mixture was transferred to ice. DNA was sheared with a Potter–Elvehjem homogenizer and the mixture was centrifuged (100 000 g, 60 min, 4 °C). Recombinant mammalian PITPs were purified from the supernatants essentially as described previously for bovine brain PITP α [12] up to and including the gel-permeation chromatography step, except that ion-exchange chromatography was performed on a Pharmacia Hi-Load Q-Sepharose (26/10) column instead of DE52. At each stage, fractions containing recombinant PITP α or PITP β were identified by Western blotting from cross-linked SDS/12 % (w/v) PAGE gels with specific antiserum. Proteins were stored at 4 °C until required.

The recombinant β ARK₁-Cp mutant WAA was expressed as a glutathione S-transferase fusion protein in *E. coli*. The proteins were purified with glutathione–Sepharose 4B beads with standard protocols. All proteins were subjected to gel filtration on a Superdex G-75 column to remove remaining impurities and high-molecular-mass aggregates.

Electrophoresis and immunoblotting

SDS/PAGE was performed by established methods [13,14]. Proteins were transferred to nitrocellulose membranes by using the Multiphor II Novablot system and further treated in accordance with established methods [15]. Detection of primary antibody binding was performed with peroxidase-conjugated or alkaline phosphatase-conjugated secondary antibodies and appropriate detection techniques.

Detection of phospholipid binding to PITP by isoelectric focusing (IEF)

Samples (between 2 and 5 μ g) of recombinant PITP protein were mixed with a 1 μ l solution of 10 mg/ml required phospholipid

[prepared in 20% (w/v) sucrose] in a total volume of 40 μ l; 5 μ l each of 50 mM MgCl_2 and 250 mM NaCl were added. After 20 min at room temperature the mixtures were centrifuged (100 000 g , 15 min, 4 $^{\circ}\text{C}$). Samples (40 μ l) of the supernatant were loaded on precast IEF gels (Ampholine PAGplate 3.5–9.5; Pharmacia) and proteins were focused at 10 $^{\circ}\text{C}$ in accordance with the manufacturer's instructions. Proteins were transferred to nitrocellulose and the blots were probed with PITP isoform-specific antisera with detection by enhanced chemiluminescence.

RESULTS

Mast cells stimulated with Ca^{2+} (pCa 5) and GTP[S] (100 μM) at the time of permeabilization release almost all of their contained hexosaminidase. When cells are stimulated after a delay, the extent of secretion decreases, as illustrated in Figure 1. In this experiment, the extent of secretion declined progressively ($\circ$) and application of the stimulus after 30 min generated no response ($t_{1/2} \approx 6$ min). The presence of neomycin (100 μM) had no discernible effect on the extent of secretion from cells subject to prompt stimulation but the run-down was accelerated ($\bullet$) so that the cells became unresponsive to stimulation after 20 min ($t_{1/2} \approx 3$ min). In this and in similar experiments, the onset of inhibition due to neomycin, which was clearly manifested by 2 min, occurred earlier than the modulation of secretion by exogenous proteins. Inhibition by RhoGDI [16] or enhancement by G-protein $\beta\gamma$ subunits [10] becomes apparent only after approx. 5 min and we conclude that the more rapid effect of neomycin is due to its faster diffusion through the permeability lesions to its intracellular targets.

The sensitivity of the system to neomycin increases with time.

Figure 1 Effect of neomycin on the loss of secretory responsiveness of permeabilized mast cells

Mast cells, pretreated with SLO at 0 $^{\circ}\text{C}$, were permeabilized by transfer to a buffer (pH 6.8) containing MgATP^{2-} (100 μM) with sufficient EGTA (0.1 mM) to maintain $[\text{Ca}^{2+}]$ below 10 nM (pCa 8). At the indicated times, the cells were stimulated to secrete by the addition of a solution containing GTP[S] (final concentration 100 μM) and Ca/EGTA buffer (3 mM) formulated to maintain pCa 5. After incubation for a further 10 min, the systems were quenched by the addition of ice-cold salts buffer containing EGTA (5 mM). The cells were sedimented and the supernatants were assayed for secreted hexosaminidase. Symbols: $\circ$, $\bullet$, cells stimulated with GTP[S] and Ca^{2+} ; $\triangle$, $\blacktriangle$, unstimulated cells; filled symbols indicate the presence of neomycin (300 μM). Results are means $\pm$ S.E.M. ($n = 4$ determinations). Similar results were obtained on five separate occasions.

Figure 2 Inhibition of secretion due to screening of phosphoinositides

Upper panel: concentration dependence of the inhibition of secretion by neomycin. Cells were stimulated at 0 ($\bullet$), 2 ($\blacktriangle$), 5 ($\blacksquare$) or 10 min ($\blacklozenge$) after permeabilization in the presence of neomycin at the concentrations indicated; $\circ$, unstimulated cells. Results are means $\pm$ S.E.M. ($n = 4$ determinations). Similar results were obtained on three separate occasions. The broken line indicates the change in EC_{50} for neomycin with time. Lower panel: concentration dependence of the inhibition of secretion by the WAA mutant of $\beta\text{ARK}_1\text{-Cp}$. Cells were run down for 10 min in the presence of WAA [as a glutathione S-transferase fusion protein ($\bullet$)] before the stimulus was applied. Results are means $\pm$ S.E.M. ($n = 4$ determinations). Heat-inactivated protein ($\circ$) was used as a control.

In cells stimulated at the time of permeabilization, inhibition requires concentrations in excess of 0.32 mM ($10^{-3.5}$ M), in agreement with previous papers [4]. However, for cells stimulated 10 min after permeabilization, neomycin is inhibitory at 18 μM ($10^{-4.75}$ M). In the experiment shown in Figure 2 (upper panel), the IC_{50} (concentration of neomycin causing 50% inhibition of secretion) declined progressively from 0.56 mM ($10^{-3.25}$ M), for cells stimulated at the time of permeabilization, to 30 μM ($10^{-4.5}$ M), for cells stimulated 10 min after permeabilization. Secretion was also inhibited by PH (pleckstrin homology)-domain peptides that bind to $\text{PtdIns}(4,5)\text{P}_2$. In particular, the WAA mutant of the

β ARK₁-Cp, which constitutes a PH domain displaying the same selectivity as neomycin for binding to PtdIns(4,5) P_2 and other phosphoinositides [17], suppressed the secretion induced by Ca^{2+} and GTP[S] (Figure 2, lower panel) [10].

Although phosphorylation reactions do not form a part of the stimulus–secretion pathway in mast cells stimulated with Ca^{2+} and GTP[S], the response became progressively dependent on the presence of ATP in the period after permeabilization. The effect of an abrupt withdrawal of ATP from cells initially permeabilized in its presence is illustrated in Figure 3 (top panel). In this experiment, hexokinase (75 units/ml) was provided at the time of permeabilization, so that when glucose (5 mM) was added after 4 min the concentration of ATP declined instantaneously to zero [18,19]. Within 20 s (the first sampling point) these cells had become virtually insensitive to stimulation, whereas the non-depleted cells remained responsive for a further 15 min (although on a declining basis) (Figure 3, top panel). Furthermore, the sensitivity of the permeabilized cells to the standard stimulus for secretion was dependent on the concentration of ATP (Figure 3, middle panel). Cells permeabilized in the absence of ATP ran down rapidly, becoming refractory to stimulation within 10 min (Figure 3, middle panel, ○). Run-down was slowed significantly by the provision of concentrations of ATP as low as 25 μ M (Figure 3, middle panel, ●) and, throughout the run-down period, the effect of ATP was maximal at 50 μ M (Figure 3, middle panel, ◆, and bottom panel).

Polyphosphoinositides are necessary adjuncts for the operation of the secretory machinery [20] and it has been suggested that, to maintain their presence, PITPs synergize with phospholipid kinases [21,22]. We therefore examined the effect of yeast Sec14p and two mammalian PITP isoforms, PITP α and PITP β , on secretion and run-down. Although all these proteins were found to support secretion when applied at concentrations in the range 30 nM to 1 μ M, the enhancement of secretion due to Sec14p was approximately double that induced by either of the mammalian PITPs applied alone (Figure 4). Because the two isoforms of mammalian PITP are thought to seek out different organelle membranes [23], we considered whether they might synergize when applied together. We found instead that their combined effect was approximately additive. As shown in Figure 4 (lower panel), the application of maximally effective concentrations of PITP α or PITP β [1 μ M; i.e. no additional effects were observed up to 30 μ M (results not shown)] enhanced the level of secretion by $11.7 \pm 3.7\%$ and $13.2 \pm 2.3\%$ respectively. When the two proteins were provided to the cells together, the enhancement approximated the sum of their individual contributions, achieving a level similar to that induced by Sec14p (1 μ M) alone or in combination with either or both of the mammalian PITPs. Furthermore, the retardation of run-down induced by these proteins was found to be independent of the identity of the loaded phospholipid [phosphatidylglycerol, PtdIns or phosphatidylcholine (PtdCho) (data not shown)].

To test the possibility that the smaller effects induced by the mammalian PITPs compared with Sec14p might have been due to less efficient lipid-transfer activity, we compared their ability to catalyse the PtdCho/PtdIns exchange reaction by IEF analysis (see the Experimental section). In the experiment illustrated in Figure 5 (lane 1), PITP α was loaded with a mixture of phosphatidylglycerol and phosphatidylethanolamine (i.e. the form isolated from *E. coli*). In lane 2, the protein was loaded with PtdIns and in lane 3 the PtdIns-loaded protein was incubated with PtdCho vesicles. The change in pI indicated that exchange of the anionic PtdIns for neutral PtdCho had occurred. The single band observed for the PtdIns-loaded protein indicates that exchange for PtdIns was efficient. Lane 3 shows that it was

Figure 3 Effect of ATP on the loss of responsiveness

Top panel: the effect of abrupt removal of ATP on the time course of run-down. Mast cells were treated under the standard conditions for run-down (see legend to Figure 1) in the presence of hexokinase (75 units/ml). Glucose (final concentration 5 mM, ○) was added 4.5 min after permeabilization to deplete the ATP. Similar results were obtained on six separate occasions. Middle panel: the effect of ATP concentration on the time course of run-down. In this experiment the cells were permeabilized in the presence of 0 (○), 25 (●), 50 (◆), 100 (▼), 500 (■) and 1000 μ M (▲) ATP and stimulated to secrete at the times indicated. Results are means $\pm$ S.E.M. ($n = 4$ determinations). Similar results were obtained on three separate occasions. Bottom panel: the same data as in the middle panel, plotted as a function of ATP concentration. Percentage secretion is plotted against [ATP] at the following times (min): 0 (○), 2 (●), 3 (△), 5 (▲), 7 (◇), 10 (◆), 15 (▽), 25 (▼) and 30 (*).

Figure 4 Effect of PI TPs

Upper panel: PIPs retard run-down. Cells were permeabilized in the presence of Sec14p or PIPα for 10 min before the stimulus was applied. Data are the differences in the extent of secretion (%) in the presence of intact compared with heat-inactivated protein (means $\pm$ S.E.M., $n = 4$ determinations). Similar results were obtained on three separate occasions. Symbols: ○, PIPα; ●, Sec14p. Lower panel: additivity of PIPα and PIPβ. Results indicate the extents of secretion induced by GTP[S] and Ca^{2+} applied 10 min after permeabilization in the presence of maximally effective concentrations (1 μM) of Sec14p, PIPα and PIPβ. Results are expressed as enhancements of secretion and are means $\pm$ S.E.M. ($n = 4$ determinations). Similar results were obtained on three separate occasions.

reversible. We therefore conclude that the PIPα was fully functional.

We have measured the levels of PtdIns4P and PtdIns(4,5) P_2 in mast cells undergoing run-down after permeabilization (Figure 6). In intact cells the two lipids were found to comprise 5.7% and 2.9% (respectively) of the total inositol lipid pool (Figures 6A and 6C). After permeabilization in the presence of ATP (hatched bars), no significant changes in the level of PtdIns4P were detected over a period of 10 min; with PtdIns(4,5) P_2 there was a

Figure 5 PtdCho/PtdIns exchange by recombinant PIPα

PIPα, as purified from bacteria (control), treated with PtdIns (+PI) or PtdIns-treated protein incubated with PtdCho (+PC), was subjected to IEF and revealed by immunoblotting. The positions of pI markers are indicated at the left. The double band revealed after incubation with PtdCho occurs because of the retention of some PtdIns due to the higher affinity of the protein for this lipid.

Figure 6 Changes in phosphoinositide levels during run-down and the effect of ATP and PIPα

Mast cells, loaded and incubated for 12 h with $\text{myo}[2\text{-}^3\text{H}]\text{inositol}$, were washed, treated with SLO and left to run down under the conditions indicated. No stimulus was applied in this experiment. The incubations were quenched by the addition of an equal volume of ice-cold, iso-osmotic buffered salts solution at the times indicated. Phospholipids were extracted, separated by TLC and assayed by phosphorescence imaging. Results are expressed as the fraction of PtdIns4P (A, B) and PtdIns(4,5) P_2 (C, D) of the total of PtdIns, PtdIns4P and PtdIns(4,5) P_2 . Open bars indicate the absence of ATP, hatched bars the presence of 100 μM ATP. Results obtained in the presence of PIPα are indicated in (B) and (D). Error bars indicate S.E.M. ($n = 4$).

small initial decrease (at 5 min). However, in cells permeabilized in the absence of ATP (Figures 6A and 6C, open bars), the fractions of both lipids declined substantially; after 10 min they contributed only 2.6% ($P < 0.0008$, $n = 4$) and 1.1% ($P < 0.004$, $n = 4$) respectively of the total inositol lipid pool. Such cells would be fully refractory to stimulation (see, for example, Figure 3, middle panel, ○). The provision of PIPα opposes the depletion of phosphoinositides. PIPα (1 μM) (Figures 6B and 6D) in the absence of ATP (open bars) slightly decreased, but

Figure 7 Sec14P^{K66,239A} fails to support secretion

The cells were left to run down in the presence of Sec14p^{WT} (○) or Sec14p^{K66,239A} (●) for 7 min before the stimulus was applied. Similar results were obtained on three separate occasions. Data are means $\pm$ S.E.M.

certainly did not abolish, the decline in the amounts of PtdIns4P and PtdIns(4,5)P₂ over the 10 min run-down period. The fraction of PtdIns4P decreased to 3.3 %, a value slightly higher than that observed in the absence of protein (2.6 %, $P < 0.04$, $n = 4$), whereas the fraction of PtdIns(4,5)P₂ decreased proportionately less, to 1.6 % (compared with 1.1 %, $P < 0.03$, $n = 4$). There is no evidence from these results that the protective effect of P1TP α was additive with that induced by ATP.

To test whether the lipid-transfer properties of P1TPs are themselves important for secretion, we compared the effects of wild-type Sec14p with a PtdIns transfer-defective mutant, Sec14p^{K66,239A}. This double mutant lacks PtdIns transfer activity but still transfers PtdCho at 60–70 % of the normal rate [24]. When applied to permeabilized mast cells (see Figure 7), it was without significant effect on the progress of run-down. By contrast, wild-type Sec14p, presented at a maximally effective concentration (1 μ M), increased the extent of secretion (stimulated 10 min after permeabilization) from 25 % to 48 % (Figure 8, upper panel). This indicates that the maintenance of the inositol lipid pool was of particular relevance to the secretory mechanism. Importantly, Sec14p also acted to reverse the inhibitory effect of neomycin. Although not great (a half log unit), the increase in IC₅₀ for neomycin (10⁻⁴ M), owing to the presence of the PtdIns exchange protein, was sufficient to allow some secretion to occur when the system would otherwise have been refractory. We obtained qualitatively similar data with recombinant P1TP α and P1TP β (results not shown). The rescue of secretion by the P1TPs from inhibition by neomycin suggests that they might act to screen the headgroup of PtdIns(4,5)P₂ from the aminoglycoside antibiotic.

To provide additional evidence that the effects of neomycin might have been due to headgroup screening, we attempted to titrate it out by the provision of a soluble analogue of the phospholipid. In the experiment illustrated in Figure 8 (lower panel), cells were left to run down for 10 min in the presence of the indicated neomycin concentrations and in the absence (○) or presence (●) of diC₈-PtdIns(4,5)P₂ (100 μ M). In the presence of the soluble lipid, neomycin was without effect at all concen-

Figure 8 Displacement of neomycin

Upper panel: concentration dependence of the inhibition of secretion by neomycin in the presence of Sec14p. Cells were left to run down for 10 min in the presence of neomycin and in the presence (●) or absence (○) of Sec14p (1 μ M) before application of the stimulus. Results are means $\pm$ S.E.M. ($n = 4$ determinations). Similar results were obtained on three separate occasions. Lower panel: displacement of neomycin by soluble diC₈-PtdIns(4,5)P₂. Cells were left to run down for 10 min in the presence of neomycin and in the presence (●) or absence (○) of diC₈-PtdIns(4,5)P₂ (100 μ M) before application of the stimulus. Results are means $\pm$ S.E.M. ($n = 4$ determinations). Similar results were obtained on three separate occasions.

trations up to 100 μ M. The apparent shift in EC₅₀ was almost equal to the concentration of supplied PtdIns(4,5)P₂, suggesting that the main target of neomycin under these conditions was PtdIns(4,5)P₂. Only when its concentration exceeded that of the exogenous lipid and it was able to gain access to the cellular lipids did inhibition become manifest.

The results presented indicate that the requirement for ATP in sustaining the secretory competence of permeabilized mast cells can be accounted for, at least in part, by the need to maintain polyphosphoinositide levels. However, earlier work has shown that secretion from permeabilized cells is sensitive to the PKC inhibitor AMG-C₁₆, a diether analogue of diacylglycerol [2,25,26]. As with neomycin (Figure 1), inhibition by AMG-C₁₆ can be partly overcome by increasing the concentration of ATP [4]. We therefore needed to address the possibility of synergy between events dictated by the state of protein and lipid

Figure 9 Lack of synergy in the inhibition of secretion by AMG-C₁₆ (PKC inhibitor) and neomycin

Upper panel: cells were stimulated 10 min after permeabilization in the presence of neomycin at the following concentrations (μM): 5 (Δ), 10 ($\circ$), 20 ($\square$), 50 ($\blacklozenge$), 100 ($\blacktriangledown$), 200 ($\blacktriangle$) and 500 ($\bullet$). AMG-C₁₆ concentrations are indicated on the abscissa. Results are means $\pm$ S.E.M. ($n = 4$ determinations). Similar results were obtained on three separate occasions. Lower panel: normalized extent of secretion (the fraction of the maximum extent of secretion, with 100% at 1 μM AMG-C₁₆). The data are plotted as a function of the concentration of neomycin. AMG-C₁₆ (μM): 1 (Δ), 2 ($\circ$), 5 ($\square$), 10 ($\blacklozenge$), 20 ($\blacktriangledown$), 50 ($\blacktriangle$).

phosphorylation. Accordingly, cells were treated with a combination of neomycin and AMG-C₁₆, the concentrations of both inhibitors being varied systematically over the range 1 μM to 0.3 mM (Figure 9, upper panel). When the inhibitors were provided together, the extent of inhibition was substantially increased but there was no evident shift in the EC₅₀ for either compound. If the results are plotted on a normalized scale (Figure 9, lower panel), it is apparent that the effects of the two inhibitors were simply additive: there is no evidence that they synergized.

DISCUSSION

In cells of haemopoietic origin (including mast cells), the key activator for exocytosis is GTP [27]. Cells of this class, stimulated with GTP[S], can undergo exocytosis in the effective absence of Ca²⁺ [28]. Moreover, just as the presence of Ca²⁺ is not an absolute requirement for exocytosis, neither is ATP [1]; from this it follows that a phosphorylation reaction cannot be a determining step in the final stages of the stimulus–secretion pathway. In

contrast, in permeabilized cells the system becomes ATP-requiring when the stimulus to secrete is applied after a delay that allows proteins to leak from the cell. This gives the clue that the maintenance of a phosphorylation state (as opposed to a phosphorylation reaction) is essential. Such a state is likely to involve both protein and lipid phosphorylations, as we show here.

Permeabilized mast cells leak soluble factors within a few minutes but their propensity for responding to stimulation by Ca²⁺ and GTP[S] declines over a period of approx. 30 min. This ‘run-down’ has been attributed to the loss of tethered proteins that might act as essential regulators or components of the exocytotic machinery [29–32]. Another component of run-down might be the depletion of phosphorylated lipids and proteins. In several other cell types, the role for polyphosphoinositides in the priming of the exocytotic mechanism has been well established [20]. In adrenal chromaffin cells, the major component of secretion has a requirement for a high concentration of ATP (5 mM), consistent with a role for PtdIns4P 5-kinase [33,34]. In PC12 and HL60 cells, a similar role for polyphosphoinositides in priming (rather than triggering) has been described [12,35]. Here, PITPs, in combination with PtdIns4P 5-kinase, provide the necessary source substrates [36,37].

In permeabilized mast cells, ATP is maximally effective at 50 μM , acting to prolong the period during which the cells remain sensitive to stimulation. In relation to phosphorylation reactions, this should be sufficient to saturate PKC [7] and also PI-4K ($K_m \approx 18 \mu\text{M}$ [5]). However, it might not be sufficient to support the further phosphorylation of PtdIns4P to PtdIns(4,5)P₂ by PtdIns4P 5-kinase ($K_m \approx 0.5 \text{ mM}$ [38]), although the cytosolic type II β PtdIns4P kinase ($K_m^{\text{ATP}} \approx 5 \mu\text{M}$ [39]) has been shown to phosphorylate its substrates PtdIns3P and PtdIns5P *in situ* at the membrane and is also active at these low ATP concentrations [40].

For mast cells stimulated at the time of permeabilization, neomycin at concentrations sufficient to impede the interaction of polyphosphoinositides with PLC has only a marginal effect on secretion [4]. However, as shown here, the inhibition becomes more potent as run-down proceeds. For cells stimulated only 2 min after permeabilization, the inhibition by neomycin is already quite evident. This is most probably due to the interaction of neomycin with the headgroups of polyphosphoinositides [3,41–43] and this also forms the basis for a number of other phenomena that are not related to secretion (e.g. decrease in protein synthesis [44]).

Not only does neomycin inhibit secretion in run-down mast cells, but so does the WAA mutant of $\beta\text{ARK}_1\text{-Cp}$. We previously reported that $\beta\text{ARK}_1\text{-Cp}$ inhibits secretion triggered by Ca²⁺ and GTP[S] when presented at concentrations in the range 10^{−7} to 10^{−5} M [10]. This PH-domain-containing peptide binds both to G-protein $\beta\gamma$ subunits and to polyphosphoinositides, mainly PtdIns(4,5)P₂ [45]. We show here that the WAA mutant (two Ala residues inserted after Trp-643), which has a low affinity for $\beta\gamma$ subunits but retains its affinity for PtdIns(4,5)P₂ ($K_d \approx 30 \mu\text{M}$ [17]), also inhibits secretion. This supports the conclusion that the inhibition of secretion by neomycin is due to its ability to screen the negatively charged lipid headgroup of PtdIns(4,5)P₂.

Although the availability of PtdIns(4,5)P₂ is important for secretion, the particular pool of polyphosphoinositides required for this purpose is likely to be distinct from that which provides the substrate for PLC. Immediately after permeabilization, the inositol lipid pool required for secretion seems to be protected and therefore not accessible to neomycin. After permeabilization, this protecting component (likely to be a protein) might detach and leak from the cells, thus exposing the polyphosphoinositides

to which it was bound, making them available for interaction with neomycin or the WAA peptide. PITP might provide this protection. We note that in A431 cells and HL60 cells, permeabilized by SLO, most of the PITP α is lost within 5 min [46,47]. Moreover, the PITPs applied at relevant physiological concentrations oppose the inhibition due to neomycin. Thus they can act not only to maintain the levels of PtdIns in appropriate membrane locations but also to screen the headgroups of the polyphosphoinositides, so preventing the access of neomycin. If the PtdIns carried by the transfer protein were to become phosphorylated *in situ*, at no point would the lipid headgroup become accessible to the inhibitor. The finding that the proposed lipid-binding site for PtdIns on Sec14p is a deep cleft within which the headgroup is retained is consistent with this proposal [22,48].

Such a mechanism raises several matters for consideration and can be discussed further, considering two models for PITP function that have been formulated [49,50]. One possibility is that the PtdIns is phosphorylated sequentially by lipid kinases while bound to PITP. The finding that phosphorylated inositol lipids bind to PITPs *in vitro* supports this model [51]. However, the fact that the lipid headgroup is buried deep inside the binding pocket makes it difficult to interpret how these phosphorylations occur. A more probable explanation is that PITP 'docks' at or near the site of a complex formed by several enzymes (e.g. PI-4K and PtdIns4P 5-kinase) and that PITP-bound PtdIns is channelled through this complex. A direct interaction of PITPs with PI-3K isoforms has been demonstrated by immunoprecipitation [52], suggesting that a similar interaction might occur with PI-4Ks, for example. In addition, we have observed that PtdIns- or PtdCho-loaded PITP α binds to PtdIns(4,5) P_2 -containing vesicles (G. M. H. Thomas, unpublished work), raising the possibility that PITPs can screen lipids other than those in their binding pocket. In any case, the screening of the inositide headgroups that prevents the access of neomycin is maintained through all steps leading to exocytosis. We conclude that the transfer-protein-lipid complex is itself an essential and intimate component of the secretory machinery.

In this system, the effects of PITP α and PITP β are additive, suggesting that these isoforms are not functionally redundant. The finding that the sum of their effects approximates that induced by Sec14p, and that there is no additional component of secretion when Sec14p is also present, could mean that Sec14p fulfils the roles of both PITP α and PITP β . Although there is some uncertainty about whether the physiological function of Sec14p in yeast is that of a lipid-exchange catalyst, it is likely that this is the way in which it operates when applied to mammalian cells [24]. Although PITP α supports the activity of PI-3K better than Sec14p does [21], the yeast protein provides better support than mammalian PITP α to secretion in mast cells (the present study) and PC12 cells [36]. For these reasons it is unlikely that the interaction between PITP α and PI-3K is directly involved in the regulation of secretion. Further, we have been unable to perceive any effect of the PI-3K inhibitors wortmannin and LY294002 [10]. There are several possibilities to explain the additive effect of PITP α and PITP β . If the isoforms work on different intracellular membranes, this could mean that either both pools of lipids are involved in the fusion machinery or that one pool is necessary for recruiting vital proteins (e.g. SNARE proteins [53]). Another possibility is that, especially in mast cells and related cell types, the second pool has a role in compound exocytosis. Finally, there is also the possibility that there are distinct pools of lipids (micro domains) on the plasma membrane or that PITP isoforms interact with slightly different complexes of kinases and regulatory factors.

Although ATP is not essential for the exocytosis reaction, at low concentrations (50 μ M) it acts to prolong the period during which permeabilized cells remain competent to respond to stimulation. Its apparent role is in the maintenance of the necessary phosphorylation status of proteins and lipids. We noted previously [10] that the enhancement of secretion by $\beta\gamma$ subunits derived from GTP-binding proteins involves both PKC-dependent and -independent pathways. However, in contrast with AMG-C₁₆, the pseudosubstrate peptide PKC₁₉₋₃₆, also an inhibitor of PKC, is without noticeable effect on secretion from run-down cells, even when applied at concentrations 10-fold the reported K_i (15 μ M [54]). Specific substrate sequence motifs of PKC isoenzymes have now been defined and it has become apparent that PKC₁₉₋₃₆ does not inhibit all forms equally [55-57]. The enhancement by exogenous $\beta\gamma$ subunits of GTP[S]-induced secretion is suggestive of roles for proteins having PH domains [10]. This report now raises the possibility that an atypical PKC or PKD (PKC μ) might operate downstream of the $\beta\gamma$ subunits in the mast cell.

In mast cells the importance of the phosphorylation state of inositol lipids and certain proteins to secretion is underlined by our findings that the PITPs and phorbol ester both act to retard run-down, whereas the relevant inhibitors act to accelerate it. The PITPs operate even in the presence of neomycin, mimicking the effect of exogenous (short-chain) PtdIns(4,5) P_2 . The possibility that the transfer activity of PITP depends on phosphorylation by PKC [58] is improbable in the light of the observation that the effects of PITP and PKC are additive, not synergistic. The simplest interpretation is that PITP protects an essential pool of phosphoinositides from neomycin without diminishing either the phosphorylation of the lipids or their ability to take part in the secretory mechanism. This raises the interesting possibility that a phospholipid-protein complex, possibly the complex of PtdIns(4,5) P_2 with one of the PITP isoforms, is itself an essential component of the exocytotic machinery.

Our observation that the inhibitory effects of neomycin and of the PKC inhibitor AMG-C₁₆ are additive and not synergistic indicates that they inhibit independent regulatory branches of the secretory process. What could these be? The extent of secretion measured as the release of hexosaminidase reports the proportion of cell-contained secretory granules (approx. 1000 per mast cell) that undergo exocytosis. For promptly stimulated cells, which respond in an all-or-none fashion, this correlates with the proportion of responding cells [59]. We do not know whether run-down cells respond in a similar all-or-none fashion but the idea that individual cells would be separately sensitive to inhibition of PKC, or blockade of lipid headgroups, seems improbable. More likely is the idea that individual granules, distinguished either by their age or by their location within the cells (cortical or inner zones), determine the non-interacting sensitivities of the secretory mechanism to reagents specific to lipid and protein phosphorylation.

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