Activation of exocytosis by cross-linking of the IgE receptor is dependent on ADP-ribosylation factor 1-regulated phospholipase D in RBL-2H3 mast cells: evidence that the mechanism of activation is via regulation of phosphatidylinositol 4,5-bisphosphate synthesis

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The physiological stimulus to exocytosis in mast cells is the cross-linking of the high-affinity IgE receptor, FcϵR1, with antigen. We demonstrate a novel function for ADP-ribosylation factor 1 (ARF1) in the regulation of antigen-stimulated secretion using cytosol-depleted RBL-2H3 mast cells for reconstitution of secretory responses. When antigen is used as the stimulus, ARF1 also reconstitutes phospholipase D activation. Using ethanol to divert the phosphatidic acid (the product of phospholipase D activity) to phosphatidylethanol causes inhibition of ARF1-reconstituted secretion. In addition. ARF1 causes an increase in phosphatidylinositol 4,5-bisphosphate (PIP_2) levels at the expense of phosphatidylinositol 4-monophosphate. The requirement for PIP_2 in exocytosis was confirmed by using phosphatidylinositol transfer protein (PITPα) to increase PIP_2 levels. Exocytosis, restored by either ARF1 or PITPα, was inhibited when PIP_2 levels were depleted by phospholipase Cδ1. We conclude that the function of ARF1 and PITPα is to increase the local synthesis of PIP_2, the function of which in exocytosis is likely to be linked to lipid–protein interactions, whereby recruitment of key components of the exocytotic machinery are targeted to the appropriate membrane compartment.

Key words: ARF1, phosphatidylinositol transfer protein, phospholipase Cδ, streptolysin O permeabilization.

INTRODUCTION

The physiological stimulus to exocytosis in mast cells is the cross-linking of the high-affinity IgE receptor, FcϵR1, by antigen [1]. Upon stimulation, multiple signalling pathways are triggered, including the activation of phosphoinositide 3-kinase, phospholipase Cγ (PLCγ) and phospholipase D (PLD). PLCγ hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate the second messengers inositol 1,4,5-trisphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate mobilizes intracellular Ca^{2+}, whereas diacylglycerol mediates the activation of protein kinase C isoenzymes. Both Ca^{2+} and protein kinase C have been implicated in the control of the exocytotic machinery by undefined mechanisms [2]. Likewise wortmannin, an inhibitor of phosphoinositide 3-kinase, also inhibits antigen-mediated exocytosis. The potential role of PLD in regulated exocytosis is indicated by the observation that ethanol can inhibit antigen-mediated exocytosis from mast cells [3].

Studies of exocytosis from cells of haematopoietic origin, including mast cells, have indicated an essential role for a G-protein (G_E) in addition to increases in Ca^{2+} [4,5]. Subsequent work has revealed several candidate G-proteins that are involved in exocytosis. These include the Rho family members Rac and Rho [6–8], and the heterotrimeric G-protein G_{i3} [9]. The constitutively active mutant proteins V14RhoA and V12Rac1 enhanced Ca^{2+}-regulated exocytosis from permeabilized mast cells by increasing the proportion of cells that were competent to respond to stimulation. In addition, inhibition of endogenous Rac and Rho activity using inhibitors (N17Rac1 and C3 transferase respectively) reduced the exocytotic response of mast cells to guanosine 5′-[γ-thio]triphosphate (GTP[S]) [6]. The effects on exocytosis were independent of changes on the cytoskeleton [7]. Independent studies using a different approach also identified Rac as an effector of exocytosis from these cells [8]. In none of these reports has the downstream component regulated by these G-proteins been identified.

PLD is activated by many cell surface receptors, including the cross-linking of the IgE receptor [10]. PLD catalyses the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) and choline. In the presence of ethanol, PA is diverted to phosphatidylethanol (PEt), in a reaction known as transphosphatidylation. Reconstitution studies using cytosol and permeabilized HL60 cells or membranes initially identified ARF1 and ARF3 as regulators of mammalian PLD [11,12]. Subsequent work has shown that all ARF proteins (ARF1–ARF6) are capable of activation. Two mammalian PLD enzymes (PLD1 and PLD2) have been cloned [13,14]. Regulation of PLD1 has been intensively studied and is known to be directly and synergistically regulated by ARF1, Rho proteins (RhoA, Rac and Cdc42) and protein kinase Cα [15,16].

Rho protein and protein kinase C, direct regulators of PLD1 [15], have already been identified as regulators of exocytosis from mast cells [7]. In the present study we have examined the requirement for ARF1 in antigen-stimulated exocytosis from cultured mast cells. We provide evidence that the function of ARF1 (via PLD) is to make PIP_2 available at localized membrane sites, which subsequently participate in the exocytotic pathway of mast cells.

Abbreviations used: ARF, ADP-ribosylation factor; PLD, phospholipase D; PEt, phosphatidylethanol; PC, phosphatidylcholine; COP, coat protomer; PA, phosphatidic acid; PITP, phosphatidylinositol transfer protein; PLC, phospholipase C, GTP[S], guanosine 5′-[γ-thio]triphosphate; ATP[S], adenosine 5′-[γ-thio]triphosphate; SLO, streptolysin O; DNP–HSA, dinitrophenol conjugated to human serum albumin; PIP, phosphatidylinositol 4-monophosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate.

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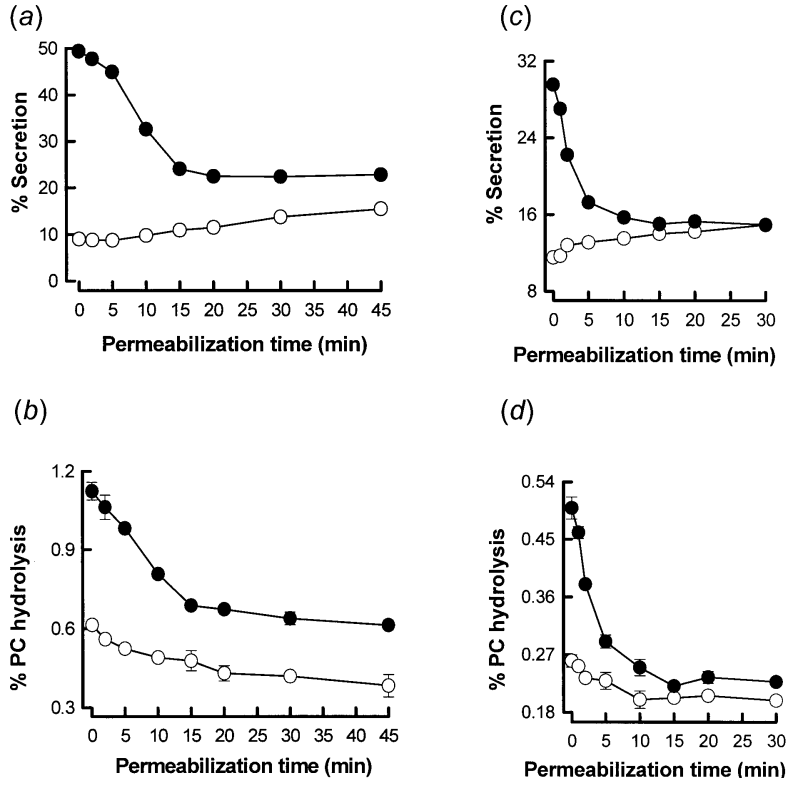

Figure 1 Time-dependent loss of exocytotic competence and PLD activation from permeabilized cells stimulated with GTP[S] (a, b) or with antigen (c, d)

[^{3}H]Choline-labelled RBL-2H3 mast cells were permeabilized in Pipes buffer containing 0.4 unit/ml SLO and 1 mM MgATP for the indicated times. The permeabilized cells were assayed in the presence (●) or absence (○) of either GTP[S] (10 μM) plus Ca^{2+} (10 μM) (**a** and **b**) or antigen (DNP–HSA; 40 ng/ml) plus Ca^{2+} (10 μM) (**c** and **d**). The cells were stimulated for 25 min at 37 °C, the samples quenched and the supernatants analysed for released β-hexosaminidase (**a** and **c**) and [^{3}H]choline release (**b** and **d**). Values are means ± range of duplicate incubations, and are taken from one of four experiments.

MATERIAL AND METHODS

Materials

The nucleotides GTP[S], adenosine 5′-[γ-thio]triphosphate (ATP[S]) and ATP were purchased from Boehringer Mannheim (Mannheim, Germany). Streptolysin O (SLO) was obtained from Murex (Dartford, U.K.) (MR16). IgE [anti-dinitrophenol (DNP)] and DNP conjugated to human serum albumin (DNP–HSA) (antigen) were purchased from Sigma. Monoclonal antibodies to phosphatidylinositol transfer protein α (PITPα) and ARF1 were prepared as detailed [17,18], and the polyclonal antibody to RhoA was purchased from Santa Cruz Biotechnology. Myristoylated ARF1 (referred to in the text as ARF1) and the myristoylated ARF1 mutant (N52R)ARF1 [referred to in the text as (N52R)ARF1] were expressed in *Escherichia coli* and purified exactly as described in [11]. Myristoylation was approximately 10 %. PITPα was expressed in *E. coli* and purified as described previously [19].

Culturing and labelling of RBL-2H3 mast cells

Rat basophilic leukemic cells, RBL-2H3 mast cells, were grown in Dulbecco's modified minimal medium supplemented with 12.5 % (v/v) fetal calf serum, 4 mM glutamine, 50 μg/ml penicillin and 50 units/ml streptomycin. The cells were grown as a monolayer in 175 cm^2 vented flasks, at 37 °C with 5 % CO_2 and 100 % humidity. Each flask contains approx. 4×10^7 cells when confluent. For measurement of PLD activity, two protocols were used [20,21]. RBL-2H3 mast cells were prelabelled with [^{3}H]choline to label the PC pool. The label (0.5 μCi/ml) was added directly to the complete medium and the cells were allowed to proliferate for 48 h to reach 80 % confluence [21]. For measurements of PEt production, the cells were prelabelled with [^{3}H]alkyl-lyso-PC (5 μCi/ml) in 2 ml for 1 h [20]. For the labelling of the inositol lipids, the cells were grown in the presence of [^{3}H]inositol (1.0 μCi/ml) for 48 h.

Assays using permeabilized cells

Cell monolayers were washed twice in Dulbecco's modified minimal medium and scraped into 5 ml of fresh medium. IgE (2 μg/ml final) was added to sensitize the cells for 1 h at 37 °C. The cells were centrifuged at 450 ***g*** for 5 min at room temperature and resuspended in Hepes buffer, pH 7.2 (for antigen stimulation), or Pipes buffer, pH 6.8 (for stimulation with GTP[S]). The composition of the Hepes and Pipes buffers was 137 mM NaCl, 3 mM KCl, 2 mM $MgCl_2$, 5.6 mM glucose and 0.1 mg/ml BSA, plus 20 mM Hepes or Pipes as indicated. The cells were washed once and then resuspended in the appropriate buffer, and 50 μl aliquots of cells were transferred to tubes containing 50 μl of a cocktail comprising SLO (0.4 unit/ml), 1 mM MgATP and calcium buffered with 3 mM EGTA, in the presence or absence of stimulus (10 μM GTP[S] or 40 ng/ml antigen, as indicated). Concentrations indicated were the final concentrations in a final 100 μl assay volume. The tubes were incubated at 37 °C for 25 min and the reaction was terminated by centrifugation of the cells at 4 °C (1800 ***g*** for 5 min). An aliquot of 50 μl of the

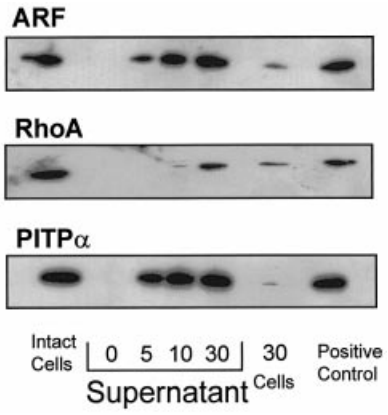

Figure 2 Leakage of endogenous ARF1, Rho and PITPα proteins from permeabilized cells over time

RBL-2H3 mast cells were permeabilized in the presence of 0.4 unit/ml SLO and 1 mM MgATP, and at the indicated times (min) the supernatants were harvested by centrifugation and analysed for leaked proteins using antibodies to ARF1, RhoA and PITPα as indicated. The cell pellet was also examined after 30 min of permeabilization. Recombinant ARF1 and PITPα and partially purified Rho from human neutrophils were used as positive controls.

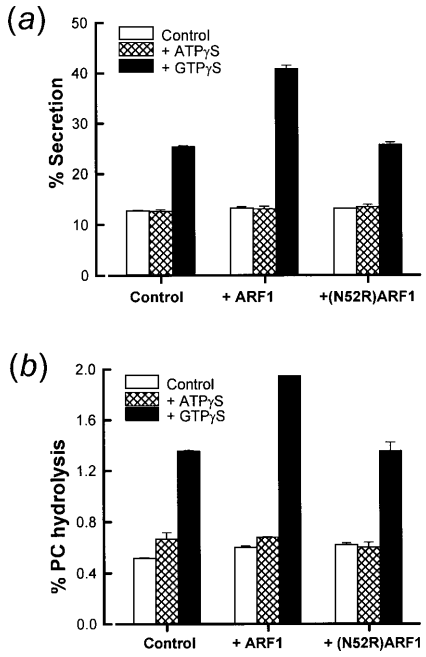

Figure 3 Specificity of the activation of secretion and of PLD activity by ARF1 and GTP[S]

[^{3}H]Choline-labelled RBL-2H3 mast cells were permeabilized with 0.4 unit/ml SLO in the presence of 2 mM $MgCl_2$ and 1 mM MgATP for 10 min, washed and reconstituted with ARF1 or with (N52R)ARF1 (100 μg/ml) in the presence of 10 μM GTP[S] (GTPγS) or ATP[S] (ATPγS). After incubating the reactions for 25 min at 37 °C, the cells were centrifuged and the supernatants analysed for released β-hexosaminidase (**a**) and [^{3}H]choline release (**b**). Values are means $\pm$ range of duplicate incubations, and are taken from one of three experiments.

supernatant was sampled to assay for released β-hexosaminidase [19]. To monitor the activation of PLD, [^{3}H]choline-labelled cells were used, and release of [^{3}H]choline was monitored as described previously [19]. This protocol is referred to as using 'acutely permeabilized' cells.

To monitor the loss of exocytotic function and the ability to activate PLD as a function of permeabilization, 4 ml of cells (10^7/ml) was added to 1 ml of permeabilization mixture containing 1 mM MgATP, 0.4 unit/ml SLO and 10 μM calcium buffered by 0.3 mM EGTA. Immediately, an aliquot of cells was transferred to assay tubes containing the appropriate stimulus at 37 °C to provide a starting point for the 'run-down'. The remaining cells were left to permeabilize at 37 °C. At timed intervals, further aliquots were removed and transferred to assay tubes. The final concentrations of the reagents in the final assay were 1 mM MgATP, 0.4 unit/ml SLO and 10 μM calcium buffered by 3 mM EGTA, plus GTP[S] (10 μM) or antigen (40 ng/ml). All assay tubes were incubated for 25 min at 37 °C, and reactions were terminated by centrifugation at 4 °C (1800 ***g*** for 5 min); 50 μl of the supernatant was sampled to assay for released β-hexosaminidase and for release of labelled choline.

Reconstitution of cytosol-depleted cells

Portions of 4 ml of cells (10^7/ml) were added to 1 ml of permeabilization buffer as described above, and aliquots were removed immediately to measure the 'acutely permeabilized' response. The remaining cells were transferred to 37 °C and allowed to permeabilize, for 5 min for reconstitution with antigen or for 10 min for reconstitution with GTP[S]. After permeabilization, the cells were pelleted by centrifugation (1800 ***g*** for 5 min) and the supernatant discarded. The cells were resuspended in buffer, and then aliquots were added to assay tubes containing the appropriate reagents, including ARF1 and PITPα, as indicated in the Figure legends. The final concentrations of the reagents in the assay were 1 mM MgATP and the indicated levels of Ca^{2+} buffered with EGTA; GTP[S] (10 μM) or antigen (40 ng/ml) was present where indicated in the individual Figure legends. The assay tubes were incubated for 25 min at 37 °C and processed as described above for secretion and for [^{3}H]choline release. For measurement of PEt production, the [^{3}H]alkyl-lyso-PC-labelled cells were used, and ethanol was present in the assay mixture as indicated in the Figure legends. PEt was analysed by TLC exactly as described previously [20].

Leakage of ARF, Rho and PITPα from permeabilized cells

Aliquots of 4 ml of cells (5×10^7 cells) were added to 1 ml of permeabilization buffer, to give final concentrations of 1 mM MgATP, 0.4 unit/ml SLO and 100 nM Ca^{2+} buffered by 0.3 mM EGTA. At the stated time points (5, 10 and 30 min), 1 ml samples were removed. After centrifugation of the cells (1800 ***g*** for 5 min), the supernatant was harvested and treated with 10% trichloroacetic acid at 4 °C for 30 min to precipitate proteins. The precipitate was recovered by centrifugation (15000 ***g*** for 15 min), resuspended in 50 μl of 1 M NaOH, and neutralized with 50 μl of 1 M Tris, pH 6.8. Precipitated proteins were separated by SDS/PAGE (14% acrylamide), transferred to PVDF and Western-blotted with appropriate antibodies. The cell pellet was resuspended in 100 μl of RIPA buffer [150 mM NaCl, 1% (v/v) Triton X-100, 0.5% (w/v) deoxycholate, 0.1% (w/v) SDS and 50 mM Tris, pH 7.5], vortexed thoroughly and placed at 4 °C for 30 min. After centrifugation at 15000 ***g*** for 15 min at 4 °C, the solubilized proteins were concentrated by treatment with trichloroacetic acid as described above.

Changes in localization of PITPα and ARF after stimulation with antigen

Intact RBL-2H3 mast cells were stimulated with antigen for 10 min at 37 °C. Cells were permeabilized with SLO for a further

10 min, and cell pellets and the leaked cytosol were separated by centrifugation (1800 ***g*** for 5 min). The presence of PITPα and ARF in the cell pellet compared with the cytosol was probed by Western blotting as described above.

Measurement of polyphosphoinositide levels in permeabilized cells

Aliquots of 4 ml of RBL-2H3 mast cells were permeabilized for 10 min to deplete the cells of cytosolic proteins and washed as described above. The cytosol-depleted cells were resuspended in buffer containing 1 mM MgATP and 10 μM Ca^{2+}. Next 20 μCi/ml [γ-^{32}P]ATP was added to the cells, aliquots of which which were immediately added to assay tubes containing the stimulus and proteins as indicated in the Figure legends. The cells (100 μl final) were incubated at 37 °C for 20 min, transferred to 4 °C and quenched with 375 μl of acidified chloroform/methanol/conc. HCl (100:200:1.5, by vol.), and the mixture was vortexed thoroughly to obtain a single phase. Then 10 μl of Folch extract was added [Folch extract (Sigma) is a brain lipid extract rich in phosphoinositides added to aid recovery of labelled phosphoinositides]. Next, 125 μl each of chloroform and 0.1 M HCl were added to obtain two phases. The samples were vortexed thoroughly and then centrifuged (1800 ***g*** for 5 min). The upper phase was removed and replaced with a 'synthetic' upper phase of chloroform/methanol/0.1 M HCl (1:1:0.9, by vol.). The upper phase was replaced twice more, to remove the majority of the [^{32}P]ATP not incorporated into lipid. Finally, all of the chloroform phase was transferred to a clean Eppendorf tube, dried under vacuum and resuspended in 50 μl of chloroform. The polyphosphoinositides were separated by TLC as described previously [22]. The labelled lipids were analysed using a Fuji Phosphorimager, and the images were quantified using appropriate software.

Studies with rat peritoneal mast cells

Mast cells were obtained and purified exactly as described previously [5]. The protocol for cytosol depletion was identical to that used for RBL-2H3 cells described above.

RESULTS

Loss of secretory function and PLD activation from permeabilized cells

Stimulation of exocytosis from intact RBL-2H3 mast cells by antigen is inhibited by ethanol, implying a role for PLD in the signalling pathway leading to secretion. PLD activity is regulated by the small GTPases ARF1 and Rho proteins; thus antigen stimulation can be bypassed by using GTP[S] as a stimulus in permeabilized cells. We initially established that both antigen and GTP[S] were capable of stimulating exocytosis and PLD activation in the permeabilized cells. We routinely used Pipes buffers, pH 6.8, for our experiments and found that secretion from antigen-stimulated cells was not well maintained in permeabilized cells. After experimenting with different conditions, we finally succeeded in identifying conditions that maintained antigen stimulation in permeabilized cells. This required working at pH 7.2.

We characterized the ability of the two stimuli, i.e. antigen and GTP[S], to elicit secretion and PLD activation with respect to MgATP requirements and Ca^{2+} requirements under conditions where the cytosolic proteins were still present. When the permeabilizing agent (SLO) and the stimulus were added simultaneously, both stimuli were capable of eliciting exocytosis optimally at 10 μM Ca^{2+} in the presence of MgATP (Figure 1, time 0). We refer to this condition as 'acutely permeabilized'. The response to both stimuli was concentration-dependent; near-maximal activation was observed with 1 μM GTP[S] and maximal activation at 10 μM. For antigen, maximal secretion occurred at 40 ng/ml. Secretion was found to plateau at 20–25 min for both stimuli. In all subsequent experiments, GTP[S] was used at 10 μM and antigen at 40 ng/ml, and the cells were stimulated for 25 min.

Permeabilization with SLO generates lesions, which allow freely diffusable proteins to exit from cells. To examine the role of cytosolic proteins in secretion and PLD activation, the cells were permeabilized for different lengths of time to deplete cytosolic proteins. Antigen or GTP[S] was added subsequently (Figure 1). Parallel declines in PLD activity and exocytosis were

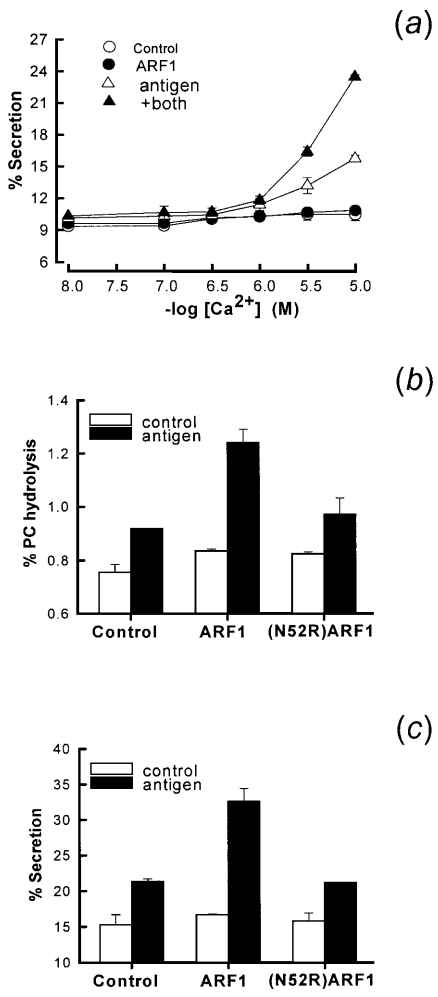

Figure 4 ARF1 reconstitutes secretion and PLD activity in permeabilized cells

(**a**) ARF1 restores antigen-meditated secretion in a Ca^{2+}-dependent manner. RBL-2H3 mast cells were permeabilized with 0.4 unit/ml SLO for 5 min in the presence of 1 mM MgATP, washed and stimulated with antigen (40 ng/ml DNP–HSA) in the presence or absence of 100 μg/ml ARF1 for 25 min at 37 °C. The supernatants were analysed for release of β-hexosaminidase. (**b**, **c**) ARF1, but not (N52R)ARF1, restores antigen-meditated secretion and PLD activity. [^{3}H]Choline-labelled RBL-2H3 mast cells were permeabilized with 0.4 unit/ml SLO for 5 min, washed and stimulated with antigen (40 ng/ml DNP–HSA) in the presence or absence of 100 μg/ml ARF1 or (N52R)ARF1 for 25 min at 37 °C. The supernatants were analysed for release of [^{3}H]choline (**b**) and β-hexosaminidase (**c**). Values are means ± range of duplicate incubations, and are taken from one of three experiments.

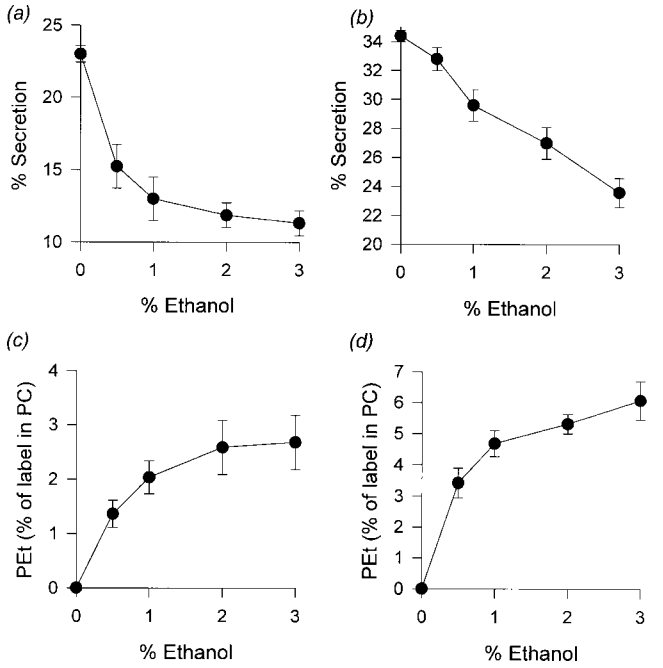

Figure 5 Ethanol inhibits ARF1-reconstituted exocytosis and increases PEt production

Ethanol inhibits secretion reconstituted with ARF1 + antigen (**a**) or ARF1 + GTP[S] (**b**), and increases PEt production in cells reconstituted with ARF1 + antigen (**c**) or ARF1 + GTP[S] (**d**). RBL-2H3 mast cells were permeabilized with 0.4 unit/ml SLO, washed and incubated with either antigen + ARF1 (**a** and **c**) or ARF1 + GTP[S] (**b** and **d**) in the presence of increasing concentrations of ethanol. For measurement of PEt, the cells were prelabelled with [^{3}H]alkyl-lyso-PC, and the amount of PEt produced was expressed as the amount of label incorporated into PC. Experiments were conducted in duplicate and were repeated on between three and seven occasions. Error bars reflect the S.E.M. values from pooled experiments: (**a**) $n = 3$; (**b**) $n = 7$; (**c**) $n = 3$; (**d**) $n = 4$.

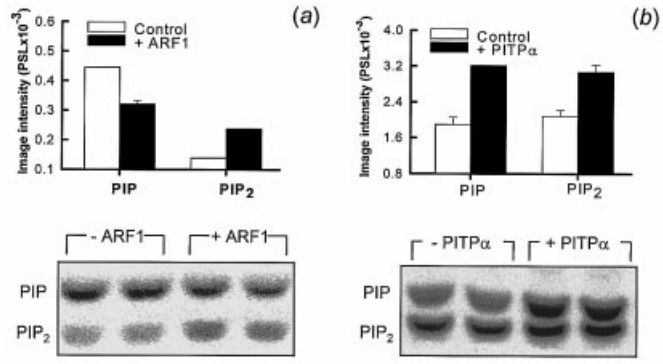

Figure 6 Effects of ARF1 and PITPα on phosphoinositide levels

(**a**) ARF1 stimulates the production of PIP_2 at the expense of PIP in permeabilized RBL-2H3 mast cells. (**b**) PITPα increases PIP and PIP_2 levels in permeabilized cells. RBL-2H3 mast cells were permeabilized with 0.4 unit/ml SLO for 10 min, washed and incubated with (**a**) 10 μM GTP[S] and 20 μCi/ml [γ-^{32}P]ATP in the presence or absence of 100 μg/ml ARF1 at 10 μM Ca^{2+}, or with (**b**) 20 μCi/ml [γ-^{32}P]ATP in the presence or absence of 50 μg/ml PITPα at 10 μM Ca^{2+}. The reactions were incubated at 37 °C for 20 min, quenched with chloroform/methanol and the polyphosphoinositides extracted and analysed by TLC. The TLC plates were analysed using a PhosphorImager plate, and the images of the plate are shown in the lower panels. The data were quantified using the appropriate software and are expressed as arbitrary units of image intensity (PSL). All experiments were conducted in duplicate and were repeated on four separate occasions with similar results. Error bars reflect the ranges of the duplicate values.

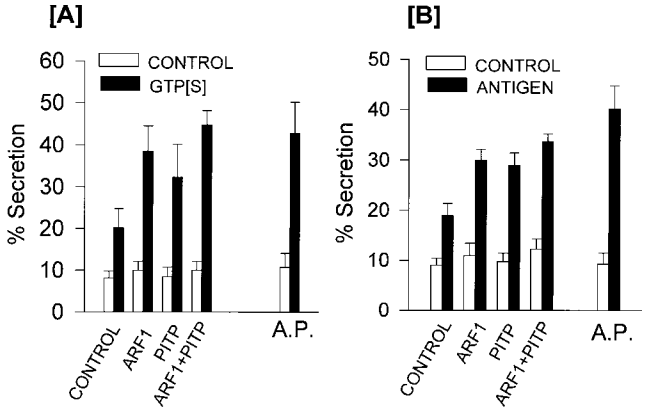

Figure 7 PITPα and ARF1 restore exocytosis

RBL-2H3 mast cells were depleted of cytosol, and reconstituted with 50 μg/ml PITPα, ARF1 or both in the presence or absence of (**A**) 10 μM GTP[S] or (**B**) antigen (40 ng/ml). The reactions were incubated for 25 min at 37 °C, and after centrifugation the supernatants were analysed for released β-hexosaminidase. Values are means ± S.E.M. from four experiments. The difference between secretion elicited by ARF1 alone or PITPα alone and that elicited by a combination of ARF1 and PITPα was analysed by a two-tailed t test, and these values were significantly different ($P < 0.02$). A.P. denotes 'acutely permeabilized' cells (see the text for details).

observed for both stimuli as the permeabilization period increased. Permeabilization for 5 min was sufficient to induce a marked loss of responsiveness to antigen, with complete loss by 10 min. In comparison, responses to GTP[S] were relatively resilient. GTP[S] was still capable of eliciting nearly 50 % of the maximal responses after 10 min of permeabilization.

To determine whether the loss of ARF1 proteins contributed to the decline in secretory and PLD responses, the leaked supernatant from the permeabilized cell preparations was examined (Figure 2). Within 5–10 min a substantial proportion of ARF1 proteins was found in the supernatant. In contrast, Rho proteins did not substantially leak out of cells, similar to results obtain previously with human neutrophils [16].

Secretion and PLD activation can be restored by ARF1 proteins in cytosol-depleted cells

To examine the possibility that ARF1 could restore the loss of secretory function and PLD activation from the cytosol-depleted cells, a reconstitution assay was used. For GTP[S] as a stimulus, the cells were permeabilized for 10 min and then washed by centrifugation to remove the leaked proteins. The depleted cells were stimulated with GTP[S] in the presence or absence of ARF1 or an ARF1 mutant, (N52R)ARF1, which can bind GTP but is unable to activate PLD1 [23] (Figure 3a). ARF1, but not (N52R)ARF1, increased both secretion and PLD activation in the cytosol-depleted cells. Optimal restoration of both responses required the presence of MgATP and 10 μM Ca^{2+}. To confirm that reconstitution of PLD activity and secretion was dependent on the activation of ARF1 by a guanine nucleotide, ATP[S] was examined; this nucleotide was not able to support ARF1-reconstituted exocytosis or PLD activity (Figures 3a and 3b).

The above experiment indicated that ARF1 could potentially mediate the activation of PLD and secretion, but did not reveal whether ARF1 is a component of the secretory pathway when a physiological stimulus is applied. To explore this possibility, RBL-2H3 mast cells were permeabilized for 5 min, washed and

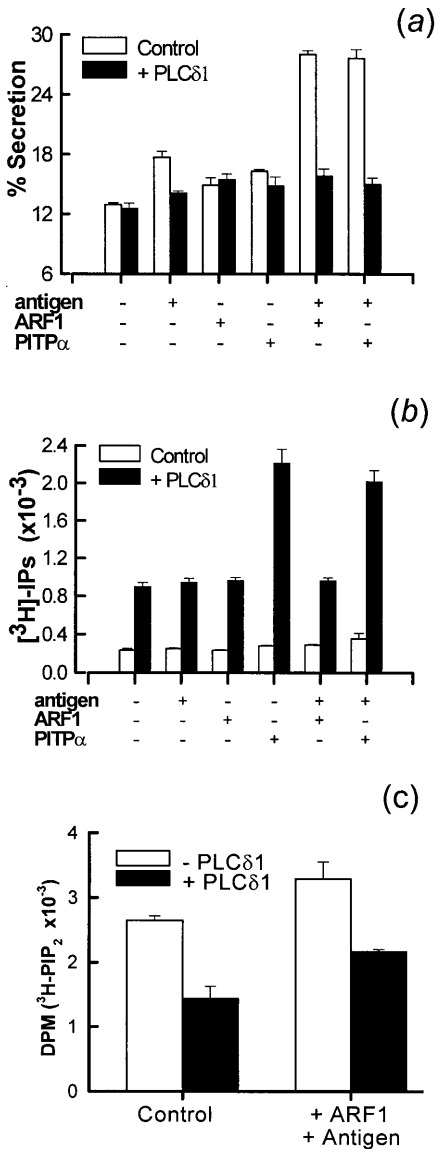

Figure 8 Depletion of phosphoinositide levels by PLCδ1 inhibits secretion reconstituted with ARF1 or PITPα

(**a**) PLCδ1 inhibits ARF1- and PITPα-mediated secretion; (**b**) PLCδ1 and PITPα enhance the amount of inositol phosphates formed; and (**c**) PLCδ1 depletes PIP_2 levels when added to permeabilized RBL-2H3 mast cells. RBL-2H3 mast cells were labelled to equilibrium with [^{3}H]inositol for 48 h. The labelled cells were permeabilized with 0.4 unit/ml SLO for 5 min, washed and stimulated with antigen (40 ng/ml) in the presence or absence of 10 μg/ml PLCδ1, 100 μg/ml ARF1 or 50 μg/ml PITPα for 25 min at 37 °C. The supernatants were analysed for released β-hexosaminidase or for released inositol phosphates (IPs), derived from hydrolysis of phosphoinositides by PLCδ1. The lipids were extracted from the cells and the levels of PIP_2 were analysed by TLC. Values are means $\pm$ range of duplicate incubations, and are taken from one of three similar experiments.

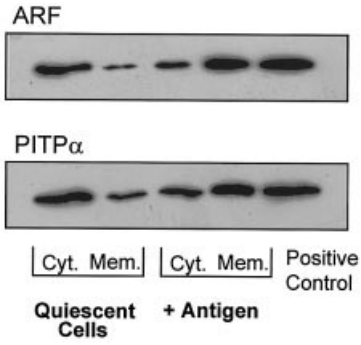

Figure 9 Antigen stimulation results in the retardation of PITPα and ARF1 leakage from permeabilized RBL-2H3 mast cells

Intact RBL-2H3 mast cells were stimulated with antigen (40 ng/ml DNP–HSA) for 10 min, and subsequently permeabilized with 0.4 unit/ml SLO for 10 min. Cellular membranes (Mem.) were separated from their leaked cytosolic contents (Cyt.) and analysed by Western blotting for ARF1 and PITPα. For the positive controls, recombinant proteins were used.

subsequently stimulated with antigen in the presence or absence of myristoylated ARF1 or the mutant (N52R)ARF1. Figure 4 illustrates that loss of secretory response to antigen was regained when ARF1 was included in the assay. Restoration was optimal at 10 μM Ca^{2+}. We noted that secretion stimulated by the antigen could only be restored provided that the permeabilization interval did not exceed 6–8 min. Restoration of the secretory process was strictly MgATP-dependent, emphasizing a requirement for phosphorylation. Specificity was tested using the inactive ARF1 mutant (N52R)ARF1. PLD activity and secretion were restored in parallel by ARF1, but not by (N52R)ARF1 (Figures 4b and 4c).

How does ARF1 restore secretory function?

A potential mechanism underlying the effects of ARF1 on secretion could be linked to its ability to stimulate PLD. Our recent analysis using ARF mutants identified a good correspondence between the ability of ARF1 to stimulate PLD activity and secretion in HL60 cells [23]. On the assumption that it is the production of PA that is required for secretory function, we used ethanol to divert the PA into production of PEt, in order to provide supporting evidence that PLD activation is required for the secretory process. Restoration of secretory function by ARF1 + GTP[S] or by ARF1 + antigen was inhibited by ethanol in a concentration-dependent manner (Figures 5a and 5b). Likewise, in the presence of increasing concentrations of ethanol, PEt production increased (Figures 5c and 5d). Ethanol at 1% (v/v) was sufficient to inhibit secretion mediated by antigen + ARF1 in the reconstituted system; this concentration effectively inhibits secretion in intact cells [3,24]. In contrast, 3% ethanol was required for inhibition of secretion mediated by GTP[S] + ARF1 in the reconstituted system (compare Figures 5a and 5b). This difference in sensitivity to ethanol could reflect the lower levels of PLD activation stimulated by antigen + ARF1 compared with GTP[S] + ARF1. When GTP[S] + ARF1 is used as a stimulus, ethanol is less effective because of the greater potency of GTP[S] as a stimulus (compare Figures 5c and 5d).

We next examined whether ARF1 has an effect on phosphoinositide levels. ARF1 has been shown to raise PIP_2 levels via direct activation of phosphoinositide 4-kinase β [25]. Alternatively, ARF1 raises PA levels via PLD, and PA can potentially activate type I phosphatidylinositol monophosphate 5-kinase, which catalyses the phosphorylation of phosphatidylinositol 4-monophosphate (PIP) to PIP_2 [26–28]. Figure 6(a) illustrates that ARF1 increased PIP_2 levels in permeabilized cells, and this was accompanied by a decrease in PIP levels. The increase in PIP_2 levels was small, but may only appear so because we are monitoring global cellular levels of PIP_2, which include multiple pools which may not be affected by ARF1. Because of the small changes, we have been unable to use ethanol to examine whether

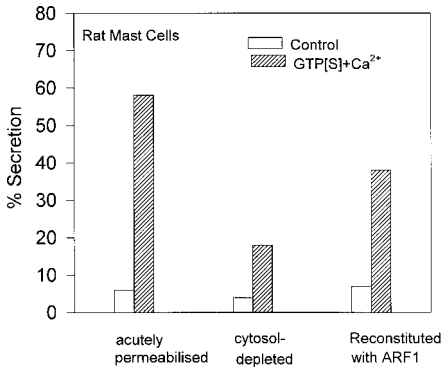

Figure 10 ARF1 restores exocytosis in rat peritoneal mast cells

Mast cells were permeabilized with 0.4 unit/ml SLO for 10 min to deplete endogenous cytosol, washed and reconstituted with 50 μg/ml ARF1 in the presence or absence of 10 μM GTP[S] and 10 μM Ca^{2+}. The reactions were incubated for 25 min at 37 °C; after centrifugation, the supernatants were analysed for released β-hexosaminidase. Values are means $\pm$ range of duplicate incubations, and are taken from one of three experiments.

the increase in PIP_2 levels is mediated directly via ARF1 or via the ARF1-stimulated PLD pathway. However, to test the possibility that increased production of PIP_2 was the essential outcome of the effect of ARF1, we examined whether PITPα, another cytosolic protein that is known to be important in PIP_2 synthesis [22], could replace ARF1 in the exocytotic process. PITPα was detected in RBL-2H3 mast cells by immunoblotting, and was found to leak out of SLO-permeabilized cells at a similar rate to ARF1 (Figure 2).

PITPα was found to restore exocytosis (Figures 7A and 7B), but not PLD activity (results not shown). Unlike ARF1, addition of PITPα increased the levels of both PIP and PIP_2 in permeabilized RBL-2H3 mast cells (Figure 6b). However, an increase in PIP_2 levels was insufficient to trigger exocytosis, as stimulation with GTP[S] or antigen was still required (see Figure 7). The effect of PITPα on secretion was both Ca^{2+}- and MgATP-dependent. The combination of PITPα and ARF1 gave a slight enhancement over the effects of each component alone, and the level of secretion attained was similar to that obtained in the acutely permeabilized cells, where the endogenous proteins were still present (Figure 7).

To reinforce the importance of PIP_2 in exocytosis, we used PLCδ1 to deplete PIP_2 levels. We confirmed that addition of PLCδ1 increased the production of inositol phosphates (Figure 8b). PLCδ1 reduced PIP_2 levels by nearly 40 % (Figure 8c), and this was sufficient to inhibit both PITPα- and ARF1-reconstituted antigen-mediated exocytosis (Figure 8a). When PITPα was present together with PLCδ1, inositol phosphate production was substantially enhanced; this is an expected result, as the transfer protein is involved in regulating substrate supply [29].

Antigen stimulation leads to recruitment of PITPα and ARF1 to membranes

Both ARF1 and PITPα have been found to reconstitute antigen-mediated secretion. To examine whether both proteins can be regulated upon antigen-mediated stimulation, we examined the translocation of the two proteins from the cytosol to the membranes. Figure 9 illustrates that when cells were activated with antigen before permeabilization, less ARF1 and PITPα exited from the cells and became associated with the membranes.

ARF1 also reconstitutes secretion from rat peritoneal mast cells

Rat peritoneal mast cells have been widely used in studies of exocytosis, and we also examined whether ARF1 proteins were capable of restoring secretory function in these cells. Rat mast cells were stimulated with 10 μM Ca^{2+} and 10 μM GTP[S] either during the initial permeabilization with SLO ('acutely permeabilized') or after cytosol depletion for 10 min. Ca^{2+} and GTP[S] enhanced secretion to a small extent from cytosol-depleted cells, and this was potentiated when ARF1 was included during the assay (Figure 10). The extent of reconstitution observed with ARF1 did not reach the level seen in acutely permeabilized cells, suggesting that other cytosolic factors may have become rate limiting.

DISCUSSION

ARF1 has a central role in constitutive membrane traffic, where it has been identified as a structural component for coat protomer (COP)-coated vesicles [30]. Additionally, ARF1 is required for recruitment of adaptor proteins AP1, AP2 and AP3 at both the plasma membrane and the *trans*-Golgi network [31–33]. Other roles for ARF1 include a requirement in vesicle formation at the *trans*-Golgi network in a reaction not necessarily requiring COPs or other identified coat proteins [34], and recruitment of paxillin to focal adhesion complexes [35]. In the present study we extend the function of ARF1 to include a role in antigen-regulated exocytosis in RBL-2H3 mast cells.

ARF1 can interact directly with several effectors, including PLD1, ARFAPTIN and the β-subunit of coatomer proteins [15,36,37]. In the present study of regulated exocytosis, the data support the possibility that part of the function of ARF1 is mediated via PLD. Previous studies using intact mast cells have shown that ethanol (a reagent that interferes with the production of PA, the product of PLD activity) inhibits secretion [3,24]. In the present study we demonstrate that the ability of ARF1 to reconstitute secretion was also inhibited by ethanol. Additional evidence that ARF1-mediated exocytosis is via PLD comes from mutational analysis of ARF1; PLD effector domain mutants have greatly extended the relationship between PLD activation and exocytosis [23]. Other activators of the exocytotic process that have been identified in mast cells are Rho proteins and protein kinase C [2,6,7]. Rho proteins are also recognized regulators of PLD activity, as are the conventional isoforms of protein kinase C [10,15,38]. Thus a common theme emerges: the proteins that have been identified previously as participants in the exocytotic function of RBL-2H3 mast cells are all activators of the PLD pathway.

We also show that ARF1 can stimulate PIP_2 synthesis at the expense of PIP in permeabilized cells. The mechanism of this stimulation may be complex. The product of ARF1-regulated PLD activity is PA, which can regulate the activity of type 1 PIP 5-kinases [26–28]. In addition, ARF1 has been shown to increase PIP and PIP_2 levels at the Golgi by recruitment of phosphoinositide and PIP kinase independently of PLD activity [39]. Although we cannot rule out this route of PIP_2 synthesis taking place at the Golgi, only PIP_2 produced at the lysosomal granule or the plasma membrane (or both) could play a role in exocytosis. PLD1 and ARF6 both localize at the lysosomes and plasma membranes, indicating that these are the relevant sites of PA production, and thus PIP_2 synthesis [40–42]. (Although we have used ARF1 for our studies, ARF6 is equally capable of restoring secretory function in permeabilized cells. Due to the instability of myristoylated ARF6, a detailed analysis has not been possible.) The ability of PITPα to replace ARF1 in the reconstitution assay

provides further support for the hypothesis that PIP_2 is required for exocytosis. PITPα is also required for the synthesis of PIP_2 as a substrate for PLCs [22,43], in the Ca^{2+}-stimulated exocytotic pathway in PC12 cells [44,45], and for vesicle formation ([46]; reviewed in [47]). Interestingly, PITPα and ARF1 can be functionally exchanged in the vesicle formation assay, just as in the secretory reconstitution assays presented here.

ARF1 and PITPα both restore exocytosis from permeabilized RBL-2H3 mast cells by stimulating the synthesis of PIP_2, and in their combined presence the response was more robust. This suggests that both proteins are physiologically relevant, and this is clearly supported by the observation that antigen stimulation causes the relocation of both proteins. Potential roles for PIP_2 in exocytosis include recruitment of specific proteins in a spatially and temporally regulated manner, and modulation of enzymic activities. There are many proteins with pleckstrin homolgy domains that interact with PIP_2 [48]. In addition, many cytoskeletal proteins undergo conformational changes in the presence of PIP_2 [49]. While the results presented here do not shed light on the mechanism of the PIP_2 requirement in exocytosis, they provide a clear indication that this lipid plays a prominent role in the process leading to exocytosis, not only in neuronal cells but also in cells of the haematopoietic origin.

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