Phospholipases Stimulate Secretion in RBL Mast Cells†

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ABSTRACT: Roles for glycerophospholipids in exocytosis have been proposed, but remain controversial. Phospholipases are stimulated following the activation of the high-affinity receptor for immunoglobulin E (IgE) in mast cells. To study the biochemical sequelae that lead to degranulation, broken cell systems were employed. We demonstrate that the addition of three distinct types of exogenous phospholipases (i.e., bcPLC, scPLD, and tfPLA_2), all of which hydrolyze phosphatidylcholine (PC), trigger degranulation in permeabilized RBL-2H3 cells, a mucosal mast cell line. Production of bioactive lipids by these phospholipases promotes release of granule contents through the plasma membrane and acts downstream of PKC, PIP_2, and Rho subfamily GTPases in regulated secretion. These exogenous phospholipase-induced degranulation pathways circumvent specific factors activated following stimulation of the IgE receptor as well as in ATP- and GTP-dependent intracellular pathways. Taken together, these results suggest that regulated secretion may be achieved in vitro in the absence of cytosolic factors via phospholipase activation and that products of PC hydrolysis can promote exocytosis in mast cells.

Allergic and immune challenges elicit the regulated secretion of bioactive molecules from mast cells during the process of degranulation. Although degranulation requires the fusion of secretory granules with the plasma membranes, it is not yet known whether changes in phospholipid composition can by themselves elicit exocytosis. Recently, SNARE fusion complexes have been proposed as being sufficient to catalyze bilayer fusion events (*1*−*3*). While these models do not directly invoke dynamic roles for phospholipid metabolism, the inclusion of specific phospholipids or cytosol in the presence of enriched endosome preparations does not rule out the possibility that phospholipid factors may participate in unappreciated ways in these systems. By contrast, other investigators have proposed that trafficking proteins participate in targeting, while changes in specific phospholipids provide the driving force for vesicle fusion (*4*).

Compensatory mutations in two genes from *Saccharomyces cerevisiae* involved in long chain fatty acid elongation and sphingolipid synthesis allowed differential recovery of secretion and viability in v-SNARE defective mutants (*5*). This suggests that long chain fatty acids are utilized during secretion, presumably by enhancing vesicle fusion, consistent with predictions from the modified stalk hypothesis (*4*, *6*) and that SNARE complexes, while specifying proper vesicle targeting, are unlikely to represent the minimal membrane fusion machinery [reviewed by Robinson and Martin (*7*)]. A report by Scheller and colleagues suggests that SNARE interactions mediate vesicle trafficking fidelity and that other target proteins may facilitate these interactions (*8*). Recent reports have demonstrated that the acylation of lysophosphatidic acid is a key step in the formation of synaptic vesicles (*9*) or vesicle biogenesis from Golgi membranes (*10*). The reconstitution of vesicles coated with either clathrin (*11*) or coatomer (*12*) on chemically defined liposomes also suggests a role for specific phospholipids in vesicle biogenesis and intracellular transport. Evidence from several groups implicates phospholipase D (PLD)[1] activity in transport-vesicle formation and secretion in mammalian cells (*13*), as well as in sec14-mediated secretory processes in yeast (*14*).

A signal-activated phospholipase D (PLD), initially described in several tissues and cell lines, hydrolyzes phosphatidylcholine (PC) into choline and phosphatidic acid (PA) [reviewed by Singer et al. (*15*) and Exton (*16*)]. This activity is stimulated by two subfamilies of GTP-binding proteins [ADP-ribosylation factor (Arf) and Rho and classical isoforms of protein kinase C (PKC)]. It requires phosphatidylinositol 4,5-bisphosphate (PIP_2) as a cofactor for activity (*15*, *16*) and has been linked to fodrin (*17*) and gelsolin (*18*), two components of the peripheral cytoskeleton. Interestingly, studies from numerous laboratories have linked these factors to secretion processes (*22*−*27*). Frohman and colleagues have

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[1] Abbreviations: RBL, rat basophilic leukemia; IgE, immunoglobulin E; bc, *Bacillus cereus*; sc, *Streptomyces chromofuscus*; tf, *Trimeresurus flavoviridis*; PLC, phospholipases C; PLD, phospholipase D; PLA_2, phospholipase A_2; TAG, triacylglycerol; PI, phosphatidylinositol; PC, phosphatidylcholine; PA, phosphatidic acid; DAG, diacylglycerol; PEtOH, phosphatidylethanol; LPC, lysophosphatidylcholine; PIP_2, phosphatidylinositol 4,5-bisphosphate; pa, palmitoyl arachidonoyl; po, palmitoyl oleoyl; Arf, ADP-ribosylation factor; PKC, protein kinase C; Cy3, carbocyanine; SLO, streptolysin O; MEM, minimum essential medium; DNP-BSA, 2,4-dinitrophenylated bovine serum albumin; ATP, adenosine 5′-triphosphate; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); AMP-PNP, adenylyl imidodiphosphate; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); GMP-PNP, guanylyl imidodiphosphate; TLC, thin-layer chromatography.

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cloned two distinct mammalian genes (PLD1 and PLD2), and extensive molecular dissection has revealed distinctions in the cellular localization and function of these two isoenzymes (*19*). Several PLD-inhibiting factors identified as amphiphysin I and II (*20*), synaptojanin, and AP180 (*21*) have also been linked to transport processes. Moreover, PLD activity has itself been correlated with secretion in some cell types (*24*). As yet, the specific roles of phospholipids in cellular secretion have not been differentiated from the potential participation of the multifunctional activator proteins.

Phospholipase A_2 (PLA_2), phosphatidylinositol phospholipase C (PI-PLC), and PLD have been shown to be activated in response to the stimulation of the high-affinity immunoglobulin E (IgE) receptor in mast cells (*28*), and phosphatidic acid derived from PLD has been implicated in immune responses in neutrophils (*29*) and rat basophilic leukemia (RBL-2H3) cells (*30*). In experiments in which liposomes were used, phospholipase treatment or the inclusion of primary metabolites derived from their activities has promoted membrane fusion. These include liposomes treated with PLC (*4*), PLD (*31*), and PLA_2 (*32*) or containing phosphatidic acid (*33*) or free fatty acid (*34*). Isolated secretory granules (*35*) and synaptic vesicles (*36*) treated with porcine PLA_2 were shown to fuse in vitro with isolated plasma membranes and presynaptic membranes, respectively. Findings further suggest that pharmacological inhibitors of PLA_2 block vesicle fusion in macrophages (*37*). Additional evidence for a central role of lipids in exocytosis was recently provided by Cockcroft and colleagues (*38*) by demonstrating that ethanol blocks ARF1-reconstituted secretion in RBL-2H3 cells. The findings imply that ARF1-regulated PLD participates in exocytosis via increased production of PIP_2. While the precise mechanisms of PIP_2 involvement in exocytosis are undefined, these insights raise questions as to whether other bioactive lipids participate directly in regulated secretion.

To address the central role of phospholipid metabolism in degranulation in RBL-2H3 cells, we have introduced two bacterial phospholipases (*Streptomyces chromofuscus* PLD and *Bacillus cereus* PLC) and snake venom-derived PLA_2 (*Trimeresurus flavoviridis*) into streptolysin O-permeabilized cells. These PC-preferring phospholipases are independent of mammalian regulation and function as constitutively active phospholipases, thereby providing a tool for examining the effects of lipid hydrolysis on regulated secretion. It is unlikely that these exogenous phospholipases participate in physiologically relevant protein−protein interactions. This approach allows us to test the relatively pure effects of bioactive lipid formation without the confounding effects of adding protein activators that themselves have been implicated in exocytotic processes.

EXPERIMENTAL PROCEDURES

Materials. Ro-31-8220, calphostin C, *B. cereus* PC-PLC, and *T. flavoviridis* PLA_2 were obtained from Calbiochem (San Diego, CA). *Clostridium difficile* toxin B was provided by K. Aktories (Mainz, Germany). Phospholipids were obtained from Avanti Polar Lipids (Alabaster, AL). Streptolysin O was obtained from Abbott Diagnostics (Abbott Park, IL). *S. chromofuscus* PLD, *B. cereus* PI-PLC, TAG lipase (*Rhizopus arrhizus* triacylglycerol acylhydrolase), and all adenosine and guanosine nucleotides and nucleotide analogues were obtained from Boehringer Mannheim (Indianapolis, IN). The lactate dehydrogenase analysis kit (Optimized, Ultraviolet, Kinetic, GSCC), β-escin, and *p*-nitrophenyl *n*-acetyl-β-D-glucosaminide were obtained from Sigma-Aldrich (St. Louis, MO). Myristic acid, [9,10-^{3}H(N)]-tetradecanoic acid, was obtained from NEN Life Science Products (Boston, MA). The 5G10 antibody was generously provided by J. Bonifacino at NICHD (Bethesda, MD). DNP-BSA (2,4-dinitrophenylated bovine serum albumin), anti-2,4-dinitrophenyl mouse IgE mAb, goat anti-mouse IgG conjugated to the amino reactive carbocyanine dye (Cy3), and RBL-2H3 cells were generous gifts from D. Holowka and B. Baird at Cornell University.

Cell Culture. RBL-2H3 cells were maintained as monolayers in 75 cm × 75 cm flasks in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD), 4 mM L-glutamine, insulin-transferrin-selenium A, and 50 μg/mL gentamicin (Gibco BRL) at 37 °C. Cells were typically used 3−5 days after passage and were grown overnight in the presence of 5% CO_2 prior to the assay, at which time 2.5×10^5 cells were plated overnight (unless indicated otherwise).

Treatment with Inhibitors and Toxins. During experiments in which inhibitors were used, agents were added directly to fresh cell medium. Cells were preincubated for 30 min with either 2 mM neomycin sulfate or 3.6 μM brefeldin A, for 60 min with either 1 μM Ro-31-8220 or 1 μM calphostin C, or for 2.5 h with 100 ng/mL *C. difficile* toxin B. Cells were washed prior to stimulation of degranulation. In cells pretreated with neomycin, the compound was also present during the degranulation assay.

Cell Permeabilization and Stimulation. Intact cells were transferred to ice and washed twice in ice-cold permeabilization buffer (buffer P) containing 10 mM HEPES, 115 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, 3 mM EGTA, and 5 mM glucose. Streptolysin O (SLO, 2 units/mL) in buffer P was added to the cells at 4 °C and the mixture incubated for 15 min. SLO was removed, and cells were washed with ice-cold buffer P to remove residual, unbound SLO. Cytosol-depleted preparations were obtained by washing cells with prewarmed (37 °C) buffer P, three times over the course of 30 min. β-Escin suspended in buffer P was added to the cells and incubated for 2 h at 4 °C followed by removal of unbound β-escin. Permeabilized cells to be stimulated with phospholipases were incubated in prewarmed buffer for 5 min to permit pore formation. Nucleotide-stimulated cells were not routinely preincubated (except where indicated) to minimize loss of cytosolic factors and maintain responses. Cells were stimulated by addition of prewarmed buffer P supplemented with 3.1 mM total $CaCl_2$ (producing a calculated free Ca^{2+} concentration of 103 μM based on Sliders version 1.00 or WINMAXC version 1.80 software using BERS constants). The buffer also contained (as indicated) 5 mM ATP, 0.5 mM GTPγS with 1 mM ATP, or 0.4 unit/mL exogenous phospholipases. In some experiments, 0.5% EtOH was added to the stimulation mixtures to increase phospholipase solubility. EtOH (3.0%) was included during nucleotide stimulation of degranulation when lipid analysis was performed to measure the amount of transphosphatidylation product. Spontaneous release (attributable to calcium-

containing buffer alone) was assessed by analysis of identically treated cells incubated in buffer alone, and the level of release was subtracted from all degranulation responses. In experimental results obtained over several months, this typically ranged between 5 and 10%. Unless otherwise indicated, all assays were carried out at pH 7.4 and 37 °C for 60 min.

Intact Cell Stimulation. Following overnight incubation with 1 μg/mL IgE, cells were washed twice with cold Tyrodes buffer [20 mM HEPES, 115 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 0.1% bovine serum albumin (BSA), and 1.8 mM $CaCl_2$ (pH 7.4)]. Cells were incubated for 15 min at 4 °C, and buffer was removed. Prewarmed Tyrodes buffer (250 μL, 37 °C) containing 0.85 μg/mL DNP-BSA was added to the cells, and release was assessed at 60 min.

β-Hexosaminidase Release Assay. The level of degranulation was determined using a modified colorimetric assay to assess release of β-hexosaminidase as previously described (*39*). Briefly, 50 μL of the sample supernatant was incubated with 200 μL of 1 mM *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide [in 0.05 M citrate buffer (pH 4.5)] for 1 h at 37 °C. The total β-hexosaminidase concentration was determined by a 1:1 extraction of the remaining buffer and cells with 1% Triton X-100; a 50 μL aliquot was removed and analyzed as described. Reactions were quenched by addition of 500 μL of 0.1 M sodium carbonate buffer. The enzyme concentration was determined by measuring the OD at 400 nm. β-Hexosaminidase release was represented as a percent of total enzyme. Unless otherwise indicated, experiments were carried out in duplicate, and each data point represents the average of two samples. The measurement of the level of degranulation coincident with lipid analysis was derived from single samples. Leakage of soluble β-hexosaminidase from β-escin-permeabilized cells was assessed by determining the level of release of the enzyme in a calcium-free buffer.

LDH Release Assay. Leakage of soluble lactate dehydrogenase from β-escin-permeabilized cells was quantitated using a modified sample start procedure with the Sigma Diagnostics LDH (optimized) kit (procedure DG1340-UV). Briefly, following permeabilization, stimulation, and incubation of cells, a 4 μL aliquot of supernatant was added to 96 μL of LDH sample start reagent and inverted. Following a 30 s incubation, absorbance (340 nm) was read at 1 min intervals. LDH activity was calculated from the average decrease in absorbance.

Lipid Analysis. RBL-2H3 cells (5×10^5) were grown overnight in growth medium as described. Cells were washed twice with serum-free MEM. [^{3}H]Myristic acid (200 μL, 20 μCi/mL) in serum-free MEM was incubated with cells at 37 °C with 5% CO_2 for 16 h. A duplicate plate for secretion analysis was treated similarly but without the label. We observed that the secretion responses to ATP and GTPγS were routinely lower following serum starvation. Cells were washed twice in ice-cold buffer P, permeabilized, and stimulated as described. Following stimulation, aliquots were taken from the duplicate plate for secretion analysis. Labeled cells were transferred to ice; stimulation buffer was removed, and a 1:1 mixture of cold methanol and 0.1 M HCl was added. Cells were collected and transferred to a tube containing $^1/_2$ volume of chloroform. Samples were vortexed and centrifuged to enhance phase separation. The aqueous phase was carefully removed, and lipids were dried under nitrogen. Samples were resuspended in chloroform and analyzed by thin-layer chromatography (TLC) using a resolving system containing a 10:2:4:2:1 chloroform/methanol/acetone/acetic acid/water mixture. The TLC plate was exposed for 4 days on a Kodak ^{3}H phosphorimager screen and quantitated using a Molecular Dynamics Storm 840 instrument. Phospholipid species were identified by comigration with the following known standards: palmitoylarachidonoyldiacylglycerol (paDAG), phosphatidylethanol (PEtOH), palmitoyloleoylphosphatidic acid (poPA), palmitoylarachidonoylphosphatidylcholine (paPC), and lysophosphatidylcholine (LPC).

Immunolocalization. Intact or SLO-permeabilized cells were either washed with buffer (as described previously) or stimulated with antigen, nucleotides, or exogenous phospholipases for 15 min. Cells were washed briefly in phosphate-buffered saline (PBS) and 1 mM EDTA and fixed for 10 min in PBS, EDTA, and 3.7% formaldehyde. Fixation was halted by washing the cells with PBS, EDTA, and 1% BSA. Fixed cells were briefly washed in PBS and EDTA and incubated with 5G10 supernatant (1:1) for 60 min. The cells were washed in PBS and EDTA and then incubated for 60 min with a Cy3-conjugated, goat anti-mouse secondary antibody (diluted 1:200 in PBS, EDTA, and 1% BSA). The secondary antibody was removed, and cells were stored in PBS and EDTA until imaging. Just prior to imaging, a fresh PBS/EDTA mixture was added. Cells were imaged using an Olympus (Melville, NY) IX70 inverted microscope, a 100× oil immersion objective lens, and a rhodamine filter cube. Images were digitally captured using Media Cybernetics (Silver Spring, MD) ImagePro version 3.0.

RESULTS

Characterization of Exogenous Phospholipase Responses. Degranulation was assessed by the release of the granule-contained enzyme, β-hexosaminidase, in streptolysin O-permeabilized RBL-2H3 cells. To examine the effects of changing lipid composition in RBL-2H3 cells, a bacterial PLD from *S. chromofuscus*, a PC-PLC from *B. cereus*, and *T. flavoviridis* PLA_2 were used to challenge cells and β-hexosaminidase release was assessed as described. As shown (Figure 1a), the degranulation responses increased with phospholipase concentration until the responses reached saturation. A phosphatidylinositol specific PLC and a triacylglycerol (TAG) lipase failed to induce a response. Additions of exogenous phospholipases that had been heat denatured failed to induce degranulation (data not shown), demonstrating that catalytic activity was essential. The exogenous phospholipases were also tested by application to the outer surfaces of intact cells. Under the conditions that were tested, phospholipid hydrolysis in the outer leaflet of the plasma membrane was insufficient to elicit degranulation (data not shown), suggesting that production of bioactive lipid on internal cytoplasmic membranes was essential.

Addition of exogenous phospholipases to cells either concurrent with permeabilization (unwashed) or following the removal of cytosol (washed) resulted in level of degranulation of similar magnitudes (Figure 1b). Previous studies have revealed two distinct pathways capable of

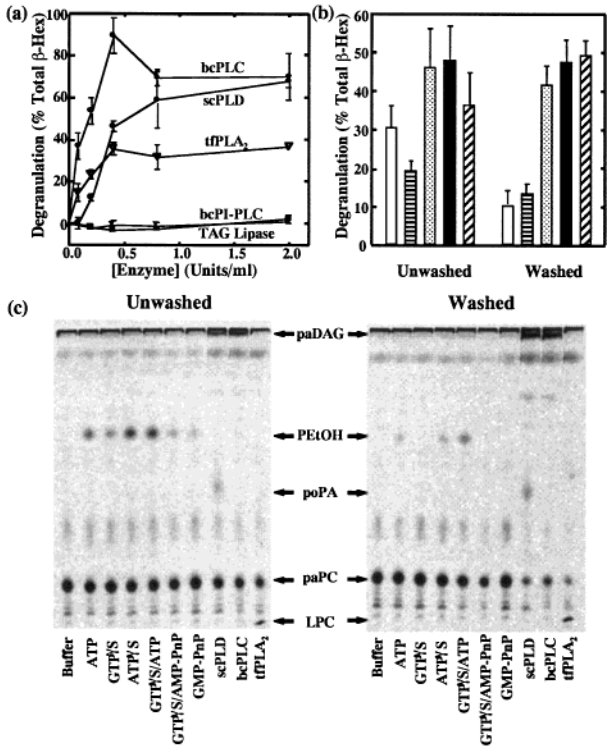

FIGURE 1: Characterization of phospholipase-mediated degranulation in permeabilized cells. (a) Samples were stimulated with increasing concentrations of scPLD (●), bcPLC (●), tfPLA$_2$ (▽), bcPI-PLC (▼), and TAG lipase (■). (b) Application of exogenous phospholipases to permeabilized RBL-2H3 cells results in degranulation independent of cytosolic factors. ATP (white), GTPγS (horizontal stripes), scPLD (dotted), bcPLC (black), and tfPLA$_2$ (diagonal stripes) were applied either concurrently with cell permeabilization (unwashed) or following the removal of cytosol (washed) for 45 min as shown. (c) Lipids were extracted from cells labeled with [^{3}H]myristic acid and stimulated with the indicated nucleotides, hydrolysis-resistant nucleotide analogues, or exogenous phospholipases in both unwashed and washed cell preparations. The samples were analyzed by TLC, and the relative migrations of phospholipid standards are indicated. The results shown in panels a and b are the mean of three independent experiments, while the results shown in panel c are from a single representative experiment ($n = 3$).

triggering degranulation that utilize a combination of calcium, ATP, and the hydrolysis-resistant GTP analogue, GTPγS (*40*). The level of stimulation of cells by one of these two pathways, termed the high-ATP (5 mM ATP) pathway or the GTPγS (0.5 mM GTPγS/1 mM ATP) pathway, was reduced in the washed RBL-2H3 cell preparations. Use of hydrolysis-resistant analogues of ATP (i.e., ATPγS) supported phospholipase activity and secretion; however, nonhydrolyzable analogues of either ATP or GTP (i.e., AMP-PNP and GMP-PNP) were less responsive. Phospholipid analysis of [^{3}H]myristic acid-labeled RBL-2H3 cells following exogenous phospholipase-stimulated degranulation revealed significant lipid metabolism under both experimental conditions (Figure 1c). In contrast, stimulation of unwashed cells with a high level of ATP or GTPγS (or other ATP and GTP analogues) stimulated an endogenous PLD activity that was partially inhibited by removal of cytosolic components (i.e., washed preparations). Our results indicated that each of the described degranulation pathways is accompanied by phospholipase activation (Figure 1c). Although different stimuli generate distinct products, one possibility is that these lipid second messengers have common effects on membrane

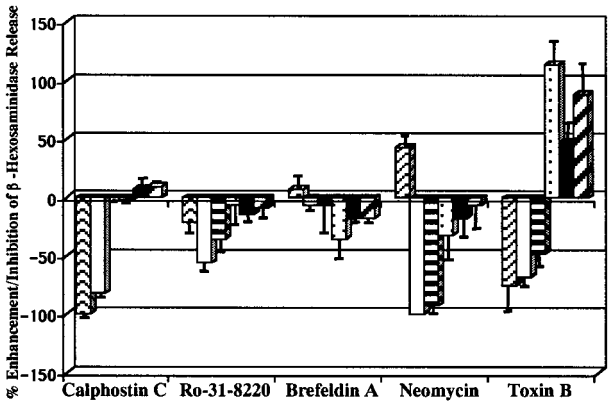

FIGURE 2: Effects of pharmacological inhibitors on mast cell degranulation. Inhibition of protein kinase C (calphostin C, Ro-31-8220), brefeldin A-sensitive Arf isoforms, PIP$_2$ binding (neomycin), and the Rho proteins (*C. difficile* toxin B) by specific inhibitors has differential effects on antigen-mediated degranulation in intact cells as well as nucleotide- and phospholipase-stimulated degranulation in permeabilized cells. Samples were stimulated with either antigen (wavy lines), ATP (white), GTPγS (horizontal stripes), scPLD (dotted), bcPLC (black), and tfPLA$_2$ (diagonal stripes). The results shown are the average of three independent experiments.

structure such that bilayer fusion is promoted. Alternatively, these phospholipase-mediated reactions may serve to generate substrates for common secondary product formation that are crucial to the fusion process. Significantly, to date we have not found a stimulus that triggers degranulation in the absence of phospholipid metabolism.

Inhibitors of Degranulation. To compare the exogenous phospholipases to well-characterized secretion pathways in mast cells, we determined the sensitivity of specific factors involved in the ATP- and GTP-dependent degranulation pathways and looked for resulting blocks in lipid metabolism. Pretreatment with calphostin C (a competitive inhibitor that binds to the DAG-binding site of PKC) markedly inhibited antigen-stimulated degranulation in intact RBL-2H3 cells and high-level ATP-dependent degranulation in permeabilized cells, but it had little effect on stimulation by GTPγS and exogenous phospholipases (Figure 2). Ro-31-8220 (an inhibitor of PKC catalytic activity) also partially blocked antigen stimulation of intact cells and nucleotide-dependent degranulation in permeabilized cells.

To further validate the in vitro system, we examined the effects of other factors, which have been shown to inhibit both agonist and calcium ionophore-induced degranulation in RBL-2H3 cells (*41*). Brefeldin A is a fungal toxin [an uncompetitive inhibitor of certain Arf exchange factors (*42*) which blocks the exchange of GTP for GDP on target Arf isoforms] often utilized to identify Arf-mediated activities. In our experiments, pretreatment of cells with brefeldin A demonstrated little inhibition of either agonist- or nucleotide-dependent degranulation (Figure 2). On the basis of findings by Cockcroft (*38*), these results suggest that IgE-mediated Arf effects are mediated by a brefeldin insensitive exchange factor.

Neomycin is an antibiotic that has been shown to bind to polyphosphatidylinositols in cell membranes. This binding has been shown to disrupt PLD activity in some cells (*43*). Addition of 2 mM neomycin inhibits degranulation in response to both GTPγS and high levels of ATP (Figure 2).

The antigen-mediated responses were modestly stimulated by neomycin treatment, although it is unknown how much of the compound enters intact cells under these conditions. Some effects were seen in response to phospholipase stimulation, but these were much smaller than that observed in the GTPγS or high ATP conditions. Although these relatively high doses of neomycin are not specific for PIP2 binding, the findings are noteworthy. In contrast to blocks in nucleotide triphosphate-stimulated degranulation, neomycin fails to inhibit exogenous phospholipase-mediated degranulation. This suggests that formation of other bioactive lipids can bypass the need for available PIP_2.

Pretreatment of cells with *C. difficile* toxin B (Figure 2), which causes glucosylation and the subsequent inactivation of Rho subtype family GTPases, resulted in inhibition of agonist-dependent degranulation in intact cells and nucleotide-dependent degranulation in permeabilized cells. In contrast, the effects of the exogenous phospholipases were enhanced by pretreatment with toxin B. The findings reveal that pathways stimulated by agonist, high ATP, and GTPγS are upstream of Rho subfamily GTPases. Although the significance of enhanced phospholipase-induced release following the *C. difficile* toxin B treatment is not understood, it may reveal some unappreciated negative regulation of exocytosis by Rho, perhaps via a cytoskeletal interaction.

Differentiating Degranulation and Vesicle Leak. We sought to exclude the possibility that exogenous phospholipase treatment was destabilizing granule membranes and causing contents to simply leak out of granules as opposed to granule fusion with the plasma membrane. Therefore, we conducted a series of experiments to distinguish the amount of lipid product generated from the extent of granule release. In this way, we determine whether lipid products can be generated under conditions in which granule fusion does not follow. Variation in either the temperature (Figure 3a−d) or pH (Figure 3e−h) was able to discriminate specific phospholipase hydrolytic activities from degranulation. In permeabilized cells stimulated at 22 °C, both high ATP and GTPγS (Figure 3a) initiated release of β-hexosaminidase. At this lower temperature (22 °C), there were decreases in the level of degranulation induced by the three phospholipases (Figure 3b−d) compared to the level of degranulation at 37 °C. While the amount of PA produced at 22 °C was only modestly reduced, the amount of degranulation was essentially eliminated. Similarly, in response to bcPLC, an increased production of diacylglycerol (DAG) was observed possibly due to a decrease in the rates of degradation, yet the resulting degranulation was reduced at the lower temperature. At pH 5.3, addition of the nucleotides (Figure 3e), bcPLC or $tfPLA_2$ (Figure 3g,h), was incapable of stimulating degranulation. scPLD addition (Figure 3f) resulted in comparable granule release at both pH 7.4 and 5.3. Interestingly, significant lipid metabolism from addition of the phospholipases was assessed by TLC analysis following incubations at the indicated temperatures and pHs. Despite reductions in the resulting levels of degranulation, PA formation following scPLD stimulation was similar at both temperatures. Lysophosphatidylcholine (LPC) levels were similar following $tfPLA_2$ stimulation at either pH despite a complete loss of degranulation. Although we found that the 16 h preincubations in serum-free medium diminished the overall level of nucleotide-mediated responses (compared to Figures 1 and 2), the differences shown for ATP- and GTPγS-mediated secretion at various temperatures and pHs were consistently observed. These findings demonstrate that simply generating the primary product lipids is not sufficient to trigger granule fusion under specific conditions. Rather, the production of these lipids is an essential intermediate step required for granule fusion.

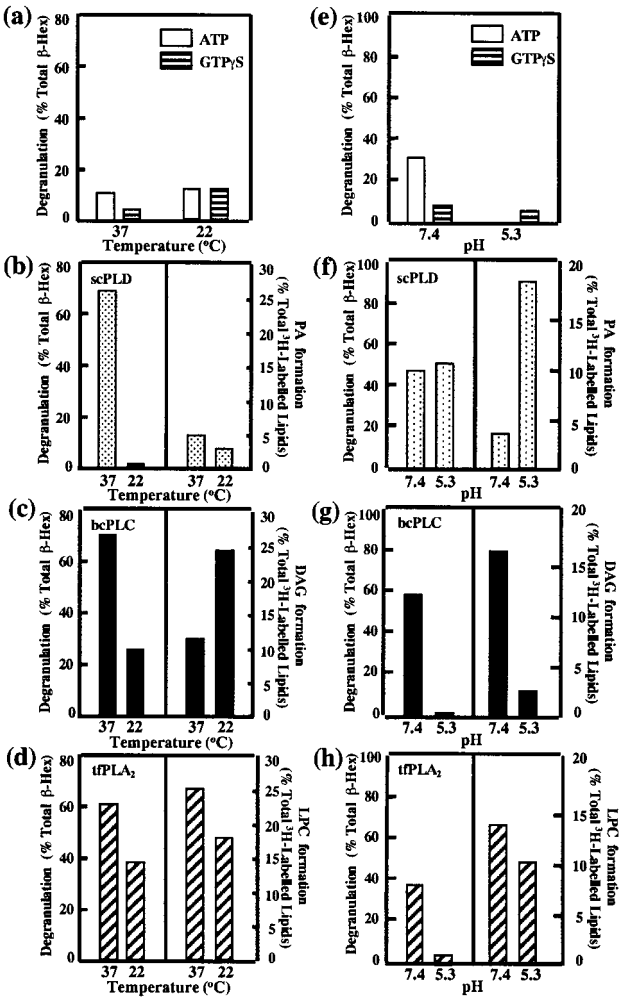

FIGURE 3: Pathways of mast cell degranulation have distinct requirements for temperature and pH. Specific exogenous phospholipase-induced degranulation in unwashed permeabilized cells can be decoupled from hydrolytic activity by incubation at 22 °C or pH 5.3. (a−d) Samples were stimulated at either 37 or 22 °C at pH 7.4 or (e−h) at either pH 7.4 or 5.3 at 37 °C. Samples were assayed as indicated with (a and e) ATP (white), (a and e) GTPγS (horizontal stripes), (b and f) scPLD (dotted), (c and g) bcPLC (black), or (d and h) $tfPLA_2$ (diagonal stripes). Primary ^{3}H-labeled lipid products generated in stimulated cells are indicated in the right-hand panel. Cells in these experiments required an extended preincubation in serum-free medium as described. Results are shown from a single representative experiment ($n = 5$).

Specificity of Granule Content Release. Further evidence for excluding the possibility that nonspecific enzyme leakage was occurring was obtained by generating intermediate-sized pores in RBL-2H3 cells using β-escin. The precise use of β-escin (30−40 μM) generates pores of sufficient diameter to allow cellular entry of ATP and the phospholipases (20, 28, and 50 kDa for bcPLC, $tfPLA_2$, and scPLD, respectively), while excluding the nonspecific diffusion of β-hexosaminidase. Such modulation of pore diameter cannot be achieved in streptolysin O-permeabilized cells. Under these conditions,

Table 1: Degranulation Induced by ATP and Exogenous Phospholipases Can Be Stimulated in β-Escin-Permeabilized Cells without Significant Leakage of LDH or Soluble β-Hexosaminidase[a]

	% degranulation[c]					
[β-escin] (μM)[b]	ATP	scPLD	bcPLC	tfPLA$_2$	% LDH leak[d]	% β-hex leak[e]
0	0	0	0	0	0	1.5 ± 0.9
20	0	3.6 ± 0.8	11.2 ± 2.6	8.3 ± 0.6	0.25	2.5 ± 0.3
30	12.5 ± 0.9	22.1 ± 2.4	11.9 ± 0.2	2.7 ± 0.1	3.1	2.2 ± 0.5
40	8.8 ± 2.4	38.4 ± 7.4	64.4 ± 7.6	55.0 ± 3.2	1.4	4.7 ± 0.8
50					6.9	12.6 ± 1.0
100					48.8	26.6 ± 2.3

[a] Results show the stimulation of β-escin-permeabilized RBL-2H3 cells. [b] Cells were permeabilized at the indicated β-escin concentrations. [c] The % degranulation was measured as described above. The results are the average of three independent experiments (±standard deviation). [d] The % LDH leak was measured as described above. The results are from a single representative experiment ($n = 3$). [e] The % β-hexosaminidase leak was measured as described above. The results are the average of three independent experiments (±standard deviation).

the pore sizes appeared to be insufficient to permit the leakage of either lactate dehydrogenase (140 kDa), a cytosolic protein used to quantitate loss of cytosol, or soluble β-hexosaminidase (120 kDa), as determined by release of the enzyme in the absence of calcium (Table 1). Calcium concentrations did not affect LDH release (data not shown). The ability to introduce ATP and the exogenous phospholipases into the β-escin-permeabilized cells without concomitant diffusion of soluble LDH and β-hexosaminidase out of the cell indicates that the release of granule contents occurs through fusion of secretory vesicles with the plasma membrane.

Immunolocalization of Granule Specific Protein. Bonifacino and colleagues have generated a monoclonal antibody raised against an 80 kDa integral membrane protein in RBL-2H3 cells. Previous reports have revealed that this 80 kDa protein localizes to secretory vesicles in unstimulated cells and translocates to the cell surface following stimulus-coupled secretion (*44*). Utilizing this antibody (Figure 4), we were able to demonstrate a significant increase relative to unstimulated cells (a and c) in the extent of plasma membrane localization of this protein following stimulation with antigen (b), high ATP (d), GTPγS (e), scPLD (f), bcPLC (g), and tfPLA$_2$ (h). In either intact or permeabilized cells (Tyrodes or buffer P, respectively), approximately 10% of the cells showed significant plasma membrane localization of the protein marker. The percentage of cells ($n > 500$) showing a plasma membrane localization increased in cells stimulated with either antigen (56%), ATP (80%), GTPγS (64%), scPLD (32%), bcPLC (31%), or tfPLA$_2$ (32%). These findings show that degranulation stimulated by either high ATP, GTPγS, scPLD, or bcPLC in permeabilized RBL-2H3 cells has an immunolocalization that is similar to that of antigen-mediated degranulation in the intact RBL-2H3 cell. While the tfPLA$_2$ also stimulated immunostaining of the cell periphery, careful examination reveals a pattern somewhat distinct from that observed via the other pathways (Figure 4). This may be the result of release from a distinct subpopulation of vesicles or an actual difference in the mechanism of release.

The ability to differentially dissociate lipid hydrolysis from degranulation under defined conditions suggests that the formation of a fusogenic lipid may be a late step, but not the final step, in regulated secretion. The ability to modulate degranulation by temperature, pH, and pore diameter demonstrates that granule contents are being released through the plasma membrane in a specific manner rather than by

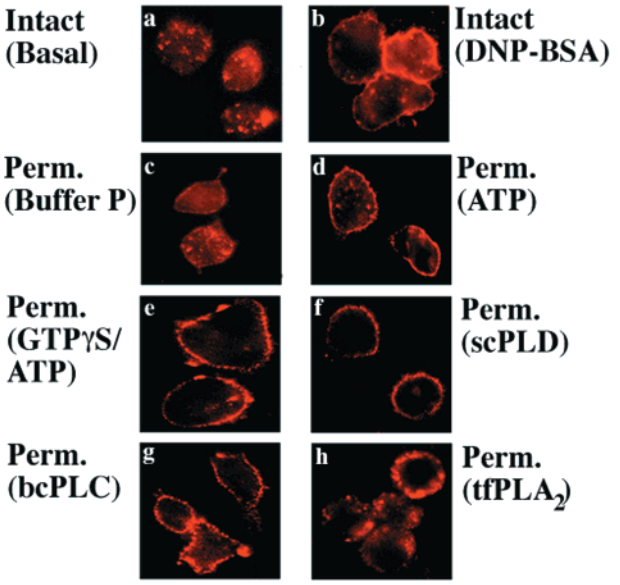

FIGURE 4: Translocation of a secretory granule specific marker is concurrent with stimulation of degranulation. The 5G10 specific protein translocates to the plasma membrane upon stimulation. (a) Intact RBL-2H3 cells incubated with Tyrodes buffer or (b) DNP-BSA (antigen). The labeling pattern observed following antigen stimulation is consistent with the fusion of secretory granules with the plasma membrane. Following permeabilization, cells were either washed in (c) buffer P or challenged with (d) ATP, (e) GTPγS, (f) scPLD, (g) bcPLC, or (h) tfPLA$_2$. Each panel illustrates the changes observed in the cellular localization of the 5G10 protein following addition of the indicated compounds ($n > 500$ cells per condition).

leakage through pores. These findings are consistent with the observation that a granule specific protein translocates to the plasma membrane following stimulation in permeabilized cells in a way that resembles antigen-mediated secretion in intact cells. Taken together, these findings suggest that high ATP, GTPγS, and exogenous phospholipases are initiating degranulation in the RBL-2H3 cells by a process dependent on the formation of bioactive lipids.

DISCUSSION

To understand the role of phospholipid metabolism in mast cell degranulation, we examined the consequences of directly altering the lipid composition within a cell in comparison to physiological methods to both activate endogenous phospholipases and stimulate regulated secretion. In characterizing these effects, we explored whether the hydrolysis of specific substrates was a prerequisite for degranulation, how alterations in phospholipid composition can be physiologically

achieved, and how these alterations might result in secretion. Moreover, we considered whether alterations in lipid composition by application of exogenous phospholipases resulted in a specific fusion event. Our goal was to illuminate the possible role of phospholipases in regulated secretion, an area in which different investigators have reached contradictory conclusions. Furthermore, we seek to apply these findings on exogenous phospholipase action to the physiological stimulation of mast cell degranulation by the high-affinity IgE receptor.

Previous work has suggested that phospholipase hydrolysis of PC was involved in degranulation (*45*). We utilized three distinct, PC-preferring phospholipases, scPLD, bcPLC, and tfPLA$_2$, that resulted in a stimulation of degranulation at increasing concentrations of phospholipase. The saturable responses indicate that a finite pool of PC is involved in the process. The method employed in our experiments preferentially labels cellular pools of PC. Therefore, it is possible that other important lipid products were not observed. However, the lack of an effect by either PI specific PLC or TAG lipase and the consistent effects of the PC-preferring phospholipases suggest that PC levels play a central role in degranulation.

To identify the cellular pathways involved in degranulation, we compared responses in both intact and permeabilized cells in parallel with either divalent antigen, high ATP, and the slow hydrolyzing analogue of GTP, GTPγS (with substimulatable concentrations of ATP). This requirement for substimulatable levels of ATP in the latter pathway likely reflects multiple roles of ATP or other nucleotide triphosphates during degranulation. On the basis of previous work in broken cells, a large group of macromolecules have been implicated in regulated secretion. Interestingly, several common factors associated with both regulated secretion and phospholipase activation have been identified. In particular, factors that activate mammalian PLD1 have been implicated in degranulation, including PKC (*25*), Arf (*24*, *38*), PIP$_2$ (*46*), and the Rho small G-protein family (*22*, *23*). Our findings suggest that the exogenous phospholipases are working independently of PKC. The inhibition of antigen and high-ATP-mediated secretion by calphostin C and Ro-31-8220 suggest a role for PKC in these two pathways. Inhibition by calphostin C may interfere with PKC activation of PLD1 (through its regulatory domain), and lipid analysis in high-ATP-stimulated cells indicates a significant reduction in PLD activity corresponding to a loss in the level of degranulation (data not shown). The lack of an effect in RBL-2H3 cells of the fungal inhibitor brefeldin A on degranulation is consistent with the involvement of Arf6 (an isoform reported to be insensitive) (*47*). Neomycin (inhibitor of PIP$_2$-related activities) inhibited degranulation in response to both high ATP and GTPγS, while not significantly disrupting secretion stimulated by exogenous phospholipases.

Cells treated with *C. difficile* toxin B have been shown to have significant decreases in the levels of both agonist-dependent degranulation in intact cells (*48*, *49*) and GTP-dependent degranulation in permeabilized cells (*49*). Previous characterizations of Rho GTPase involvement in degranulation focused on stimulation by GTPγS. Our findings suggest an involvement by Rho GTPases in the high-ATP degranulation pathway as well. A central role for Rho GTPases is consistent with a model for mast cell exocytosis

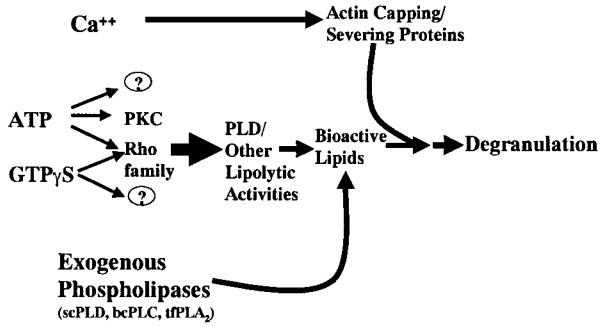

FIGURE 5: Model of degranulation in permeabilized RBL-2H3 cells. The roles of Rho and PKC include activation of endogenous PLD. These factors can be bypassed and degranulation achieved by the introduction of three types of exogenous, active phospholipases. The production of bioactive lipids appears to be essential, but not sufficient, for regulated secretion to proceed.

recently proposed by Koffer and colleagues (*22*). Incorporation of our findings into this paradigm suggests that an important target of the Rho GTPases in regulated secretion is PLD (see Figure 5). The high-ATP- and GTPγS-mediated pathways appear to be dependent on formation of PA (measured as PEtOH in lipid analysis) or by other bioactive lipids as well.

Our data show that phospholipase-induced degranulation is not the result of a nonspecific, hydrolytic leakage phenomenon. We addressed this possibility in three distinct ways. First, we identified conditions that decoupled phospholipase catalytic activity from release of β-hexosaminidase. By decreasing the incubation temperature to 22 °C, we showed that degranulation was reduced for all three phospholipases compared to samples incubated at 37 °C despite comparable levels of lipid hydrolysis. Whereas the change in temperature resulted in a modest decline in the level of PA formation by scPLD, the degranulation response was essentially ablated. ATP- and GTPγS-stimulated secretion was minimally affected by the reduced temperature even though the responses were lower than the corresponding bcPLC and tfPLA2 responses. Similarly, incubations at pH 5.3 showed a loss in the level of β-hexosaminidase release compared to incubations at pH 7.4 despite comparable lipid hydrolysis by tfPLA$_2$ (and some residual activity by bcPLC). In the second series of studies, we modulated the pore size of the permeabilized cells to allow the entry of the relatively small exogenous phospholipases and nucleotides, but to retain the relatively larger β-hexosaminidase. Intermediate-sized pores were generated by permeabilizing cells with β-escin, and at moderate concentrations (30−40 μM) the cytosolic pools of lactate dehydrogenase (140 kDa) and soluble β-hexosaminidase (120 kDa) were largely retained inside the permeabilized cells in the presence or absence of calcium. In the absence of stimulators, the soluble pools of these enzymes were released at increasing pore sizes. This suggests that the stimulated release of β-hexosaminidase from the granules occurs through the plasma membrane. Last, using immunolocalization, we observed that the nucleotides and exogenous phospholipases trigger the translocation of a granule membrane marker protein to the plasma membrane. Stimulation of degranulation in intact RBL-2H3 cells via the high-affinity receptor for IgE looks quite similar to stimulation in permeabilized cells whether induced by high ATP, GTPγS, or addition of the exogenous phospholipases. Taken

together, these results suggest that the granule content release in the permeabilized cells occurs by membrane fusion.

Several models have been proposed that require an active role for lipid metabolism in membrane biogenesis and exocytosis. There are strong experimental and theoretical indications that lipid composition is directly involved in fusion processes. The stalk−pore theory of bilayer fusion as proposed by Kozlov and Markin (*50*) and modified by Siegel (*6*) predicts the formation of cone-shaped structures with small polar headgroups relative to hydrophobic acyl chains (e.g., PE, cis-unsaturated fatty acids, and PA) as an intermediate step in membrane fusion. Factors that promote formation of nonbilayer (H_{II} phase) structure appear also to induce fusion between membranes [reviewed by Cullis, Fenske, and Hope (*51*)]. Production of LPC has been reported to interfere with the formation of fusion intermediates (*52*), although Marquardt and Walker (*53*) reported that addition of micromolar concentrations of LPC stimulated mast cell degranulation by modulation of the intracellular calcium level. The data suggest that $tfPLA_2$ does not mediate secretion either through classical isoforms of PKC (Figure 2) or through modulation of intracellular calcium levels. It is possible that the release of the free fatty acid by PLA_2 is the predominant factor in RBL-2H3 cell degranulation. Our findings in permeabilized cells that addition of nucleotides that activate endogenous PLD and exogenous phospholipases are capable of initiating degranulation via changes in membrane lipid composition are consistent with these models (*51*) of membrane fusion (Figure 5).

The observation that regulators of mammalian PLD activity are involved in mast cell degranulation suggests an intimate relationship between lipid metabolism and mast cell degranulation. Identification of specific phospholipids that mechanistically mediate degranulation has been complicated by the parallel generation of both primary and secondary products produced from hydrolysis of PC by mammalian phospholipases as well as exogenous scPLD, bcPLC, and $tfPLA_2$. PA and DAG are metabolically interconvertable through the opposing activities of lipid phosphate phosphatase (formerly known as PA phosphohydrolase) and diacylglycerol kinase. TLC analysis reveals conversion of PA to DAG in PLD-stimulated samples; however, we observed no evidence of PA formation from DAG in this preparation. Since the exogenous phospholipases can stimulate degranulation in the absence of supplementary ATP, it is unlikely that the DAG conversion to PA is a relevant pathway in the RBL-2H3 cells. LPC may be playing either direct or indirect roles in mediating secretion as it has been shown to enhance the binding of DAG to some other signaling proteins. Although DAG may function as a secretory mediator, the accumulation of a free fatty acid that can be detected by TLC analysis in samples treated with all three phospholipases and GTPγS suggest that some common fusogenic lipid may be responsible for secretory granule fusion with the plasma membrane. In this case, the phospholipases are all functioning to produce substrates that will be metabolized to liberate a common fusogenic lipid mediator. Although only $tfPLA_2$ directly generates a free fatty acid, PA and DAG have both been linked to the stimulation of cytosolic PLA_2 activity (*54*). Possibly, this is the convergence step of the exogenous phospholipases and GTPγS. The lack of an observed accumulation of free fatty acid in response to high-ATP stimulation may be attributed to differences in substrate specificity of downstream phospholipases or granule subpopulation.

We propose that these exogenous phospholipases generate bioactive lipids that function as either primary products or substrates leading to formation of secondary metabolic products that trigger membrane fusion. The immunolocalization data indicate that granules fuse with the plasma membrane in nucleotide- and phospholipase-mediated degranulation in a manner similar to that of antigen-stimulated mast cells. The initiation of granule content release by bcPLC, scPLD, and $tfPLA_2$ may suggest that mast cells have multiple pathways that utilize different lipids to trigger degranulation, perhaps to shuttle distinct subpopulations of granules to the plasma membrane. On the basis of findings reported by Wakelam and colleagues (*55*), the primary site of action by PLD in RBL-2H3 cells may be the secretory vesicles themselves. The production of PA in the presence of PIP_2 has been implicated in coat assembly and other transport processes (*9*, *56*). Modulation of the levels of DAG, PA, and LPC alters membrane curvature and fluidity (*2*, *4*, *9*). This may lead to membrane remodeling and trigger fusion events. It is also possible that these various pathways are producing a common lipid species via subsequent metabolism that has not been identified. Our findings demonstrate that the production of lipid second messengers by phospholipases is capable of initiating degranulation and implicates formation of lipids as essential steps in regulated secretion. Defining the protein−protein interactions and the lipid species generated in the cascade downstream of the high-affinity IgE receptor will be central to understanding regulated exocytosis.

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REFERENCES

1. Weber, T., Zemelman, B. V., McNew, J. A., Westermann, B., Gmachl, M., Parlati, F., Söllner, T. H., and Rothman, J. E. (1998) *Cell 92*, 759−772.
2. Christoforidis, S., McBride, H. M., Burgoyne, R. D., and Zerial, M. (1999) *Nature 397*, 621−625.
3. McBride, H. M., Rybin, V., Murphy, C., and Zerial, M. (1999) *Cell 98*, 377−386.
4. Basáñez, G., Goñi, F. M., and Alonso, A. (1998) *Biochemistry 37* (11), 3901−3908.
5. David, D., Sundarababu, S., and Gerst, J. E. (1998) *J. Cell Biol. 143* (5), 1167−1182.
6. Siegel, D. P. (1993) *Biophys. J. 65*, 2124−2140.
7. Robinson, L. J., and Martin, T. F. (1998) *Curr. Opin. Cell Biol. 10* (4), 483−492.
8. Scales, S. J., Chen, Y. A., Yoo, B. Y., Patel, S. M., Doung, Y. C., and Scheller, R. H. (2000) *Neuron 26* (2), 457−464.
9. Schmidt, A., Wolde, M., Thiele, C., Fest, W., Kratzin, H., Podtelejnikov, A. V., Witke, W., Huttner, W. B., and Söling, H.-D. (1999) *Nature 401*, 133−141.
10. Weigert, R., Silletta, M. G., Spanò, S., Turacchio, G., Cericola, C., Colanzi, A., Senatore, S., Mancini, R., Polishchuk, E. V., Salmona, M., Facchiano, F., Burger, K. N. J., Mironov, A., Luini, A., and Corda, D. (1999) *Nature 402*, 429−433.
11. Takei, K., Haucke, V., Slepnev, V., Farsad, K., Salazar, M., Chen, H., and De Camilli, P. (1998) *Cell 94* (1), 131−141.
12. Matsuoka, K., Orci, L., Amherdt, M., Bednarek, S. Y., Hamamoto, S., Scheckman, R., and Yeung, T. (1998) *Cell 93*, 263−275.

13. Jones, D., Morgan, C., and Cockcroft, S. (1999) *Biochim. Biophys. Acta 1439* (2), 229−244.
14. Li, X., Routt, S. M., Xie, Z., Cui, X., Fang, M., Kearns, M. A., Bard, M., Kirsch, D. R., and Bankaitis, V. A. (2000) *Mol. Biol. Cell 11* (6), 1989−2005.
15. Singer, W. D., Brown, H. A., and Sternweis, P. C. (1997) *Annu. Rev. Biochem. 66*, 475−509.
16. Exton, J. H. (1999) *Biochim. Biophys. Acta 1439* (2), 121−133.
17. Lukowski, S., Mira, J. P., Zachowski, A., and Geny, B. (1998) *Biochem. Biophys. Res. Commun. 248* (2), 278−284.
18. Steed, P. M., Nagar, S., and Wennogle, L. P. (1996) *Biochemistry 35*, 5229−5237.
19. Frohman, M. A., Sung, T. C., and Morris, A. J. (1999) *Biochim. Biophys. Acta 1439* (2), 175−186.
20. Lee, C., Kim, S. R., Chung, J. K., Frohman, M. A., Kilimann, M. W., and Rhee, S. G. (2000) *J. Biol. Chem. 275* (25), 18751−18758.
21. Lee, C., Kang, H. S., Chung, J. K., Sekiya, F., Kim, J. R., Han, J. S., Kim, S. R., Bae, Y. S., Morris, A. J., and Rhee, S. G. (1997) *J. Biol. Chem. 272* (25), 15986−15992.
22. Sullivan, R., Price, L. S., and Koffer, A. (1999) *J. Biol. Chem. 274* (53), 38140−38146.
23. Hong-Geller, E., and Cerione, R. A. (2000) *J. Cell Biol. 148* (3), 481−493.
24. Caumont, A. S., Galas, M. C., Vitale, N., Aunis, D., and Bader, M. F. (1998) *J. Biol. Chem. 273* (3), 1373−1379.
25. Ozawa, K., Szallasi, Z., Kazanietz, M. G., Blumberg, P. M., Mischak, H., Mushinski, J. F., and Beaven, M. A. (1993) *J. Biol. Chem. 268* (3), 1749−1756.
26. Hay, J. C., Fisette, P. L., Jenkins, G. H., Fukami, K., Takenawa, T., Anderson, R. A., and Martin, T. F. J. (1995) *Nature 374*, 173−177.
27. Borovikov, Y. S., Norman, J. C., Price, L. S., Weeds, A., and Koffer, A. (1995) *J. Cell Sci. 108* (2), 657−666.
28. Garcia-Gil, M., and Siraganian, R. P. (1986) *J. Immunol. 13* (1), 259−263.
29. Zhou, H. L., Chabot-Fletcher, M., Foley, J. J., Sarau, H. M., Tzimas, M. N., Winkler, J. D., and Torphy, T. J. (1993) *Biochem. Pharmacol. 46*, 139−148.
30. Ali, H., Choi, O. H., Fraundorfer, P. F., Yamada, K., Gonzaga, H. M. S., and Beaven, M. A. (1996) *J. Pharmacol. Exp. Ther. 276*, 837−845.
31. Blackwood, R. A., Smolen, J. E., Transue, A., Hessler, R. J., Harsh, D. M., Brower, R. C., and French, S. (1997) *Am. J. Physiol. 272* (4, Part 1), C1279−C1285.
32. Blackwood, R. A., Transue, A. T., Harsh, D. M., Brower, R. C., Zacharek, S. J., Smolen, J. E., and Hessler, R. J. (1996) *J. Leukocyte Biol. 59* (5), 663−670.
33. Brock, T. G., Nagaprakash, K., Margolis, D. I., and Smolen, J. E. (1994) *J. Membr. Biol. 141*, 139−148.
34. Zellmer, S., Cevc, G., and Risse, P. (1994) *Biochim. Biophys. Acta 1196* (2), 101−113.
35. Nagao, T., Kubo, T., Fujimoto, R., Nishio, H., Takenuchi, T., and Hata, F. (1995) *Biochem. J. 307* (Part 2), 563−569.
36. Nishio, H., Takenuchi, T., Hata, F., and Yagasaki, O. (1996) *Biochem. J. 318* (Part 3), 981−987.
37. Mayorga, L. S., Colombo, M. I., Lennartz, M., Brown, E. J., Rahman, K. H., Weiss, R., Lennon, P. J., and Stahl, P. D. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 10255−10259.
38. Way, G., O'Luanaigh, N., and Cockcroft, S. (2000) *Biochem. J. 346*, 63−70.
39. Pierini, L., Holowka, D., and Baird, B. (1996) *J. Cell Biol. 134*, 1427−1439.
40. Koffer, A., and Churcher, Y. (1993) *Biochim. Biophys. Acta 1176* (3), 222−230.
41. Nakamura, Y., Nakashima, S., Kumada, T., Ojio, K., Miyata, H., and Nozawa, Y. (1996) *Immunobiology 195* (2), 231−242.
42. Chardin, P., and McCormick, F. (1999) *Cell 97*, 153−155.
43. Pertile, P., Liscovitch, M., Chalifa, V., and Cantley, L. C. (1995) *J. Biol. Chem. 270*, 5130−5135.
44. Bonifacino, J. S., Perez, P., Klausner, R. D., and Sandoval, I. V. (1986) *J. Cell Biol. 102* (2), 516−522.
45. Dinh, T. T., and Kennerly, D. A. (1991) *Cell Regul. 2* (4), 299−309.
46. Toker, A. (1998) *Curr. Opin. Cell Biol. 10* (2), 254−261.
47. Cavenagh, M. M., Whitney, J. A., Carroll, K., Zhang, C.-j., Boman, A. L., Rosenwald, A. G., Mellman, I., and Kahn, R. A. (1996) *J. Biol. Chem. 271* (36), 21767−21774.
48. Prepens, U., Just, I., Eichel-Streiber, C., and Aktories, K. (1996) *J. Biol. Chem. 271*, 7324−7329.
49. Ojio, K., Banno, Y., Nakashima, S., Kato, N., Watanabe, K., Lyerly, D. M., Miyata, H., and Nozawa, Y. (1996) *Biochem. Biophys. Res. Commun. 224*, 591−596.
50. Kozlov, M. M., and Markin, V. S. (1983) *Biofizika 28* (2), 242−247.
51. Cullis, P. R., Fenske, D. B., and Hope, M. J. (1996) in *Biochemstry of lipids, lipoproteins and membranes* (Vance, J. E., and Vance, D. E., Eds.) Vol. 31, pp 541, Elsevier Science B. V., Amsterdam.
52. Yeagle, P. L., Smith, F. T., Young, J. E., and Flanagan, T. D. (1994) *Biochemistry 33* (7), 1820−1827.
53. Marquardt, D. L., and Walker, L. L. (1991) *J. Allergy Clin. Immunol. 88* (5), 721−730.
54. Bauldry, S. A., and Wooten, R. E. (1997) *Biochem. J. 322* (Part 2), 353−363.
55. Brown, F. D., Thompson, N., Saqib, K. M., Clark, J. M., Powner, D., Thompson, N. T., Solari, R., and Wakelam, M. J. (1998) *Curr. Biol. 8* (14), 835−838.
56. Chen, Y. G., Siddhanta, A., Austin, C. D., Hammond, S. M., Sung, T. C., Frohman, M. A., Morris, A. J., and Shields, D. (1997) *J. Cell Biol. 138* (3), 495−504.

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