Distinct Phosphoinositide 3-Kinases Mediate Mast Cell Degranulation in Response to G-protein-coupled Versus FceRI Receptors*

David A. Windmiller‡ and Jonathan M. Backer§

From the Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461

Phosphoinositide (PI) 3-kinases are critical regulators of mast cell degranulation. The Class IA PI 3-kinases p85/p110β and p85/p110δ but not p85/p110α are required for antigen-mediated calcium flux in RBL-2H3 cells (Smith, A. J., Surviladze, Z., Gaudet, E. A., Backer, J. M., Mitchell, C. A., and Wilson, B. S., (2001) J. Biol. Chem. 276, 17213–17220). We now examine the role of Class IA PI 3-kinases isoforms in degranulation itself, using a single-cell degranulation assay that measures the binding of fluorescently tagged annexin V to phosphatidylserine in the outer leaflet of the plasma membrane of degranulated mast cells. Consistent with previous data, antibodies against p110α and p110β blocked FceRI-mediated degranulation in response to FceRI ligation. However, antigen-stimulated degranulation was also inhibited by antibodies against p110α, despite the fact that these antibodies have no effect on antigen-induced calcium flux. These data suggest that p110α mediates a calcium-independent signal during degranulation. In contrast, only p110β was required for enhancement of antigen-stimulated degranulation by adenosine, which augments mast cell-mediated airway inflammation in asthma. Finally, we examined carbachol-stimulated degranulation in RBL2H3 cells stably expressing the M1 muscarinic receptor (RBL-2H3-M1 cells). Surprisingly, carbachol-stimulated degranulation was blocked by antibody-mediated inhibition of the Class III PI 3-kinase hVPS34 or by titration of its product with FYVE domains. Antibodies against Class IA PI 3-kinases had no effect. These data demonstrate: (a) a calcium-independent role for p110α in antigen-stimulated degranulation; (b) a requirement for p110β in adenosine receptor signaling; and (c) a requirement for hVPS34 during M1 muscarinic receptor signaling. Elucidation of the intersections between these distinct pathways will lead to new insights into mast cell degranulation.

Mast cells are important cellular mediators of allergic responses in humans (1). Moreover, increased levels of mast cells and mast cell-derived inflammatory mediators are found in bronchoalveolar lavage fluid from asthmatics, suggesting a role for mast cells in the etiology of clinical asthma (2–4). Cross-linking of cell surface FceRI receptors leads to the release of pre-formed mediators present in mast cell granules, as well as the induction of cytokines and bioactive lipids (5). Release of these inflammatory molecules in the lung is likely to contribute to inflammation and vasoconstriction during asthma. Antigen-mediated degranulation is enhanced by co-stimulation of mast cells with adenosine, which is an important contributor to airway inflammation in asthma (6).

The initial signaling events during antigen-stimulated degranulation have been well studied. FceRI cross-linking leads to recruitment and activation of lyn and syk tyrosine kinases, with subsequent phosphorylation of tyrosine residues in the FceRI γ-chain (5, 7). This leads to the recruitment, phosphorylation and activation of phospholipase Cγ, and generation of inositol trisphosphate and diacylglycerol from the hydrolysis of plasma membrane phosphatidylinositol (4,5)-bisphosphate. Inositol trisphosphate-mediated release of intracellular calcium stores and activation of classical and novel isoforms of protein kinase C (8) are required for the opening of plasma membrane calcium channels. The increase in intracellular calcium levels is critical for degranulation, as evidenced by the fact that thapsigargin or calcium ionophores can induce mast cell degranulation in the absence of additional stimuli.

G-protein-coupled receptors (GPCRs)1 also regulate mast cell degranulation. In bone marrow-derived mast cells and in a cell culture model, the RBL-2H3 basoleukemic line, adenosine can synergistically enhance degranulation in response to FceRI crosslinking, although it is not sufficient to stimulate degranulation (6, 9, 10). Adenosine signaling in RBL-2H3 cells is primarily mediated by the A3 adenosine receptor, a Gαs-coupled GPCR (9). In addition, Bean and colleagues (11) demonstrated that stable expression in RBL-2H3 cells of a heterologous GPCR, the M1 muscarinic acetylcholine receptor, leads to carbachol-stimulated degranulation. Carbachol versus antigen stimulation of RBL-2H3-M1 cells lead to similar changes in calcium mobilization and activation of Erk and phospholipase A2 (12–14), although carbachol-stimulated degranulation used PLCβ rather than PLCγ to trigger PKC activation and calcium flux (13).

The phosphoinositide 3-kinase inhibitor wortmannin is a potent inhibitor of mast cell degranulation (15), and deletion of the gene for the phosphatidylinositol trisphosphate-phospho-
tase SHIP markedly enhances mast cell degranulation (16). These data demonstrate that PI 3-kinases are important regulators of antigen-stimulated degranulation. Microinjection of isoform-specific inhibitory antibodies to p110β and p110δ reduce antigen-stimulated calcium flux and membrane ruffling in RBL-2H3 cells (17). In addition, recent studies on a mouse knockout of the Class IB PI 3-kinase, p110γ, suggest that p110γ is important in both FceRI and adenosine-mediated degranulation (18). However, the role of Class IA and Class III PI 3-kinases in degranulation itself has not been directly examined. In this paper, we use specific inhibitory antibodies against Class IA and Class III PI 3-kinases to test the requirement for these enzymes during degranulation. Using a single-cell degranulation assay in RBL-2H3 cells, we show that the three Class IA PI 3-kinases are all required for optimal antigen-stimulated degranulation. In contrast, synergistic enhancement of antigen-stimulated degranulation by adenosine specifically requires p85/p110β. Surprisingly, carbachol-stimulated degranulation in RBL-2H3-M1 cells does not require Class I PI 3-kinases and instead requires the Class III enzyme hVPS34. The utilization of PKC isoforms in antigen versus carbachol-stimulated degranulation was also different. These data suggest a novel role of hVPS34 in GPCR signaling and highlight the regulatory complexity of ligand-stimulated degranulation in mast cells.

EXPERIMENTAL PROCEDURES

Cell Culture—RBL-2H3 and RBL-2H3 cells expressing the M1 muscarinic receptor (RBL-2H3-M1) were cultured in Iscove’s modified Dulbecco’s medium containing 15% fetal bovine serum on Nun culture dishes or fibronectin-coated coverslips.

Antibodies, cDNA Constructs, and Inhibitors—Isoform-specific inhibitory antibodies against p110α, p110β, and p110δ, and hVPS34 have been described previously (19–21). All antibodies were affinity-purified and concentrated to 4 mg/ml in phosphate-buffered saline. The eGFP-2X-FYVE construct (22) was obtained from Dr. Harold Stenmark, The Norwegian Radium Hospital, Norway. Wortmannin was obtained from Sigma, and rottlerin and Ge6976 were obtained from Calbiochem.

Microinjections and Transfections—Cells were microinjected using an Eppendorf 5171/5242 semi-automatic micromanipulator/microinjector as described previously (19). Cells were allowed to recover for 2 h after injection prior to stimulation as described below.

Degranulation Assays—RBL-2H3 cells were incubated overnight in 0.1 µg/ml anti-DNP IgG. The cells were washed in Hank’s basic salt solution (HBSS) and stimulated for 45 min at 37°C with HBSS, 1 mM calcium containing 10 ng/ml DNP-albumin. For adenosine experiments, cells were stimulated with 0.5 mM DNP-albumin in the absence or presence of 10 µM adenosine or carrier. Alternatively, RBL-2H3-M1 cells were stimulated for 45 min at 37°C with HBSS containing 100 µM carbachol or carrier. In each case, the supernatant was removed and brought to 100 mM citrate, pH 4.5, 1 mM 4-methylumbelliferyl-N-acetyl glucosamine (Sigma). After 15 min at 37°C, the reaction was stopped with 1/10 volume of 200 mM Na3CO3, glycine, pH 10.7, and substrate hydrolysis measured using a fluorescence spectrophotometer (360 excitation/465 emission).

For detection of degranulated cells using annexin V, cells grown on fibronectin-coated coverslips were preloaded with Alexa 488-annexin V for 45 min. A 1:10 dilution of Alexa 488-annexin V reagent (Molecular Probes, Eugene, OR). The cells were washed, fixed in 3.7% formaldehyde for 10 min at 22°C, and mounted. Cells were scored for annexin V staining using a Nikon Eclipse 400 upright microscope with an Epiplan 1.4 N.A. plan-apo infinity-corrected objective. Each measure- ment reflects ~100 injected cells per condition, and the data are the means of three to five separate experiments. When indicated, cells were transfected with LipofectAMINE Plus according to manufacturer’s instructions (Invitrogen). Images were acquired using a Cohu 4910 B/W CCD camera with NIH Image 1.62 analysis software.

RESULTS

A Single-cell Assay for Mast Cell Degranulation—We and others (19–21) have previously characterized specific inhibitory antibodies to Class I and Class III PI 3-kinases. To use these reagents to study mast cell degranulation in single cells, we modified a flow cytometry assay developed by Demo et al. (23), in which the binding of fluorescently labeled annexin V is used to identify degranulated cells. As shown in Fig. 1, quiescent anti-DNP IgE-loaded RBL-2H3 cells show no staining with FITC-annexin V (Fig. 1B). In contrast, after stimulation of FceRI receptors with DNP-albumin for 45 min, a clear increase in FITC-annexin V staining is seen in most cells (Fig. 1D). To validate the use of annexin V staining as a single-cell assay, we used the percentage of FITC-annexin V-positive cells as a measure of degranulation, and compared it with degranulation as determined by a biochemical assay of degranulation, the release of β-hexosaminidase activity. Both assays showed a similar dose response for degranulation in DNP-albumin-stimulated cells (Fig. 1E). In addition, both assays showed similar inhibition by the PI 3-kinase inhibitor wortmannin (Fig. 1F) and by the pan-PKC inhibitor Bisindolmaleimide (data not shown). Thus, the annexin V-based assay accurately reflects ligand-stimulated degranulation of RBL-2H3 cells.

Role of Class IA PI 3-Kinases during FcεRI-mediated Degranulation—It was previously shown that specific inhibition of two isoforms of Class IA PI 3-kinase, p85/p110β and p85/p110δ, diminishes antigen-stimulated calcium flux and membrane ruffling in RBL-2H3 cells (17). To directly examine their role in degranulation, anti-DNP IgE-loaded RBL-2H3 cells were microinjected with inhibitory antibodies to p110α, p110β,
Adenosine is not sufficient to induce degranulation but can contribute to airway inflammation and mast cell activation in asthma by the adenosine receptor, a physiologically important contributor to carbachol-stimulated degranulation. The cells were then stimulated with 100 µM carbachol in the presence of Alexa 488-annexin V for 45 min at 37 °C, and degranulation was determined as described in the legend to Fig. 2. Data are the mean ± S.E. from three experiments. B. RBL-3H3-M1 cells were transfected with expression plasmids for either eGFP or eGFP-FYVE as indicated. After 6 h, the cells were stimulated with 100 µM carbachol in the presence of Alexa 488-annexin V for 45 min at 37 °C. Degranulation in eGFP-labeled cells was determined as described above and expressed as a percentage of degranulation in untransfected cells. Data are the mean ± S.E. from three experiments.

M1 acetylcholine receptor, was developed as a model system to study cholinergic regulation of secretion and neurotransmitter release (11). Carbachol elicits a robust degranulation response in these cells. To examine the role of PI 3-kinases in M1 muscarinic receptor signaling, we microinjected RBL-2H3-M1 cells with inhibitory anti-PI 3-kinase antibodies and stimulated the cells with 100 µM carbachol for 45 min. Unlike antigen-stimulated degranulation, carbachol-stimulated degranulation was unaffected by inhibition of any of the Class I PI 3-kinases (Fig. 3A). Surprisingly, carbachol-stimulated degranulation was markedly reduced by inhibition of the Class III enzyme, hVPS34 (Fig. 3A). To confirm the role of hVPS34 in carbachol-stimulated degranulation, we transiently transfected RBL-2H3 or RBL-2H3-M1 cells with an eGFP-linked construct containing two FYVE domains from hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) (22). Although this construct is useful as a marker for PI(3)P-containing membranes when expressed at low levels, high level overexpression of FYVE domain-containing proteins has been shown to disrupt PI(3)P-mediated signaling, presumably by titration of PI(3)P (24). We therefore used high level expression of the construct to disrupt PI(3)P-dependent signaling. We found that overexpression of the eGFP-FYVE in mast cells had no effect on FceRI-mediated degranulation in RBL-2H3 cells, but it inhibited carbachol-stimulated degranulation in the RBL-2H3-M1 cells (Fig. 3B). Thus, both hVPS34 and its lipid product, PI(3)P, are required for carbachol-stimulated degranulation.
PI 3-Kinase in Mast Cell Degranulation

Differential Utilization of PKC Isoforms in FcεRI Versus M1-Mediated Degranulation—To further characterize differential signaling during antigen versus carbachol-stimulated degranulation, we examined the requirement for PKCβ and PKCδ. These isoforms have previously been shown to be stimulatory for degranulation in RBL-2H3 cells in response to antigen (8). We found that the PKCδ inhibitor rottlerin had similar inhibitory effects on both antigen and carbachol-stimulated degranulation, with IC_{50} values of ~3–10 μM in the two cell lines (Fig. 4B). In contrast, the PKCα inhibitor Go6976 potently inhibited antigen-stimulated degranulation in RBL-2H3 cells (IC_{50} 10 nm), but had no effect on carbachol-stimulated degranulation in the RBL-2H3-M1 cells (Fig. 4A). These data show that the utilization of both PKC and PI 3-kinase isoforms is different in carbachol versus antigen-stimulated degranulation.

DISCUSSION

In this report we have used well characterized isoform-specific anti-PI 3-kinase antibodies (19–21), in conjunction with a single-cell assay for degranulation, to identify the roles of distinct PI 3-kinases during mast cell degranulation. The assay is based on a previously published flow cytometry assay (23), which used fluorescently labeled annexin V to quantitate the increase in exofacial phosphatidylinerse that occurs in the plasma membrane of degranulated cells. A similar increase in exofacial phosphatidylinerse occurs in apoptotic cells, and fluorescent annexin V is a commonly used assay for apoptosis (25). In the single cell assay presented here, degranulation is expressed as the percentage of annexin V-positive cells per field. The dose-response curve for antigen-stimulated degranulation using this assay correlates well with biochemical assays for degranulation and shows similar sensitivity to wortmannin. This assay provides a useful adjunct to previous single-cell studies in RBL-2H3 cells, which focused on calcium flux and membrane ruffling (17).

The importance of examining degranulation itself, as opposed to degranulation-related events, is shown in the experiments on the role of Class IA PI 3-kinases during antigen-stimulated degranulation. Antigen-stimulated calcium flux has previously been shown to require p85/p110β and p85/p110α but not p85/p110α (17). The role of these enzymes in calcium flux is likely to be mediated by the potenctial of PLCγ activation by phosphatidylinositol trisphosphate, either through Tec-family tyrosine kinases (26), Vav1 (27), or via direct effects on PLCγ (28, 29). A direct effect of phosphatidylinositol trisphosphate on calcium uptake has also been demonstrated (30). However, an additional calcium-independent role of PI 3-kinases has been suggested by the fact that thapsigargin or calcium ionophore-mediated degranulation is still sensitive to PI 3-kinase inhibitors (15, 31–33). Our data show that p85/p110α is required for degranulation despite the fact that it is not required for antigen-stimulated calcium flux in RBL-2H3 cells (17). This suggests that p85/p110α is a candidate for the wortmannin-sensitive, calcium-independent factor in antigen-stimulated degranulation.

Our experiments also reveal a role for the p85/p110β PI 3-kinase during adenosine-stimulated degranulation in cells treated with sub-optimal doses of antigen. The specific requirement for p85/p110β in signaling by this Gα_{i}-coupled receptor is consistent with reports that p110β is activated by βγ subunits from trimeric G-proteins (34, 35) and is required for lysophosphatidic acid-mediated signaling (36). In addition to p110β, p110γ is also required for adenosine secretion by mast cells and adenosine-mediated autocrine stimulation of degranulation in antigen-stimulated mast cells (18). The coordination of these two Gβγ-regulated PI 3-kinases during degranulation, and their potentially different downstream effectors, will be a subject of interest for future studies.

We also studied degranulation in a heterologous system, the RBL-2H3-M1 cell line (11), which expresses the Go,q-coupled M1 muscarinic receptor. Previous studies have shown that carbachol-stimulated degranulation in these cells is different in several respects from FcεRI-stimulated degranulation, for example with regard to the role of Src-family tyrosine kinases (37). Similarly, we find that inhibitors of PKCα block degranulation in antigen-stimulated RBL-2H3 cells but not carbachol-stimulated RBL-2H3-M1 cells. Even given these differences, the requirement for hVPS34 during carbachol-stimulated degranulation of RBL-2H3-M1 cells is a surprising finding. The best documented roles for hVPS34 in mammalian cells are in the regulation of traffic through the endocytic or phagocytic systems (19, 38–43), and during the sorting of newly synthesized membrane proteins in polarized cells (44). These reports are consistent with the well characterized role of VPS34p in vesicular trafficking in yeast (45). However, a role for hVPS34 during regulated secretion has not been demonstrated. It seems unlikely that hVPS34 is involved in degranulation at the level of granule fusion, since it would then also be required for degranulation in response to FcεRI receptor activation. In-
steady, our data suggest a novel role for hVPS34 in Go-q-coupled receptor signaling.

How might hVPS34 modulate signaling from the M1 acetylcholine receptor? hVPS34 signals via the production of PI(3)P and the recruitment and/or activation of proteins containing FYVE or PX domains, which specifically bind to PI(3)P (46–49). In both mammalian cells and yeast, hVPS34 is targeting to membranes along with an associated protein kinase (VPS15/p150) (40, 45, 50); in mammalian cells the Rab5 GTPase also regulates hVPS34 targeting to endosomes (38, 40). Disruption of hVPS34 alters the post-endocytic trafficking of cell surface receptors (19, 41) and therefore could affect the post-endocytic sorting of carbachol-stimulated M1 receptors. This could alter the signaling properties of the receptor, as has been suggested in the β-adrenergic receptor system (51). Alternatively, a recently described RGS protein, RGS-PX1, is a G-protein-coupled receptor synergy. We also find an unexpected requirement for hVPS34 in carbachol-stimulated degranulation. These latter data suggest an unappreciated role for hVPS34 in signaling by Go-q-coupled receptors. It will be important to determine whether hVPS34 also plays a role in signaling by other G-protein-coupled receptors.

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