Activation of $\beta\gamma$ Subunits of $G_{i2}$ and $G_{i3}$ Proteins by Basic Secretagogues Induces Exocytosis Through Phospholipase C$\beta$ and Arachidonate Release Through Phospholipase C$\gamma$ in Mast Cells

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Activation of $\beta\gamma$ Subunits of $G_{i2}$ and $G_{i3}$ Proteins by Basic Secretagogues Induces Exocytosis Through Phospholipase C$\beta$ and Arachidonate Release Through Phospholipase C$\gamma$ in Mast Cells

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Mast cells are activated by Ag-induced clustering of IgE bound to FcεRI receptors or by basic secretagogues that stimulate pertussis toxin-sensitive heterotrimeric $G$ proteins. The cell response includes the secretion of stored molecules, such as histamine, through exocytosis and of de novo synthesized mediators, such as arachidonate metabolites. The respective roles of $G$ proteins $\alpha$ and $\beta\gamma$ subunits as well as various types of phospholipase C (PLC) in the signaling pathways elicited by basic secretagogues remain unknown. We show that a specific Ab produced against the C-terminus of $G_{i3}$ and an anti-recombinant $G_{i3}$ Ab inhibited, with additive effects, both exocytosis and arachidonate release from permeabilized rat peritoneal mast cells elicited by the basic secretagogues mastoparan and spermine. A specific Ab directed against $G_{i2}$ dimers prevented both secretions. Anti-PLC$\beta$ Abs selectively prevented exocytosis. The selective phosphatidylinositol 3-kinase inhibitor LY 294002 prevented arachidonate release without modifying exocytosis. The selective phosphatidylinositol 3-kinase inhibitor LY 294002 prevented arachidonate release without modifying exocytosis. The selective phosphatidylinositol 3-kinase inhibitor LY 294002 prevented arachidonate release without modifying exocytosis.

A crucial characteristic of the effect of basic secretagogues on mast cells is its sensitivity to pertussis toxin (8, 12–14), which is known to ADP-ribosylate a cysteine residue in the carboxyl terminus of $G_{i2}$ subunits from $G_{i2}$ and $G_{i3}$ proteins (15). Two pertussis toxin substrates have been proposed in rat peritoneal mast cells (16) and were identified as the $G_{i2}$ and $G_{i3}$ proteins (17, 18). The $G_{i3}$ protein has been proposed to be responsible for histamine secretion, since an Ab directed toward a decapptide corresponding to the carboxyl terminus of its $\alpha$ subunit ($G_{i3}$) inhibited mast cell exocytosis (17). The involvement of $G_{i2}$ protein was unlikely, since an Ab directed against the decapetide of the carboxyl terminus of $G_{i2}$ considered an analog of $G_{i2}$ with 90% identity between them, failed to inhibit exocytosis (see Fig. 1). The respective roles of $\alpha$ and $\beta\gamma$ subunits of pertussis toxin-sensitive $G$ proteins in the signaling pathways of basic secretagogues have not been investigated. $G_{i2}$ subunits introduced into permeabilized mast cells amplified secretion induced by $Ca^{2+}$ and GTP$\gamma$S, whereas $G_{i3}$ subunits had no effect (19).

The mechanism by which basic secretagogues activate exocytosis and release of stored mediators appears to be distinct from the IgE-dependent pathway. Unlike IgE-induced exocytosis, secretion elicited by basic secretagogues is faster (seconds vs minutes). Antigenic cross-linking of IgE/FcεRI initiates the activation of Lyn by FcεRI, considered an analog of $G_{i2}$ with 90% identity between them, failed to inhibit exocytosis (see Fig. 1). The respective roles of $\alpha$ and $\beta\gamma$ subunits of pertussis toxin-sensitive $G$ proteins in the signaling pathways of basic secretagogues have not been investigated. $G_{i2}$ subunits introduced into permeabilized mast cells amplified secretion induced by $Ca^{2+}$ and GTP$\gamma$S, whereas $G_{i3}$ subunits had no effect (19).

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The activation of cPLA2 was mediated by release of intracellular calcium stores and protein kinase C (PKC) activation, which are both involved in exocytosis (2). Basic secretagogues initiate a rapid (5-s) production of IP3, indicating concomitant PLC activation and histamine secretion (14). The activation of PLC is not preceded by protein tyrosine phosphorylation (20). The subtype of PLC involved in this process remains unknown.

Following cytosolic phospholipase A2 (cPLA2) activation, arachidonate release reaches a maximum after 20- to 30-min incubation with IgE/FceRI-dependent (21) or G protein-mediated triggers (22). The activation of cPLA2 was first considered to be a consequence of the increase in cytosolic calcium elicited by basic secretagogues and was proposed to be a prerequisite for histamine secretion (14, 23). However, alternative regulatory pathways can lead to cPLA2 activation, which requires calcium increase or phosphorylation by various protein kinases, including PKC and mitogen-activated protein kinase (MAPK) isoforms (24). FceRI-induced cPLA2 activation is achieved by the p42 MAPK, independently of PKC (25, 26). Basic secretagogue-dependent activation of cPLA2 does not require p42/44 MAPK (20, 22), but is preceded by phosphatidylinositol 3-kinase (PI3K) and PKC-dependent activation of Syk kinase (27). The involvement of PLCs has not been studied in this pathway.

The present study was undertaken to assess the roles of both pertussis toxin-sensitive substrates Gαi2 and Gαi3 heterotrimeric G proteins, and their respective α and βy subunits in the secretion of histamine (exocytosis) and arachidonate release induced by basic secretagogues in mast cells. Putative relationships between the pathways of stored and de novo-synthesized mediators were also considered through the involvement of phosphatidylinositol-3 kinase (PI3K), PLCβ, PLCγ1, and cPLA2 to define the bifurcation point of these pathways. Here, we show that both Gαi2 and Gαi3 proteins are activated by basic secretagogues, allowing Gβy subunits to initiate exocytosis through the activation of PLCβ, on the one hand, and the release of arachidonate through the activation of PI3K leading to PLCγ1 activation, on the other.

Materials and Methods

Materials

Mastoparan and spermine were purchased from Sigma (St. Louis, MO). Pertussis toxin was obtained from List Biological Laboratory (Campbell, CA). [3H]Arachidonic acid was purchased from Amersham Pharmacia Biotech (Little Chalfont, U.K.). Protein A-Sepharose and protein G-Sepharose beads were obtained from Amersham Pharmacia (Uppsala, Sweden). Protease inhibitor tablet cocktails were purchased from Roche Diagnostics – Mannheim, Germany). Anti-Gαi2 (carboxyl-terminal 10 aa residues) Abs were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-Gαi2 (carboxyl-terminal 10 residues) Ab and mAbs against p-Tyr (PY20) were purchased from Transduction Laboratory (Lexington, KY). Anti-GB (carboxyl-terminal 20 aa of β1 of mouse origin, with broad specificity to mouse, rat and human GB1 to GB4), anti-cPLA2 (amino-terminal domain), anti-PLCβ (carboxyl-terminal 10 aa), and anti-PLCγ1 (epitope corresponding to aa residues 530–850 mapping within SH2-SH3 domains) Abs were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Akt (Ser473) was purchased from Cell Signaling Technology (Beverly, MA).

Isolation and purification of mast cells

Male Wistar rats (Iffa-Credo, L’Arbesle, France), weighing 300–500 g, were stunned and bled. Twelve milliliters of balanced salt solution (HEPES buffer) containing 137 mM NaCl, 2.7 mM KCl, 0.3 mM CaCl2, 1.0 mM MgCl2, 0.4 mM NaH2PO4, 5.6 mM glucose, and 10 mM HEPES, NaOH to pH 7.4, and supplemented with 0.1% BSA were injected into the peritoneal cavity. The peritoneal fluid was collected and centrifuged for 3 min at 180 × g. The pellet was suspended in the same buffer, and mast cells were purified on a discontinuous BSA gradient (30 and 40%, w/v) as previously described (8). The pellet was resuspended in HEPES buffer, and cells were examined under a light microscope for purity (>97%). The trypan blue exclusion test indicated a viability >95%.
Permeabilization and determination of histamine release

Purified mast cells (3 × 10⁶ cells/assay) were preincubated for 5 min at 37°C before permeabilization by adding streptolysin-O (0.4 U/ml). After 1 min Abs were added for 2 min. Then cells were triggered by basic secretagogues. Reactions were terminated 2 min later by addition of ice-cold buffer. The passive histamine release, in the absence of secretagogue, was <10% of the total content. The amount of histamine secretion was determined fluorometrically according to the method of Shore et al. (28) but without the extraction step.

Determination of arachidonate release

Purified mast cells were suspended in HEPES buffer (5 × 10⁶ cells/ml) and incubated with 5 μCi/ml [3H]arachidonic acid for 2 h at 37°C. The cells were washed twice, resuspended in HEPES buffer (10⁶ cells/ml), preincubated for 10 min, and triggered for 10 min at 37°C. The reaction was terminated by adding ice-cold buffer and placing the tubes on ice. Supernatants following centrifugation (180 × g, 3 min, 4°C) were collected and used to determine by liquid scintillation the amount of arachidonate released.

Determination of PI3K activation through Akt phosphorylation

Purified mast cells (5 × 10⁶ cells/assay) were preincubated for 15 min at 37°C with vanadate (0.1 mM) in HEPES buffer and triggered with secretagogues. Reactions were terminated by adding ice-cold buffer and placing the tubes on ice. Cell pellets obtained after centrifugation (3 min, 180 × g, 4°C) were treated by adding lysis buffer (150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, protease inhibitor cocktail, and 20 mM Tris-HCl) and centrifuged (20 min, 12,000 × g, 4°C). Supernatants were suspended in 5× Laemmli buffer and boiled for 15 min. Then supernatants were resolved by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes (Hybond ECL, Amersham). Membranes were saturated by incubation overnight in a blocking solution containing 100 mM NaCl and 0.1% casein (w/v), washed twice, and incubated for 1 h with primary Ab raised against p-Tyr. After incubation with secondary Ab (anti-mouse IgG Ab conjugated to horseradish peroxidase) for 1 h, membranes were incubated for 2 min in ECL reagents, and bound Abs were visualized by contact for 2 min with Kodak X-OMAT films.

Immunoprecipitation of Gβ and PLCβ or PI3K

Cell pellets were prepared and lysed as described above. Supernatants, mixed with 15 μl anti-PLCβ, anti-Gi2, or anti-PLCγ1 Abs and with protein A-Sepharose beads were incubated overnight at 4°C. Immunocomplexes were washed, treated, and resolved by SDS-PAGE as described above. Nitrocellulose membranes were incubated for 1 h with primary Abs (anti-Gβ, anti-PI3K, or anti-p-Tyr Abs). After incubation with secondary Ab (anti-rabbit or anti-mouse IgG conjugated to HRP) for 1 h, membranes were incubated for 2 min in ECL reagents, and bound Abs were visualized.

Determination of protein tyrosine phosphorylation

Supernatants of stimulated mast cell were prepared as described above and incubated for 24 h with 15 μl p-Tyr Ab (1 mg/ml); 40 μl protein G-Sepharose beads were added and incubated for 2 h at 4°C. Immunocomplexes were washed twice with lysis buffer, resuspended in 5× Laemmli buffer, boiled for 15 min, and centrifuged (2 min, 20,000 × g, 4°C). Supernatants were resolved by 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. Membranes were saturated by incubation overnight in a blocking solution containing 100 mM NaCl and 0.1% casein (w/v), washed twice, and incubated for 1 h with primary Ab (anti-mouse IgG Ab conjugated to horseradish peroxidase) for 1 h, membranes were incubated for 2 min in ECL reagents, and bound Abs were visualized by contact for 2 min with Kodak X-OMAT films.

FIGURE 3. Effects of anti-Gi2 recombinant protein and anti-Gi2 carboxyl-terminus Abs on histamine secretion induced by basic secretagogues. Experiments were performed as detailed in Fig. 2. A, One hundred percent of histamine secretion in the absence of Ab corresponded to 24.3 ± 5.8% (mastoparan) or 21.8 ± 1.8% (sperrmine) of the total histamine content; controls are the Ab effect on unstimulated permeabilized cells (7 ± 3%). B, Effects of anti-recombinant Gi2 and anti-Gi2 Abs (dilution, 1/1000) added alone or simultaneously. Histamine secretion induced by each compound in permeabilized cells without Ab was 40.8 ± 1.1% (mastoparan) or 28.3 ± 3.3% (sperrmine) of the total histamine content. C, One hundred percent histamine secretion in the absence of Ab corresponded to 24.3 ± 5.8% (mastoparan) or 21.8 ± 1.8% (sperrmine) of the total histamine content; controls are the Ab effect on unstimulated permeabilized cells calculated as a percentage of the total histamine content. D, Effect of pretreatment (2 h at 37°C) of mast cells with pertussis toxin (PTx; 50 ng/ml), and the effect of anti-GB Ab (40 μg/ml; see Fig. 4) on histamine secretion induced by the Ab of the carboxyl terminus of Gi2 (shown as control in C). Values are the mean ± SEM of four independent experiments.
Results

The in vitro activation of heterotrimeric G proteins by basic secretagogues involves their interaction with the carboxyl terminus of G protein α subunits (29, 30), explaining the interest in using selective Abs of corresponding peptide sequences. We chose the wasp venom peptide mastoparan (7, 8) as a member of cationic peptide and the natural polyamine spermine (9, 10) to represent other cationic secretagogues. Streptolysin-O creates pores through the cell membrane and allows entry of Abs into mast cells (31). Permeabilization was controlled in each experiment (results not shown) by monitoring secretion elicited by GTPγS, a nonhydrolysable analog of GTP that triggers heterotrimeric and small G proteins in permeabilized mast cells (32).

Involvement of $G_{i2}$ and $G_{i3}$ proteins in mast cells exocytosis and arachidonate release

We studied the effects of Abs directed against the C-terminal decapeptides (sequences shown in Fig. 1) from $G_{i2}$, $G_{i3}$, and $G_{s}$ on permeabilized mast cells. Anti-$G_{i2}$ Abs dose-dependently inhibited approximately 60% of histamine secretion elicited by mastoparan or spermine (Fig. 2A). Anti-$G_{i3}$ Abs did not modify the exocytotic response of mast cells to mastoparan or spermine (Fig. 2B). Similarly, Aridor et al. (17) showed that anti-$G_{i3}$ Abs inhibited exocytosis triggered by GTPγS, whereas anti-$G_{i2}$ Abs were inefficient. The $G_{s}$ subtype of heterotrimeric G proteins may regulate mast cell exocytosis (33), but we observed no effect of anti-$G_{i2}$ Ab on the response to basic secretagogues (Fig. 2C).

The above results confirmed the involvement of $G_{i3}$ in the triggering effects of basic secretagogues. To determine whether $G_{i2}$, the second pertussis toxin substrate characterized in rat peritoneal mast cells (17), was also involved, we used two different anti-$G_{i2}$ Abs (Fig. 3, A and C). The anti-recombinant $G_{i2}$ protein Ab (Fig. 3A) prevented histamine secretion induced by basic secretagogues without modifying controls in the absence of mastoparan or spermine. These results allowed us to propose both $G_{i2}$ and $G_{i3}$ as targets of basic secretagogues. Accordingly, the simultaneous inactivation of $G_{i2}$ and $G_{i3}$ proteins by anti-$G_{i2}$ Abs provided additive inhibition of histamine secretion elicited by basic secretagogues (Fig. 3B).

The anti-$G_{i2}$ C-terminus Ab (Fig. 3C) was apparently less efficient in preventing induced histamine secretion. However, this Ab led to histamine secretion in the absence of cationic secretagogues (control, Fig. 3C). This observation suggested that the anti-$G_{i2}$ C-terminus Ab might stimulate heterotrimeric G proteins, mimicking the basic secretagogue effect. To study this hypothesis we pretreated mast cells with pertussis toxin, which is known to ADP-ribosylate a cysteine residue in the $G_{i2}$ protein carboxyl terminus (see Fig. 1). Pertussis toxin pretreatment decreased the secretory response of mast cells (Fig. 3D).

Thus, the anti-$G_{i2}$ C-terminus Ab mimics the effect of basic secretagogues characterized by pertussis toxin sensitivity. Also, histamine secretion elicited by this Ab (Fig. 3D) was prevented by anti-$G_{i3}$ Abs. Similarly, an anti-$G_{i3}$ C-terminus Ab has been reported to activate $G_{i}$ protein (34).

FIGURE 4. Effect of anti-$G_{i2}$ recombinant protein and anti-$G_{i3}$ carboxyl-terminus protein on arachidonate release (A and B) and anti-$G_{i3}$ Abs on histamine secretion and arachidonate release (C and D) induced by basic secretagogues. A, One hundred percent induced arachidonate release from permeabilized cells in the absence of Ab was 1086 ± 52 dpm (mastoparan) or 1331 ± 63 dpm (spermine). B, One hundred percent induced arachidonate release from permeabilized cells in the absence of Ab was 1055 ± 26 dpm (mastoparan) or 1057 ± 20 dpm (spermine). C, One hundred percent induced histamine secretion in the absence of Ab was 39.7 ± 12.7% (mastoparan) or 38.6 ± 3.8% (spermine) of the total histamine content. D, One hundred percent of induced arachidonate release from permeabilized cells without Ab was 778 ± 21 dpm (mastoparan) or 813 ± 29 dpm (spermine). The inset in D shows the effects of pretreatment (2 h at 37°C) of mast cells with pertussis toxin (PTx; 50 ng/ml) and the effect of anti-$G_{i3}$ Ab (40 μg/ml) on arachidonate release. Controls stand for the Ab effect on unstimulated permeabilized cells (A, 486 ± 32 dpm; B, 487 ± 30 dpm; C, 7.0 ± 3.1%; D, 435 ± 14 dpm). Values are the mean ± SEM of four independent experiments.
The anti-recombinant Go12 protein Ab and the anti-Go13 C-terminus Ab both prevented arachidonate release induced by basic secretagogues (Fig. 4, A and B) with additive effects (inset in Fig. 4D). These data indicate that Gi2 and Gi3 are similarly involved in exocytosis and arachidonate release, indicating that Gi2 and Gi3 activation is a common step of the corresponding signaling pathways.

Involvement of βγ subunits of G proteins in exocytosis and arachidonate release

Both α and βγ subunits of heterotrimeric G proteins can stimulate effectors (see Ref. 35 for review). To address the question of whether Giβ subunits were involved in signal transduction elicited by basic secretagogues, we studied the effect of an anti-Gi2 Ab with broad specificity to mouse, human, and rat G1 to G4 subunits. This Ab did not elicit mast cell secretory responses (controls, Fig. 4, C and D), but strongly inhibited histamine and arachidonic acid release (Fig. 4, C and D). These observations strongly suggest that βγ dimers of pertussis toxin-sensitive G proteins are involved in both exocytosis and arachidonic acid release induced by basic secretagogues in connective tissue mast cells.

Role of PLCβ, PLCγ1, and cPLA2 in exocytosis and arachidonate release

The involvement of PLC in the secretory responses of mast cells to cationic triggers has been proposed by Nakamura and Ui (14), but the subtype of PLC has not been characterized. Considering the role of βγ dimers of heterotrimeric G proteins suggested by the above data, we assessed the involvement of PLCβ subtypes, with anti-PLCβ1, -β2, and -β3 Abs. We obtained similar results with all three Abs, calling into question their subtype selectivity. Although

FIGURE 5. Effects of anti-PLCβ3, anti-PLCγ1, and anti-cPLA2 Abs on exocytosis and arachidonate release induced by basic secretagogues. Cells were incubated with streptolysin-O (0.4 U/ml) for 1 min at 37°C, and Abs were added. Mast cells were stimulated by mastoparan (0.1 mM) or spermine (3 mM). Controls are the Ab effect on unstimulated permeabilized cells. One hundred percent values correspond to induced histamine secretion or induced arachidonate release from permeabilized cells in the absence of Ab: A, 6.8 ± 0.7% (control), 36.9 ± 4.2% (mastoparan), and 28.3 ± 4.9% (spermine) of total histamine content; B, 534.2 ± 23.4 dpm (control), 944.5 ± 28.5 dpm (mastoparan), and 1106.8 ± 34.1 dpm (spermine); C, 9.6 ± 0.5% (control), 31.0 ± 5.2% (mastoparan), and 33.2 ± 5.9% (spermine) of total histamine content; D, 476 ± 16 dpm (control), 1020 ± 33 dpm (mastoparan), and 1118 ± 16 dpm (spermine); E, 6.7 ± 0.7% (control), 25.9 ± 7.9% (mastoparan), and 29.5 ± 1.2% (spermine) of total histamine content; F, 667 ± 29 dpm (control), 1003 ± 32 dpm (mastoparan), and 1291 ± 26 dpm (spermine). Insets in A and B show the respective effects of anti-PLCβ1, -β2, and -β3 Abs (40 µg/ml) on secretions triggered by 0.1 mM mastoparan. Values are the mean ± SEM of four independent experiments.
all PLCβ Abs were seen to prevent basic secretagogue-induced histamine secretion (Fig. 5A), none of them was able to modify induced arachidonic acid release (Fig. 5B). On the contrary, anti-PLCγ1 Abs did not alter exocytosis (Fig. 5C), but prevented arachidonate release (Fig. 5D) triggered by mastoparan or spermine. The various PLC Abs studied had no effect on mast cells in the absence of cationic triggers (controls, Fig. 5).

The activity of cPLA2 generates arachidonate from membrane phospholipids. The activation of cPLA2 has been proposed to be a prerequisite for histamine secretion induced by basic compounds, suggesting its involvement in both secretion types (14, 23). However, we observed that anti-cPLA2 Abs selectively prevented arachidonate release without affecting exocytosis or cells in the absence of trigger (Fig. 5, E and F). These results show that the involvement of PLCβ is restricted to the exocytotic pathway, and that PLCγ1 and cPLA2 are selectively involved in the arachidonate release pathway.

Sequence of events leading to exocytosis and arachidonate release

The above results suggested that the early step of basic secretagogue transduction is common to both signaling pathways, involving βγ subunits of G12 and G13 proteins. Gβγ is known to be able to interact with PLCβ (35) and PI3K (36), increasing their activities. This suggests that PI3K might be one of the direct effectors of basic secretagogue-activated G proteins. Activation of PI3K results in local accumulation of PIP3 at the plasma membrane, allowing the recruitment of cytosolic proteins characterized by a pleckstrin domain (37). However, the involvement of PI3K in mast cell secretion is poorly documented.

The selective inhibitor of PI3K, LY294002 (38), inhibited arachidonate release, but did not modify histamine secretion (Fig. 6, A and B). The activation of PI3K by basic secretagogues is further demonstrated in Fig. 6C by the LY29402-sensitive phosphorylation of Akt (PKB), a common substrate for PI3K (39).

The interaction of Gβγ with PLCβ and PI3K is confirmed by the experiments represented in Fig. 7. We observed by Western blotting the communoprecipitation of Gβ and PLCβ with the PLCβ serum (Fig. 7A), and the communoprecipitation of PI3K and Gβ with the anti-Gβ serum (Fig. 7B). These observations allow us to propose Gβγ as the bifurcation point of the two secretory pathways, with PLCβ as the first following member of the exocytotic pathway and PI3K as the first following member of the pathway leading to de novo-synthesized mediators.

The activation of PLCγ1 can be achieved through its binding to membrane PIP3 generated by PI3K and/or through tyrosine phosphorylation (40–42). PLCγ1 immunoprecipitate is shown in Fig. 7C. We did not observe any binding of anti-phosphotyrosine Abs to PLCγ1, suggesting that tyrosine phosphorylation was not involved in its activation by spermine. This observation strongly suggested that protein tyrosine kinases (PTK) might be downstream of PLC.

The activation of mast cells by basic secretagogues in the presence of vanadate to inhibit protein tyrosine phosphatases leads to tyrosine phosphorylation of several cellular proteins with M, of 26–100 kDa (20). These phosphoproteins included the p42/p44 MAPKs (22). Fig. 9 shows that tyrosine phosphorylation of proteins of 36–57 kDa was prevented by anti-Gβ Abs, LY294002, and anti-PLCγ1 Abs. These data demonstrate that G proteins, PI3K, and PLCγ are upstream of PTK.

Discussion

The sensitivity to pertussis toxin of the secretory responses of mast cells to the basic secretagogue compound 48/80 has been the first indication of the involvement of G proteins in this process (13, 14). This property has been extended to other basic triggers considered to stimulate mast cells in a receptor-independent manner by directly activating pertussis toxin-sensitive G proteins (4, 6–9, 11, 12, 16, 17, 29, 30, 43–45). The direct activation of G proteins involves the ability of basic peptides and drugs to translocate across the plasma membrane into mast cells to reach their targets. This has been demonstrated for the neuropeptide substance P (46), but experimental evidence is lacking for other basic secretagogues.

FIGURE 6. Effects of LY294002, a PI3K inhibitor, on exocytosis, arachidonate release, and Akt (PKB) phosphorylation induced by basic secretagogues. A, Cells were preincubated for 15 min at 37°C with vanadate (0.1 mM) and the indicated concentrations of LY294002 and were stimulated for 10 min by spermine (3 mM) or mastoparan (0.1 mM). One hundred percent histamine secretion in the absence of LY294002 corresponds to 50.5 ± 2% (mastoparan) and 50.6 ± 3% (spermine) of the total histamine content. B, Cells were labeled with [3H]arachidonate as detailed in Materials and Methods. Then cells were preincubated for 15 min at 37°C with vanadate (0.1 mM) and the indicated concentrations of LY294002 and stimulated for 20 min by spermine (3 mM) or mastoparan (0.1 mM). One hundred percent arachidonate release in the absence of LY294002 treatment corresponds to 1153 ± 45 dpm (mastoparan) and 1098 ± 51 dpm (spermine). C, Phosphorylation of Akt (PKB) due to PI3K activation. Cells were preincubated for 15 min at 37°C with vanadate (0.1 mM) and the indicated concentrations of LY294002 and were stimulated for the indicated time with spermine (3 mM). Cell extracts were lysed by adding lysis buffer and were subjected to Western blotting with anti-phospho-Akt Abs. Data are representative of three separate experiments.
The participation of PLA₂ in exocytosis was first proposed by Nakamura and Ui (14). This was based on the inhibitory effect of mepacrine and \( p \)-bromophenacyl bromide, which are considered to be selective inhibitors of this enzyme, on both histamine secretion and arachidonate release triggered by compound 48/80. However, higher drug concentrations were required to inhibit histamine secretion than arachidonate release (14). Alternatively, Churcher et al. (50) proposed that PLA₂ activation is not an essential precursor of histamine secretion, considering that under some circumstances exocytosis was observed without measurable release of arachidonate. The present results confirm the latter view; anti-cPLA₂ Ab prevented arachidonate release without affecting histamine secretion (Fig. 5). Thus, the bifurcation point of the two pathways is localized upstream of cPLA₂.

Basic secretagogues induce a rapid production of IP₃, indicating concomitant PLC activation and histamine secretion (6, 8, 9, 14, 16). The present results confirm the participation of the PLCβ role of α subunits cannot be excluded. Such a role would not involve adenylate cyclase inhibition, since pertussis toxin did not increase the cAMP level in mast cells (13).

The present results allow us to propose both G₁₂ and G₁₃ as targets for basic secretagogues. G₁₂ had been previously excluded (17), since anti-Gα Ab Abs were unable to inhibit exocytosis, considering that anti-Gα Ab Abs might recognize Gα₁₂ due to the structural analogy of G₁ and G₁₂ C-terminals (Fig. 1). G₁₃ was considered to mediate PLC-independent exocytosis, thus acting downstream of PLC, fulfilling the properties of the putative G₁ protein proposed by Gomperts (47, 48). It would be tempting to propose G₁₂ and G₁₃ as the Gα and Gβ₁ proteins acting in series to control stimulus-secretion coupling in mast cells (47). However, several observations argue against this being the case. Firstly, small G proteins, Rac2 and cdc42, have recently been considered as candidates for Gα₁₂ (49). Secondly, the cumulative inhibitory effect of anti-G₁₂ and -G₁₃ Abs (Fig. 3) is not compatible with the two proteins acting in series. Thus, we believe that both G₁₂ and G₁₃ correspond to the putative G₁ protein, acting upstream of PLC and leading to exocytosis and arachidionate release.

Anti-Gβ Abs fully inhibited exocytosis and arachidionate release (Fig. 4). This observation strongly suggests that βγ subunits of G₁₂ and G₁₃ play a major role in the transduction pathway. A regulatory

Thus, a role for some undetermined membrane receptor with low ligand selectivity, cannot be formally excluded. However, the receptor-mediated or receptor-independent stimulation of pertussis toxin-sensitive G proteins is an early step in the response of mast cells to basic secretagogues.
family in this process, as could be predicted from its ability to be activated by heterotrimeric G proteins (35). The coimmunoprecipita-
tion of PLCβ and Gβγ (Fig. 7A) confirms that PLCβ interacts
with Gβ subunits of Gi proteins. However, we were unable to dis-
tinguish between the different subtypes of PLCβ due to the lack of
selectivity of the available Abs. More interestingly, the anti-PLCβ
Abs were unable to prevent arachidonic acid release elicited by
mastoparan or spermine. In contrast, PLCγ1 appeared to be selec-
tive for the arachidonate release pathway initiated by basic triggers
(Fig. 5). This constitutes a major difference from secretory pro-
cesses elicited by Ags, where PLCγ1 controls both exocytosis and
arachidonate release (2). The involvement of PLCβ or PLCγ1 in
exocytosis triggered by basic secretagogues is observed in each case. The direct coupling of PLCβ induces
exocytosis within seconds, whereas the indirect coupling of
PLCγ1 to FceRI receptors leads to a delayed exocytosis. PLCγ2
does not have a major role in the IgE/FceRI pathway (42). Exper-
iments are in progress to determine its putative involvement in
the basic secretagogue pathway.

At this point of our study PLCγ1 can be placed upstream of
cPLA2 and PKC in the activation order. The activation of PLCγ1
can be achieved by tyrosine phosphorylation or by the interaction
of its pleckstrin domain with membrane PIP3 generated by PI3K
(35, 36). We did not detect any tyrosine phosphorylation of PLCγ1
following mast cell stimulation by basic secretagogues or Ags, respec-
tively, activated through its interaction with
PI3K. This is compatible with the recent observation that PI3K can be
phosphorylated as a consequence of basic secretagogue pathway.

Proposed pathways for exocytosis and arachidonate release
induced by basic secretagogues in serosal mast cells.

![Proposed pathways for exocytosis and arachidonate release induced by basic secretagogues in serosal mast cells.](image)

In summary, we propose a sequence of signaling events (Fig. 9)
initiated by basic secretagogues in mast cells with the respective
roles of PLCβ and PLCγ1 in the control of exocytosis and arachid-
onate release. These two pathways elicited by a common stim-
ulus suggest a selective subcellular localization for each PLC
comparing with their respective roles. PLCs are known to generate IP3
and diacylglycerol, leading to the increase in cytosolic Ca2+ and
PKC activation. As we show that PLCγ1 precedes PTK, the activa-
tion of PTK might require Ca2+ increase and/or PKC activation.
These points need to be thoroughly studied.

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