Activation of $\beta_3\gamma$ 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

Xavier Ferry, Virginie Eichwald, Laurent Daeffler and Yves Landry

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Activation of βγ Subunits of G_{i2} and G_{i3} Proteins by Basic Secretagogues Induces Exocytosis Through Phospholipase Cβ and Arachidonate Release Through Phospholipase Cγ in Mast Cells

Xavier Ferry, Virginie Eichwald, Laurent Daeflner, and Yves Landry

Mast cells are activated by Ag-induced clustering of IgE bound to FceRI 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 α and βγ 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_{i2} 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 Gβγ dimers prevented both secretions. Anti-PLCBAbs selectively prevented exocytosis. The selective phosphatidylinositol 3-kinase inhibitor LY294002 prevented arachidonate release without modifying exocytosis. Gβγ immunoprecipitated with PLCβ and phosphatidylinositol 3-kinase. The anti-PLCγ1 and anti-phospholipase A₂ Abs selectively blocked arachidonate release. Protein tyrosine phosphorylation was inhibited by anti-GβγAbs, LY294002, and anti-PLCγ1 Abs. These data show that the early step of basic secretagogue transduction is common to both signaling pathways, involving βγ subunits of G_{i2} and G_{i3} proteins. Activated Gβγ interacts, on one hand, with PLCβ to elicit exocytosis and, on the other hand, with phosphatidylinositol 3-kinase to initiate the sequential activation of PLCγ1, tyrosine kinases, and phospholipase A₂, leading to arachidonate release. The Journal of Immunology, 2001, 167: 4805–4813.

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 α subunits from G_{i1}, 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 decapetide corresponding to the carboxyl terminus of its α subunit (Gα_{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} was neutralized by 90% identity between them, failed to inhibit exocytosis (see Fig. 1). The respective roles of α and βγ subunits of pertussis toxin-sensitive G proteins in the signaling pathways of basic secretagogues have not been investigated. Gβγ subunits introduced into permeabilized mast cells amplified secretion induced by Ca²⁺ and GTPγS, whereas Gα_{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/FceRI initiates the activation of Lyn and Syk tyrosine kinases followed by the Syk-mediated phosphorylation of phospholipase Cγ1 (PLCγ1).² PLCγ1, in turn, catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to

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² Abbreviations used in this paper: PLC, phospholipase C; cPLA₂, cytosolic phospholipase A₂; IP₃, inositol-1,4,5-trisphosphate; G protein, heterotrimeric GTP-binding protein; MAP kinase, mitogen-activated protein kinase; PKA, protein kinase A; PKC, protein kinase C; PTK, protein tyrosine kinase.
The activation of cPLA₂ was phosphorylation by various protein kinases, including PKC and mito-proteins, and their respective units to initiate exocytosis through the activation of PLC. 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 be considered through the involvement of phosphatidylinositol-3 kinase (PI3K) and PKC-dependent activation of PLC (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 G₁₂ and G₁₃ heterotrimeric G proteins, and their respective α and βγ 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 G₁₂ and G₁₃ proteins are activated by basic secretagogues, allowing Gβγ 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-Gα₁₃ (carboxyl-terminal 10 aa residues) and anti-recombinant Gα₃ protein Abs were purchased from Chemicon International (Temecula, CA). Anti-Gα₁₃ and anti-Gα₃ (carboxyl-terminal 10 residues) Abs were obtained from Upstate Biotechnology (Lake Placid, NY). Anti-Go₃ (carboxyl-terminal 10 residues) Ab and mAbs against p-Tyr (PY20) were purchased from Transduction Laboratory (Lexington, KY). Anti-βG (carboxyl-terminal 20 aa of β 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’Arbresle, 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%.

FIGURE 2. Effect of anti-Gα₁₃ (A), Gα₁ (B), or Gα₃ (C) carboxy-terminal Abs on histamine secretion induced by basic secretagogues. Cells were incubated with streptolysin-O (0.4 U/ml) for 1 min at 37°C before the addition of Ab. Mast cells were stimulated 2 min later by adding mastoparan (0.1 mM) or spermine (3 mM). One hundred percent of each stimulation stands for histamine secretion induced by each compound on permeabilized cells in the absence of Ab (A, mastoparan, 17.2 ± 0.6%; spermine, 32.5 ± 4.9%; B, mastoparan, 29.2 ± 1.7%; spermine, 21.5 ± 3.5%; C, mastoparan, 30.4 ± 6.4%; spermine, 21.1 ± 0.9% of total histamine content). Controls show the Ab effect on unstimulated permeabilized cells (A, 7.9 ± 1.8%; B, 7.9 ± 1.8%; C, 14.2 ± 8.0%). Values are the mean ± SEM of four independent experiments.

Gα₁₂ : Lys–Asn–Asn–Leu–Lys–Asp–Cys–Gly–Leu–Phe
Gα₁₃ : Lys–Glu–Asn–Leu–Lys–Asp–Cys–Gly–Leu–Phe
Permeabilization and determination of histamine release

Purified mast cells (3 × 10^6 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 × 10^6 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 (10^6 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 × 10^6 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 10% 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 with 15 μl anti-PLCγ1, anti-PI3K, or anti-p-Tyr Abs. After incubation with secondary Ab (anti-mouse IgG Ab conjugated to HRP) 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-PLCγ1, anti-Gβγ, or anti-PI3K 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.

**FIGURE 3.** Effects of anti-Gα12 recombinant protein and anti-Gα12 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 Gα12 and anti-Gα12 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-Gβ Ab (40 μg/ml; see Fig. 4) on histamine secretion induced by the Ab of the carboxyl terminus of Gα12 (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 Gi2 and Gi3 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 Goi2, Gαi, and Goi3 on permeabilized mast cells. Anti-Goi2 Abs dose-dependently inhibited approximately 60% of histamine secretion elicited by mastoparan or spermine (Fig. 2A). Anti-Gα Abs did not modify the exocytotic response of mast cells to mastoparan or spermine (Fig. 2B). Similarly, Aridor et al. (17) showed that anti-Gαi Abs inhibited exocytosis triggered by GTPγS, whereas anti-Gαi Abs were inefficient. The Gi subtype of heterotrimeric G proteins may regulate mast cell exocytosis (33), but we observed no effect of anti-Gα Ab on the response to basic secretagogues (Fig. 2C).

The above results confirmed the involvement of Gi3 in the triggering effects of basic secretagogues. To determine whether Gi3, the second pertussis toxin substrate characterized in rat peritoneal mast cells (17), was also involved, we used two different anti-Gi2 Abs (Fig. 3, A and C). The anti-recombinant Goi2 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 Gi2 and Gi3 as targets of basic secretagogues. Accordingly, the simultaneous inactivation of Gi2 and Gi3 proteins by anti-Gα Abs provided additive inhibition of histamine secretion elicited by basic secretagogues (Fig. 3B).

The anti-Goi2 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-Gi2 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 Gα protein carboxyl terminus (see Fig. 1). Pertussis toxin pretreatment decreased the secretory response of mast cells (Fig. 3D). Thus, the anti-Goi2 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-Gβ Abs. Similarly, an anti-Gαi C-terminus Ab has been reported to activate Gi protein (34).
The anti-recombinant \( G_{i2} \) protein Ab and the anti-\( G_{i3} \) 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 \( G_{i2} \) and \( G_{i3} \) are similarly involved in exocytosis and arachidonate release, indicating that \( G_{i2} \) and \( G_{i3} \) activation is a common step of the corresponding signaling pathways.

**Involvement of \( \beta \gamma \) subunits of \( G \) proteins in exocytosis and arachidonate release**

Both \( \alpha \) and \( \beta \gamma \) subunits of heterotrimeric \( G \) proteins can stimulate effectors (see Ref. 35 for review). To address the question of whether \( \beta \gamma \) subunits were involved in signal transduction elicited by basic secretagogues, we studied the effect of an anti-\( G_{i2} \) Ab with broad specificity to mouse, human, and rat \( G_{i1} \) to \( G_{i4} \) subunits. This Ab did not elicit mast cell secretory responses (controls, Fig. 4, C and D), but strongly inhibited histamine and arachidonate release (Fig. 4, C and D). These observations strongly suggest that \( \beta \gamma \) 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 PLC\( _{\beta} \), PLC\( _{\gamma} \), and cPLA\( _{2} \) 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 \( \beta \gamma \) dimers of heterotrimeric \( G \) proteins suggested by the above data, we assessed the involvement of PLC\( _{\beta} \) subtypes, with anti-PLC\( _{\gamma} \), -PLC\( _{\beta} \), and -cPLA\( _{2} \) Abs. We obtained similar results with all three Abs, calling into question their subtype selectivity. Although

**FIGURE 5.** Effects of anti-PLC\( _{\beta} \), anti-PLC\( _{\gamma} \), and anti-cPLA\( _{2} \) 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-PLC\( _{\beta} \), -PLC\( _{\gamma} \), and -cPLA\( _{2} \) Abs (40 \( \mu \)g/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 PLCγ2 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 Gi2 and Gi3 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.
The participation of PLA$_2$ 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 PLA$_2$ 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-cPLA$_2$ Ab prevented arachidonate release without affecting histamine secretion (Fig. 5). Thus, the bifurcation point of the two pathways is localized upstream of cPLA$_2$.

Basic secretagogues induce a rapid production of IP$_3$, indicating concomitant PLC activation and histamine secretion (6, 8, 9, 14, 16). The present results confirm the participation of the PLC$\beta$

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.

The present results allow us to propose both G$_{12}$ and G$_{13}$ as targets for basic secretagogues. G$_{12}$ had been previously excluded (17), since anti-G$_{12}$ Abs were unable to inhibit exocytosis, considering that anti-G$_{12}$ Abs might recognize G$_{12}$ due to the structural analogy of G$_i$ and G$_{12}$ C-terminals (Fig. 1). G$_{13}$ was considered to mediate PLC-independent exocytosis, thus acting downstream of PLC, fulfilling the properties of the putative G$_i$ protein proposed by Gomperts (47, 48). It would be tempting to propose G$_{12}$ and G$_{13}$ as the G$_i$ and G$_{12}$ 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 cdc-42, have recently been considered as candidates for G$_{12}$ (49). Secondly, the cumulative inhibitory effect of anti-G$_{12}$ and -G$_{13}$ Abs (Fig. 3) is not compatible with the two proteins acting in series. Thus, we believe that both G$_{12}$ and G$_{13}$ correspond to the putative G$_i$ protein, acting upstream of PLC and leading to exocytosis and arachidonate release.

Anti-G$_{12}$ Abs fully inhibited exocytosis and arachidonate release (Fig. 4). This observation strongly suggests that $\gamma$ subunits of G$_{12}$ and G$_{13}$ play a major role in the transduction pathway. A regulatory role of $\alpha$ subunits cannot be excluded. Such a role would not involve adenylyl cyclase inhibition, since pertussis toxin did not increase the cAMP level in mast cells (13).

The present results confirm the latter view; anti-cPLA$_2$ Ab prevented arachidonate release without affecting histamine secretion (Fig. 5). Thus, the bifurcation point of the two pathways is localized upstream of cPLA$_2$.
family in this process, as could be predicted from its ability to be activated by heterotrimeric G proteins (35). The co-immunoprecipitation of PLCβ and Gβγ (Fig. 7A) confirms that PLCβ interacts with Gβ subunits of Gβγ proteins. However, we were unable to distinguish 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 selective for the arachidonate release pathway initiated by basic triggers (Fig. 5). This constitutes a major difference from secretory processes elicited by Ags, where PLCγ1 controls both exocytosis and arachidonate release (2). The involvement of PLCβ or PLCγ1 in exocytosis triggered by basic secretagogues or Ags, respectively, might be responsible for the different kinetics of histamine secretion 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). Experiments 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 (Fig. 7C), suggesting that the major stimulation of PLCγ1 was achieved through its recruitment at the membrane to PIP3-rich domains. However, we cannot exclude a minor participation of tyrosine phosphorylation in the activation of PLCγ. We propose that PLCγ1 is localized downstream of PI3K, which generates PIP3. This is compatible with the recent observation that PI3K can be activated through its interaction with βγ subunits of trimeric G proteins (51, 52). The co-immunoprecipitation of PI3K and Gβγ (Fig. 7B) prompts us to propose a direct interaction between Gβγ and PI3K (Fig. 9).

The participation of PTKs has recently been proposed in the arachidonate pathway (20, 53), including Syk kinase (27). The observation that anti-PLCγ1 Abs decrease tyrosine phosphorylation (Fig. 8) indicates that PLCγ1 precedes PTK.

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 arachidonate release. These two pathways elicited by a common stimulus suggest a selective subcellular localization for each PLC compatible with their respective roles. PLCs are known to generate IP3 and diacylglycerol, leading to the increase in cytosolic Ca2+ and to PKC activation. As we show that PLCγ1 precedes PTK, the activation of PTK might require Ca2+ increase and/or PKC activation. These points need to be thoroughly studied.

**References**


