Exocytosis in Mast Cells by Basic Secretagogues: Evidence for Direct Activation of GTP-binding Proteins

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Abstract. Histamine release induced by the introduction of a nonhydrolyzable analogue of GTP, GTP-γ-S, into ATP-permeabilized mast cells, is associated with phosphoinositide breakdown, as evidenced by the production of phosphatidic acid (PA) in a neomycin-sensitive process. The dependency of both PA formation and histamine secretion on GTP-γ-S concentrations is bell shaped. Whereas concentrations of up to 0.1 mM GTP-γ-S stimulate both processes, at higher concentrations the cells' responsiveness is inhibited. At a concentration of 1 mM, GTP-γ-S self-inhibits both PA formation and histamine secretion. Inhibition of secretion can, however, be overcome by the basic secretagogues compound 48/80 and mastoparan that in suboptimal doses synergize with 1 mM GTP-γ-S to potentiate secretion. Secretion under these conditions is not accompanied by PA formation and is resistant both to depletion of Ca^{2+} from internal stores and to pertussis toxin (PtX) treatment. In addition, 48/80, like mastoparan, is capable of directly stimulating the GTPase activity of G-proteins in a cell-free system. Together, our results are consistent with a model in which the continuous activation of a phosphoinositide-hydrolyzing phospholipase C (PLC) by a stimulatory G-protein suffices to trigger histamine secretion. Basic secretagogues of mast cells, such as compound 48/80 and mastoparan, are capable of inducing secretion in a mechanism that bypasses PLC by directly activating a G-protein that is presumably located downstream from PLC (G_E). Thereby, these secretagogues induce histamine secretion in a receptor-independent manner.

SECRETION of inflammatory mediators such as histamine by rat peritoneal mast cells has been extensively used as a model system to study exocytosis (20). This response can be triggered either by aggregation of specific receptors for IgE (39) or by other secretagogues including a family of basic molecules such as compound 48/80, polymers of basic amino acids and neurotransmitters such as substance P (reviewed in reference 19). The early events after stimulation of mast cells include the breakdown of phosphoinositides (PIP_2) (4), a rise in internal Ca^{2+} concentrations (42) and the generation of arachidonic acid (22). However, the mode of activation of these signaling pathways and their relative contributions to secretion have not yet been resolved.

When combined, Ca^{2+} ionophores and phorbol esters, which activate protein kinase C (PKC),[1] elicit a maximal secretory response (18, 33). This observation suggests that a rise in the internal Ca^{2+} concentration, in conjunction with PKC activation is sufficient to induce exocytosis. However, whereas both IgE-mediated and basic secretagogues-induced exocytosis are accompanied by phosphoinositide breakdown (4), there are a number of important differences in their mode of action. IgE-dependent exocytosis is relatively slow, extending over a period of ~2 min, is strictly dependent on the presence of external Ca^{2+} (9), and is pertussin toxin (PtX) insensitive (38). In contrast, secretion by basic secretagogues is far more rapid (~10 s), is independent of external Ca^{2+}, and is PtX sensitive (38). Hence, basic secretagogues appear to induce secretion by a different mechanism to that mediated by IgE.

The introduction of nonhydrolyzable analogues of GTP that persistently activate GTP-binding proteins (G proteins) into mast cells induces exocytosis (7, 11). This implicates G proteins in playing a role in the cascade of events leading to histamine secretion. Studies using streptolysine O-permeabilized mast cells have suggested the involvement of two different G proteins in stimulus–secretion coupling, one coupled to phospholipase C (PLC) activation (G_P), and the other coupled directly to exocytosis (G_E) (15). However, these putative G proteins have not yet been identified. In the present work, we have attempted to further characterize these putative G proteins by gaining better insight into their mode of activation and their role in controlling mast cell exocytosis. For this purpose, we have examined the effects of G protein activators introduced into ATP-permeabilized mast cells on exocytosis induced by secretagogues that act up or downstream to PLC activation.

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1. *Abbreviations used in this paper*: NAD, nicotinamide adenine dinucleotide; PA, phosphatidic acid; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; PtX, pertussis toxin.

Materials and Methods

Materials

[^{32}P] Orthophosphate (9,400 Ci/mmol) was purchased from the Nuclear Center (Negev, Israel). ^{125}I-donkey anti-rabbit IgG was from Amersham International (Amersham, UK); [γ-^{32}P]-GTP (3,000 Ci/mmol) and [γ-^{32}P]-NAD (800 Ci/mmol) were from New England Nuclear (Boston, MA) GTP-γ-S and GDP-β-S were from Boehringer (Mannheim, FRG). PtX was from List Biological Laboratories. Unless otherwise specified all reagents were from Sigma Chemical Co. (St. Louis, MO).

Cells

Mast cells from the peritoneal cavity of male Wistar rats (250–300 g body wt) were isolated and purified over a Ficoll gradient as previously described (32). Purity of the cell population was determined using toluidine blue staining and cell viability was determined using trypan blue exclusion. Under these conditions a homogeneous mast cell population is obtained (>90%) with 95% viability.

Cell Permeabilization

Purified mast cells were permeabilized essentially as described in reference 11. Briefly, mast cells ($\sim$1 $\times$ 10^6 cells/ml) were suspended in a divalent cation-free salt solution composed of 137 mM NaCl, 2.7 mM KCl, 20 mM Hepes, 5.6 mM glucose and 1 mg/ml BSA, pH 7.7 (buffer A). The cells were permeabilized at 30°C with ATP (3 μM) in the presence of EGTA (15 μM) for 6 min and resealed by the addition of 2 mM $MgAc_2$. After a further 10-min incubation, the cells were transferred to tubes containing 2 mM Ca^{2+} and the indicated agonists. At the end of 20 min incubation at 30°C, the reactions were quenched by the addition of Ca^{2+}- and BSA-free ice-cold buffer A. Under these conditions the cells were viable and responsive to the agonists tested.

Histamine Secretion

Histamine secretion was measured as previously described (34). Each data point represents the average of duplicate measurements, which did not vary by >3%.

Phospholipid Analysis

Purified mast cells (10^6 cells/ml) were incubated for 2 h in a phosphate- and divalent cation-free buffer in the presence of [^{32}P]orthophosphate (100 μCi/ml). They were subsequently subjected to permeabilization and resealing. Phospholipids were extracted as described in reference 3. Briefly, cells were resuspended in 200 μl of H_2O and 750 μl of chloroform/methanol/HCl (20:40:1). After 30 min, 250 μl of 2 M KCl and 250 μl of chloroform were added and the lower chloroform phase (450 μl) was collected, dried, and analyzed by thin layer chromatography on silica gel-precoated plates as described in reference 40. The solvent system comprised chloroform/methanol/water/acetic acid (75:25:3:8). Phospholipid markers were identified by iodine staining. After visualization by autoradiography the spots were cut and the incorporated radioactivity determined by liquid scintillation spectrometry.

PtX-catalyzed ADP-Ribosylation

Purified mast cells (5 $\times$ 10^6 cells) were lysed in 0.125 ml of a lysis buffer containing 50 mM Na·Hepes pH 7.4, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 20 μg/ml soy bean trypsin inhibitor, and 1% Triton X-100. The lysate was centrifuged at 12,000 *g* for 15 min at 4°C and the Triton-soluble proteins were incubated for 30 min at 30°C with 1 mg of insoluble nicotinamide adenine dinucleotide-glycohydrolase followed by centrifugation at 1,000 *g* for 5 min. 40-μl aliquots of the NAD-depleted supernatant were ADP-ribosylated by incubating them for 30 min at 30°C with a reaction mixture yielding the final concentrations of 1 mM EDTA, 10 mM thymidine, 100 mM Tris buffer pH 8.0, 1 mM DTT, 2.5 μM NAD, and 1 μCi of [α-^{32}P]NAD in a final volume of 0.1 ml. Where indicated, 2.4 μg PtX, that was activated by incubation for 15 min at 30°C with 50 mM Tris·HCl pH 7.5, 10 mM DTT, and 50 μM ATP, was added. The reaction was stopped by adding concentrated Laemmli's sample buffer. The proteins were separated by SDS-PAGE and the gels dried and autoradiographed.

Western Blot Analysis

Purified mast cells (5 $\times$ 10^6 cells) were lysed in 0.125 ml of the lysis buffer described above, except that 0.1% of SDS was also included. The lysate was clarified by centrifugation at 12,000 *g* for 15 min at 4°C. Both the soluble and insoluble proteins were separated by electrophoresis in SDS gels (10% acrylamide) and transferred to 0.2 μm nitrocellulose filters by blotting at 200 mA for 3 1/2 h in 15.6 mM Tris, 120 mM glycine. Blocking was for 2 h at 22°C with 1% BSA in 10 mM Tris·HCl pH 8, 150 mM NaCl, 0.05% Tween 20 (TBST), followed by probing with primary antibody, diluted as indicated in TBST/0.1% BSA overnight at 4°C. Blots were then washed four times with TBST 10 min each and then incubated with ^{125}I-donkey anti-rabbit IgG (0.2 μCi/ml) in TBST/0.1% BSA for 2 h at 22°C. After four 10-min washes in TBST and two in distilled water, blots were dried and autoradiographed.

Rat Brain Membrane Preparation

Rat brain membranes were prepared according to reference 17. Briefly, whole brains from Sprague-Dawley rats were homogenized at 4°C with a Dounce homogenizer in a buffer containing 20 mM Tris·HCl pH 7.5, 250 mM sucrose, 2 mM EDTA, 2 mM EGTA, 1 mM PMSF, and 10 mM mercaptoethanol. Tissue debris was removed by centrifugation at 200 *g* at 4°C for 5 min. The membranes were then collected, washed twice by centrifugation (20,000 *g*, 30 min at 4°C), and resuspended in the homogenization buffer. For solubilization, the membranes were washed in TED buffer (20 mM Tris·HCl pH 8.0, 1 mM EDTA, 1 mM DTT) supplemented with 0.1 M NaCl, 0.1% cholate, and 25 kallikrein inhibitory units/ml of aprotinin and solubilized in TED buffer supplemented with 1% cholate and aprotinin for 1 h at 4°C. The extract was centrifuged for 1 h at 100,000 *g* at 4°C and supernatants were collected.

GTPase Assay

The GTPase assay was performed according to reference 13. 20-μg aliquots of membranes or cholate solubilized membranes were incubated in the presence of the indicated reagents for 10 min at 25°C in a final volume of 100 μl. Reaction mixture contained final concentrations of 0.1 mM ATP, 0.3 μM GTP, 0.1 mM EGTA, 1 mM DTT, 2 mM $MgAc_2$, 5 mM phosphocreatine, 0.4 mg/ml creatine kinase, 20 mM Tris·HCl pH 7.5 and 0.1 μCi of [γ-^{32}P]-GTP. For cholate-solubilized membranes, 0.6% cholate was included. [^{32}P]P_i released was determined according to (29). Specific GTPase activity was calculated from the difference between [^{32}P]P_i released in the absence or presence of 100 μM GTP.

Results

GTP-γ-S-induced Histamine Secretion and Phosphatidic Acid (PA) Formation

Previous reports (11) have shown that introduction of GTP-γ-S into the cytosol of mast cells by permeabilization with ATP evokes histamine secretion. To determine whether this secretion was associated with activation of PLC, the effect of an internal application of GTP-γ-S on both histamine secretion and PA formation was determined.

Introduction of GTP-γ-S (0.1 mM) into [^{32}P]-labeled cells resulted in both histamine secretion (Fig. 1 *A*, compare lane *b* with lane *a*), and enhanced formation of PA ([^{32}P]-PA) (Fig. 1 *B*, compare lane *f* with lane *e*). Both GTP-γ-S-induced histamine release and PA formation were completely blocked by the simultaneous entrapment of GDP-β-S (1 mM) during the permeabilization period (Fig. 1, lanes *c* and *g*). Inhibition by GDP-β-S was dose dependent, with half maximal inhibition obtained with 0.5 mM GDP-β-S and maximal inhibition at a concentration of 1 mM GDP-β-S (Fig. 2). Thus release induced by GTP-γ-S involves a G-protein-dependent activation of a phospholipase that leads to PA formation.

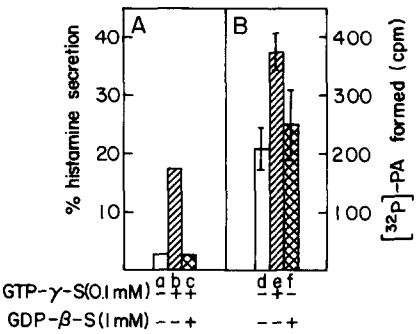

Figure 1. Effects of GTP-γ-S on histamine secretion and PA formation. Purified mast cells (1 × 10^6 cells/ml) were [^{32}P]-labeled for 2 h. They were subsequently permeabilized in the absence (*a* and *d*) or presence of GTP-γ-S (0.1 mM) (*b* and *e*) or GTP-γ-S (0.1 mM) plus GDP-β-S (1 mM) (*c* and *f*). After resealing, the cells were transferred to Ca^{2+}-containing buffers and further incubated for 20 min at 30°C. The reactions were quenched by adding ice-cold buffer. After centrifugation, histamine and [^{32}P]-labeled PA were determined as described in Materials and Methods. The results (mean ± SE) are means of duplicate determinations. Similar results were obtained on three occasions.

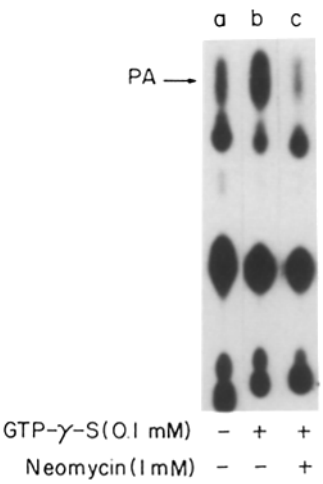

Figure 3. Effect of neomycin on GTP-γ-S-induced PA formation. Purified mast cells (1 × 10^6 cells/ml) were ^{32}P-labeled for 2 h and subsequently permeabilized in the absence (*a*) or presence (*b*) of GTP-γ-S (0.1 mM) or GTP-γ-S (0.1 mM) plus neomycin (1 mM) (*c*). The cells were subsequently resealed and transferred to Ca^{2+}-containing buffers. After 20 min incubation at 30°C, the reaction was stopped and the cells were centrifuged. Cell pellets were processed for PA formation as described in Materials and Methods. Similar results were obtained on three occasions.

The PA formed in response to GTP-γ-S could be the phosphorylation product of diacylglycerol (DAG) generated after PIP_2 breakdown. As such, PA formation could reflect the activation of a PIP_2-hydrolyzing PLC. Alternatively, PA could be formed directly by the action of a phospholipase D (PLD) enzyme that hydrolyzes phosphatidylcholine (2). To distinguish between these possibilities, the effect of neomycin on GTP-γ-S-induced PA formation was examined. This aminoglycoside antibiotic inhibits phosphoinositide metabolism in mast cells (5). As shown in Fig. 3, introduction of 1 mM neomycin into the cells during their permeabilization, completely blocked GTP-γ-S-induced PA formation, indicating that the source of the PA formed was PIP_2 metabolism.

Both histamine secretion and PA formation induced by GTP-γ-S were dose dependent and subject to self inhibition (Fig. 4). A concentration of 0.1 mM GTP-γ-S caused an optimal release (20–40%) of total cellular histamine as well as a two- to fourfold stimulation in PA formation. At higher concentrations responsiveness was lower and at a concentration of 1 mM, GTP-γ-S induced virtually no response (Fig. 4).

Commercially available GTP-γ-S can be contaminated with GDP (8). This contamination could not, however, account for the inhibitory phase of GTP-γ-S action because a 10-fold excess of GDP was required to completely inhibit the GTP-γ-S-induced responses (Fig. 2). In certain cellular systems (e.g., platelets [41]), cAMP has been shown to inhibit PLC activation. However, the reduced secretion and PA formation in response to higher concentrations of GTP-γ-S were not due to an elevation of the intracellular levels of cAMP by GTP-γ-S. This is based on the observations that the addition of cAMP (50 μM) during the permeabilization period or Bt_2cAMP (50 μM) after permeabilization, had no effect on either basal or GTP-γ-S (0.1 mM) -induced responses

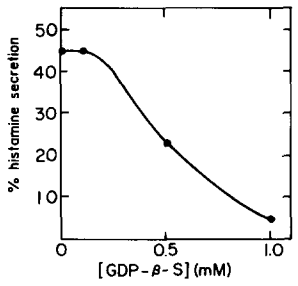

Figure 2. Effects of GDP-β-S on GTP-γ-S-induced histamine secretion. Purified mast cells (1 × 10^6 cells/ml) were permeabilized in the presence of 0.1 mM GTP-γ-S and the indicated concentrations of GDP-β-S. After resealing, the cells were transferred to Ca^{2+}-containing buffers and further incubated at 30°C. Reactions were stopped, the cells centrifuged and release of histamine was determined. The results of a representative experiment are presented. Similar results were obtained on five occasions.

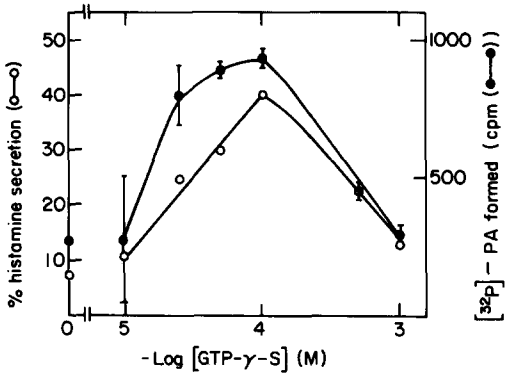

Figure 4. GTP-γ-S-induced histamine secretion and PA formation. Purified mast cells (1 × 10^6 cells/ml) in a final volume of 0.1 ml were [^{32}P]-labeled and permeabilized in the presence of the indicated GTP-γ-S concentrations. The cells were subsequently resealed and transferred to Ca^{2+}-containing tubes. After 20 min incubation at 30°C, the reaction was quenched by adding ice-cold buffer and the cells were centrifuged. The supernatants were saved for determination of histamine (○) and the cell pellets were processed for PA formation (●) as described in Materials and Methods. The data points indicate means of duplicate determinations. Similar results were obtained on three occasions.

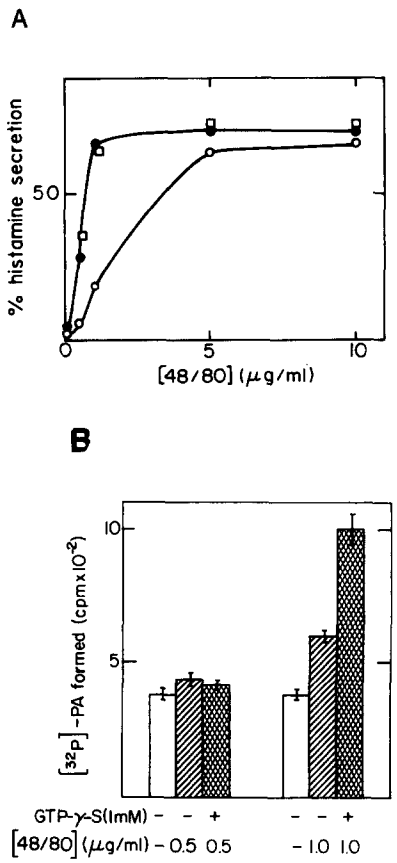

Figure 5. Effect of GTP-γ-S on 48/80-induced histamine secretion and PA formation. (*A*) Purified intact mast cells (1 × 10^6 cells/ml) (□) or cells permeabilized in the absence, (○) or presence (●) of 1 mM GTP-γ-S and resealed were subsequently treated with the indicated concentrations of 48/80. After 20 min incubation at 30°C, in a Ca^{2+}-containing buffer, the reaction was quenched and histamine was determined. Similar results were obtained on five occasions. (*B*) Purified mast cells (1 × 10^6 cells/ml) in a final volume of 0.1 ml were [^{32}P]-labeled and permeabilized in the presence or absence of 1 mM GTP-γ-S. The cells were subsequently resealed and transferred to Ca^{2+} containing buffers in the presence or absence of the indicated concentrations of 48/80. After 20 min incubation at 30°C the reaction was quenched and the cells were centrifuged. The supernatants were saved for determination of histamine and the cell pellets were processed for PA formation as described in Materials and Methods. Means of duplicate determinations are presented. Similar results were obtained on two occasions.

(not shown). Thus, the mechanism underlying the inhibitory phase in GTP-γ-S action is presently unknown.

Activation of GTP-γ-S-dependent Responses by Compound 48/80

The inhibition of secretion by 1 mM GTP-γ-S could be overcome by the addition of the basic secretagogue compound 48/80. As illustrated in Fig. 5 *A*, 48/80 triggered histamine secretion from cells that were permeabilized and resealed with a dose-response curve that was shifted to the right compared with that of intact, nonpermeabilized cells. Introduction of GTP-γ-S into the permeabilized cells increased the effective affinity for 48/80, decreasing the concentration of 48/80 required to elicit half-maximal secretion from 1 μg/ml for the cells permeabilized in the absence of GTP-γ-S to 0.2 μg/ml for cells permeabilized in its presence. Thus, 48/80 and GTP-γ-S, at concentrations that by themselves are ineffective, synergize to elicit secretion. It should be noted that the dose-response curve obtained for 48/80 in the presence of GTP-γ-S was similar to that of intact, nonpermeabilized, cells (Fig. 5 *A*). Moreover, GTP-γ-S (1 mM), could be replaced by the internal application of GTP (1 mM), yielding the same results (not shown). These results are, therefore, compatible with the previously described washout phenomenon (27), where patch clamped mast cells lose their ability to secrete in response to 48/80 due to diminution of intracellular GTP. Their responsiveness can be maintained by addition of GTP to the internal solution.

We then examined whether this synergistic interaction between 48/80 and GTP-γ-S involved the reactivation of PLC. Indeed, 48/80 stimulated PA formation in cells that were loaded with GTP-γ-S (1 mM) (Fig. 5 *B*). However, a subdose of 48/80 (0.5 μg/ml) that elicited 40% of maximal histamine secretion from GTP-γ-S (1 mM) -loaded cells, failed to stimulate PA formation and higher concentrations of 48/80 (1 μg/ml) were required (Fig. 5 *B*). It therefore appears that the synergism between GTP-γ-S (1 mM) and 48/80 in inducing histamine secretion can be dissociated from PLC activation and is therefore unlikely to be mediated by activation of PKC or a rise in the internal Ca^{2+} concentrations. This was directly tested by examining whether a rise in internal Ca^{2+} concentrations or PKC activation could mimic the effect of GTP-γ-S (1 mM) and stimulate secretion in the presence of a subdose (0.5 μg/ml) of 48/80. For this purpose, the effects of 2,3 DPG, that is known to inhibit the dephosphorylation of inositol trisphosphate (28) and may thereby lead to a rise in the internal Ca^{2+} concentrations and of the phorbol ester TPA that activates PKC, were tested.

Entrapment of 2,3 DPG in mast cells resulted in stimulation of histamine secretion in a dose-dependent manner (Fig. 6). This result suggests, though does not prove, that exocytosis of mast cells can be induced by directly increasing internal inositol trisphosphate levels. 2,3 DPG had no effect on 48/80-induced release (not shown) supporting the notion that the potentiating effect of 1 mM GTP-γ-S is not mediated by increasing internal Ca^{2+} concentrations. Similarly, 100 ng/ml TPA, that potentiated histamine secretion (18% above basal), failed to shift the dose-response curve for 48/80 (not shown), revealing that the potentiating effect of 1 mM GTP-γ-S was not due to activation of PKC.

Finally, to determine whether a rise in the intracellular concentration of Ca^{2+} is at all required for the potentiating effect exerted by 1 mM GTP-γ-S, cells were partially de-

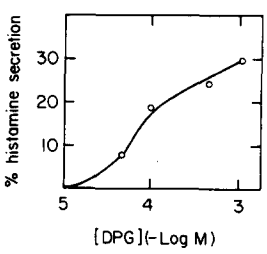

Figure 6. Histamine secretion induced by internally applied 2,3 DPG. Purified mast cells (1 × 10^6 cells/ml) were permeabilized in the presence of the indicated concentrations of 2,3 DPG. The cells were resealed and after 20 min incubation at 30°C in the same buffer, histamine released was determined. Similar results were obtained on two occasions.

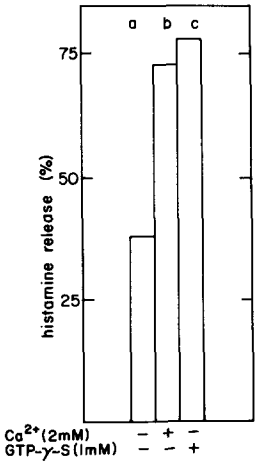

Figure 7. Effect of Ca^{2+} and GTP-γ-S on 48/80-induced histamine secretion from Ca^{2+}-depleted cells. Purified mast cells (1×10^6 cells/ml) were incubated for 1 h at 30°C in a divalent cation-free buffer supplemented with 0.1 mM EDTA. The cells were washed and permeabilized in the absence (*a* and *b*) or presence (*c*) of GTP-γ-S (1 mM). After resealing the cells were transferred to tubes containing 48/80 (5 μg/ml) (*a–c*) and Ca^{2+} (2 mM) (*b*). After incubation for 20 min at 30°C the reactions were quenched and histamine was determined. Similar results were obtained on two occasions.

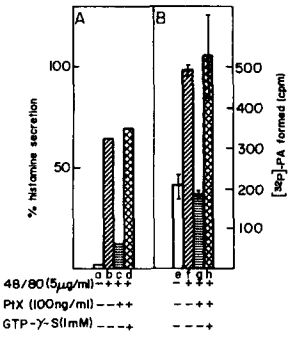

Figure 9. Effect of PtX on 48/80-induced histamine secretion and PA formation. Purified mast cells were [^{32}P]-labeled in the absence (*a*, *b*, *e*, and *f*) or presence (*c*, *d*, *g*, and *h*) of PtX. Cells were permeabilized in the absence (*a–c*, *e*, and *g*) or presence (*d* and *h*) of GTP-γ-S (1 mM). The cells were subsequently transferred to Ca^{2+}-containing buffers in the absence (*a* and *e*) or presence of 48/80 (5 μg/ml) (*b–d*, *f–h*). After 20 min incubation at 30°C, the reactions were quenched and histamine and [^{32}P]-PA were determined as described in Materials and Methods. Mean values of duplicate determinations are presented. Similar results were obtained on five occasions.

pleted of Ca^{2+}, by incubating them for 1 h in the presence of EDTA (0.1 mM) before their permeabilization and resealing. In agreement with previous data (6), these conditions resulted in inhibition of 48/80-induced release that could be recovered by the addition of external Ca^{2+} (Fig. 7, lanes *a* and *b*). Secretion of histamine could also be restored, in the absence of external Ca^{2+}, by introducing 1 mM GTP-γ-S into the cells (Fig. 7, lane *c*). Thus, these results further demonstrate that the introduction of GTP-γ-S bypasses the requirement for a rise in the intracellular concentration of Ca^{2+} for 48/80-induced release.

The stimulatory action of 48/80 on histamine secretion from 1 mM GTP-γ-S loaded cells could not be duplicated by activating PKC by 100 ng/ml TPA or by increasing the internal Ca^{2+} concentrations by means of Ca^{2+} ionophore (A23187, 4 μM) or introduction of 1 mM 2,3 DPG (Fig. 8). These experiments establish that 48/80 synergizes with GTP-γ-S to stimulate secretion in a mechanism that bypasses PLC activation and suggest that 48/80 can trigger secretion by activating a G-protein, that is located at a step distal to PLC. A possible candidate is the previously suggested G_E protein (1, 15).

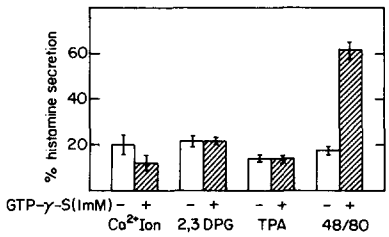

Figure 8. Modulation of GTP-γ-S-dependent secretion. Purified mast cells (1×10^6 cells/ml) permeabilized in the absence or presence of 1 mM GTP-γ-S or 2,3 DPG, as indicated, were resealed and further incubated for 20 min at 30°C in a Ca^{2+}-containing buffer in the presence of either the Ca^{2+} ionophore A23187 (4 μM), TPA (100 ng/ml), or 48/80 (1 μg/ml). At the end of the incubation period, the reaction was quenched, the cells were centrifuged, and histamine secretion was determined. The mean values of duplicate determinations are presented. Similar results were obtained on three occasions.

Effect of PtX on 48/80-activated Responses by Control and GTP-γ-S-loaded Cells

In an attempt to determine whether G_E is a PtX substrate, we compared the PtX sensitivity of 48/80-induced release from control cells and cells loaded with 1 mM GTP-γ-S. Histamine release from permeabilized and resealed cells, induced by an optimal dose of 48/80 (5 μg/ml) was significantly inhibited (>80%) after a 2-h treatment with PtX (100 ng/ml) (Fig. 9, compare lane *c* with lane *b*). In contrast, histamine secretion from cells that were loaded with 1 mM GTP-γ-S and triggered with 48/80 (5 μg/ml) was insensitive to PtX treatment (Fig. 9, lane *d*). However, in a similar manner, PA formation in response to 48/80 (5 μg/ml) that was highly sensitive to PtX in permeabilized and resealed cells (Fig. 9, compare lane *g* with lane *f*), became PtX insensitive in GTP-γ-S-loaded cells (Fig. 9, lane *h*). These results, therefore, reveal that GTP-γ-S is able to relieve inhibition by PtX by dissociating the ADP-ribosylated G protein (16). A lack of sensitivity to PtX of any biochemical process, as determined in the presence of GTP-γ-S, therefore cannot be taken as an indication for the involvement of a PtX-insensitive G protein in this process (21). In agreement, PA formation and histamine secretion induced by an optimal dose of GTP-γ-S (0.1 mM) are both insensitive to PtX treatment (not shown).

Effect of GTP-γ-S on Mastoparan-induced Secretion

48/80 could activate the G-proteins that are coupled to PLC

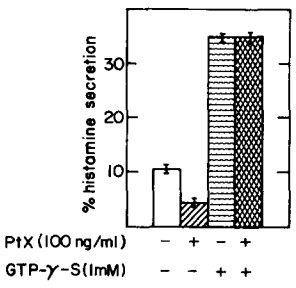

Figure 10. Effect of PtX on mastoparan-induced histamine secretion. Purified mast cells (1×10^6 cells/ml) were incubated for 2 h at 30°C in the absence or presence of PtX (100 ng/ml) as indicated. Cells were subsequently permeabilized in the absence or presence of 1 mM GTP-γ-S. After their transfer to Ca^{2+}-containing tubes, the cells were further incubated for 20 min at 30°C with mastoparan (100 μg/ml). The reaction was stopped, cells centrifuged and histamine determined. Similar results were obtained on three occasions.

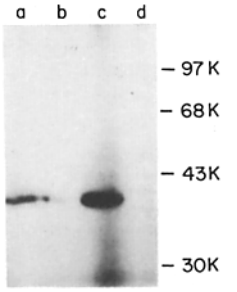

Figure 11. PtX-catalyzed ADP-ribosylation of mast cell proteins. Purified mast cells (5×10^6 cells) were solubilized in 0.125 ml of lysis buffer as described in Materials and Methods. The Triton soluble proteins were separated and subjected to ADP-ribosylation in the absence (*b*) or presence (*a*) of PtX. Rat brain membranes (0.5 mg/ml) were ADP-ribosylated under identical assay conditions in the absence (*d*) or presence (*c*) of PtX. Similar results were obtained on three occasions.

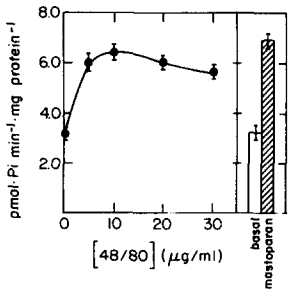

Figure 13. Activation of GTPase activity by 48/80 and mastoparan. Rat brain membranes (5 mg/ml) were solubilized with 1% cholate as described in Materials and Methods. 20-μg aliquots were incubated with the indicated concentrations of 48/80 or 150 μg/ml of mastoparan and GTPase activity was determined as described in Materials and Methods. The data points indicate means of duplicate determinations. Similar results were obtained on five occasions.

(G_P) and to exocytosis (G_E) by binding to 48/80 receptor subtypes that couple independently to G_P and G_E (26). However, receptors for polybasic substances, including 48/80, have not yet been definitively identified. Alternatively, 48/80 may be capable of directly activating G proteins in a receptor-independent manner. Mastoparan, another basic secretagogue of rat peritoneal mast cells (24), has been previously shown to directly activate G proteins and stimulate their GTPase activity (14). Therefore, we examined whether mastoparan can mimic the action of 48/80 and activate secretion from GTP-γ-S (1 mM)-loaded cells. As shown in Fig. 10, 100 μg/ml mastoparan synergized with 1 mM GTP-γ-S. While cells that were permeabilized and resealed in the absence of GTP-γ-S showed only a limited response to mastoparan, GTP-γ-S (1 mM) -loaded cells exhibited a fourfold higher response. Thus, like 48/80, mastoparan-induced secretion was inhibited by the dilution of the internal GTP concentration during the permeabilization period and this response was restored by the application of 1 mM GTP-γ-S. Moreover, like 48/80, the PtX-sensitivity of mastoparan-induced secretion was eliminated in GTP-γ-S-loaded cells (Fig. 10).

Analysis of Mast Cell G Proteins

The sensitivity to PtX of histamine secretion, induced by basic secretagogues, implicates the involvement of G proteins, that serve as PtX substrates, in the control of mast cells exocytosis. In an attempt to identify these putative G proteins, the profile of proteins modified by toxin catalyzed ADP-ribosylation was studied.

Mast cells homogenate was solubilized in Triton X-100 (1%) and the soluble material, depleted of endogenous NAD, was subjected to ADP-ribosylation in the absence or presence of PtX. As illustrated in Fig. 11, PtX catalyzed the ADP-ribosylation of a 40-kD protein that was present in the Triton-soluble fraction (compare lane *a* with lane *b*). This protein comigrated on the SDS gel with the PtX substrates present in rat brain membranes (compare lane *c* with lane *d*). The Triton-soluble fraction of mast cells also contains a higher molecular weight protein (~115 kD), that is, however, weakly ADP-ribosylated in a toxin-independent manner (Fig. 11, lanes *a* and *b*).

That mast cells indeed contain G_i-like proteins, was further supported by the immunoblot analysis presented in Fig. 12. A 40-kD protein present in the detergent-soluble fraction of mast cells was recognized by antibodies directed against a common sequence in the GTP-binding site as well as by antibodies directed against a decapeptide that corresponds to the COOH-terminal ends of the alpha subunits of transducin (TD) and G_i-α (one amino acid substitution) (Fig. 12, lanes *d* and *h*, respectively). No immunoreactive protein was detected in the detergent-insoluble fraction (lanes *c* and *g*, respectively). As expected, these antibodies also bound to TD-α present in rod outer segment membranes (lanes *a* and *e*) and to G_i-α in rat brain membranes (lanes *b* and *f*).

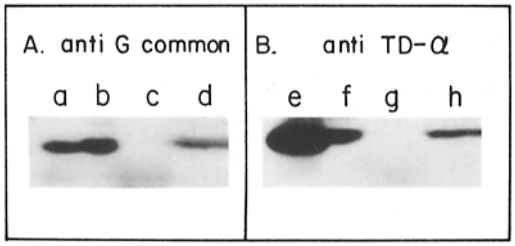

Figure 12. Immunoblot analysis with anti-G protein antibodies. Purified mast cells (5×10^6 cells) were solubilized in 0.125 ml of a lysis buffer described in Materials and Methods, and separated into detergent-soluble and -insoluble material by centrifugation. Rod outer segment membranes (10 μg) (lanes *a* and *e*), rat brain membranes (80 μg) (lanes *b* and *f*) and mast cell detergent insoluble (lanes *c* and *g*) and soluble (lanes *d* and *h*) material were analyzed by the immunoblotting protocol described in Materials and Methods. The immunoreactive proteins were visualized after reaction with a 1:100 dilution of anti G common antibodies (lanes *a–d*) or 5 μg/ml anti-TD-α antibodies (lanes *e–h*).

Effects of Mastoparan and 48/80 on GTPase Activity of Rat Brain Membranes

Because 48/80 and mastoparan seem to share a common

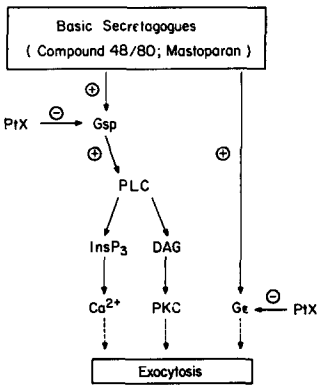

Figure 14. Model of stimulus-secretion coupling in mast cells. The model assumes that exocytosis of mast cells is induced by the synergistic interaction of Ca^{2+}, PKC, and the activated G protein G_E. Basic secretagogues stimulate secretion by activating directly PtX-sensitive G proteins that couple to PLC (Gsp) and exocytosis (G_E).

mechanism of activating histamine secretion, we examined the possibility that 48/80, like mastoparan, may be capable of directly activating the GTPase activity of G proteins. For this purpose, we used brain membranes enriched in G_i/G_o proteins as a model, and measured the GTPase activity of both intact and cholate-solubilized membranes in the presence or absence of either mastoparan (taken as a positive control) or 48/80. Consistent with previous results (14), mastoparan (150 μg/ml) stimulated the GTPase activity of intact rat brain membranes by 3.5-fold (not shown). Under these conditions, 48/80 (5 μg/ml) had no effect on GTPase activity (not shown). In contrast, both 48/80 and mastoparan stimulated twofold the GTPase activity of cholate-solubilized membranes (Fig. 13). This effect of 48/80 was dose-dependent, with the half-maximal effect obtained at 2.5 μg/ml and maximal effect at 10 μg/ml (Fig. 13). These results strongly suggest that 48/80 can directly activate G proteins. However, it appears that the lipid composition of the membranes determines its accessibility to the G-protein (Aridor, M., L. Traub, and R. Sagi-Eisenberg, unpublished results).

Discussion

Permeabilization of mast cells by ATP has proven to be a powerful technique for introducing impermeable solutes into the interior of cells (12). A special aspect of this technique is in the fact that it bypasses the requirement for applying detergents that result in permanent permeabilization. In contrast, after the ATP permeabilization period, the cells can be resealed and their responsiveness to external agonists is retained.

We have used this technique to examine the role of G proteins in the regulation of histamine secretion from mast cells. In agreement with previous data (11), the introduction into the cytosol of mast cells of a nonhydrolyzable analogue of GTP, GTP-γ-S, at concentrations <0.1 mM, led to histamine secretion (Fig. 1). We demonstrated that this release was associated with the production of PA (Fig. 1). Moreover, PA formation, induced by GTP-γ-S, was completely blocked by neomycin (Fig. 3). This implies that it was the consequence of phosphoinositide breakdown and therefore, most probably reflects the activation of a phosphoinositide (PIP_2) hydrolyzing PLC by a G protein. Indeed, both PA formation and histamine secretion could be blocked by the simultaneous entrapment of GDP-β-S in the cells (Fig. 1).

The effect of GTP-γ-S was, however, biphasic and at high concentrations it neither stimulated PA formation nor histamine secretion (Fig. 4). The inhibitory phase was not due to elevated cAMP levels as cAMP had no effect on the release induced by an optimal (0.1 mM) concentration of GTP-γ-S. The reason for the bell-shaped dependency on GTP-γ-S concentrations of both the PA response and histamine secretion is presently unknown. This observation may suggest, although it does not prove, that the PIP_2-hydrolyzing PLC in mast cells is dually regulated by stimulatory and inhibitory G proteins. In other cellular systems the existence of a PLC-inhibiting G protein has been indicated (23).

Previous studies have indicated the involvement of a G protein (G_E) located distal to PLC in exocytosis of mast cells (15). G_E could, in principle, be activated by any of the second messengers formed after PLC activation and PIP_2 breakdown. In the presence of 1 mM GTP-γ-S, conditions that result in inhibition of PLC, the activation of G_E may be abolished thereby resulting in inhibition of secretion. However, elevation of the internal Ca^{2+} concentrations or activation of PKC failed to increase the effective affinity of this putative G_E protein for GTP-γ-S to facilitate secretion in cells loaded with 1 mM of GTP-γ-S (Fig. 8).

In contrast to agonists, that act by elevating internal Ca^{2+} concentrations or by activating PKC, the two basic secretagogues of mast cells, compound 48/80 and mastoparan, taken at concentrations that by themselves are ineffective, synergized with 1 mM GTP-γ-S to induce exocytosis (Figs. 5 *A* and 10). Moreover, 48/80 also potentiated histamine secretion at concentrations that did not stimulate PA formation (Fig. 5 *B*). This suggests that 48/80 acts at a step distal to PLC. This is further supported by the finding that 48/80 could stimulate secretion from Ca^{2+}-depleted cells replacing the need for Ca^{2+} (Fig. 7). These results are consistent with a recent report demonstrating that persistent activation of PKC by TPA inhibits 48/80-induced Ca^{2+} transients without affecting the secretory response (26). Hence, like the IgE-receptor (32, 36), 48/80 may also activate an additional signal that can synergize with PKC to elicit histamine secretion in the absence of Ca^{2+} transients. The strong dependency of 48/80-induced secretion on the internal application of GTP, as evidenced by the washout phenomena in dialyzed mast cells (27) and by the effects of GTP-γ-S on the effective affinity of the cells for 48/80 (Fig. 5 *A*), suggests that the additional signal involves a G-protein that is located downstream from PLC (see model, Fig. 14).

The polybasic secretagogues of mast cells appear to release histamine by a common mechanism (35, 37). Most share similar structural features, that are essential for their activity. These include positive charges associated with a hydrophobic structure such as the COOH-terminus of substance P or the aromatic rings of 48/80. However, there is no evidence for the presence of specific receptors in mast cells for most of these ligands. In fact, it has been questioned recently whether their action involves binding to "classical" cell surface receptors at all (30, 31). The finding that mastoparan activates G-proteins directly (14) raises the intriguing possibility that histamine secretion induced by polybasic molecules also results from their direct interaction and subsequent activation of G proteins. Indeed, the cellular response to both mastoparan and 48/80 was markedly reduced after depletion of GTP during the permeabilization period, and both were capable of synergizing with 1 mM GTP-γ-S to elicit secretion (Figs. 5 and 10). Furthermore, 48/80, like mastoparan, activated the GTPase activity of cholate-solubilized rat brain membranes (Fig. 13). As this activation occurs in the presence of detergent, under conditions that do not facilitate receptor–G protein interactions (10), it appears that 48/80 directly activates G proteins present in this preparation. Hence, based on these studies, we suggest that the activation of mast cells by peptides and other polybasic molecules involves the insertion of their hydrophobic moiety into the membrane. This then enables the positively charged domain to interact and directly activate G proteins in a receptor-independent manner. Indeed, spectroscopic studies on binding properties of spin-labeled 48/80 have indicated that 48/80 does not transverse the cell membrane but it does penetrate to a point where it is not exposed to the external aqueous environment (25).

Our findings also imply that these ligands can interact with more than one G protein in the signal transduction cascade yet with different dose response relationships. As they directly activate the putative G_E protein, bypassing PLC activation, their sensitivity to PtX indicates that G_E is also a PtX substrate (see model, Fig. 14). We further demonstrated that the internal application of 1 mM GTP-γ-S reversed the PtX inhibition of both 48/80 and mastoparan induced release (Figs. 9 and 10). These results demonstrate that GTP-γ-S is able to dissociate the ADP-ribosylated G protein and that the dissociated but ADP-ribosylated G_E is still functionally active. We, therefore, predict that G_E may be a member of the G_i G protein family. Indeed, subjection of mast cell proteins to PtX-catalyzed ADP-ribosylation reveals the presence of a substrate or substrates in the 40-kD range (Fig. 11). A protein of a similar molecular weight also reacts on immunoblots with an antisera that recognizes G_i-α (Fig. 12). Moreover, a 40-kD protein seems to be the only protein that is recognized by antibodies directed against a common sequence within the GTP-binding site of G-proteins (Fig. 12). Thus, although these observations do not exclude the involvement of a yet unidentified G protein in the control of exocytosis, they are consistent with the proposed model.

It is presently unknown by what mechanism the receptor for IgE activates G_E. Clearly, the receptor for IgE activates multiple signaling pathways (26), where activation of G_E represents one of them. Hence, basic secretagogues, that may activate G_E directly should serve as useful tools in studies aimed at identifying G_E and the cellular targets for its action.

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