

Two G-Proteins Act in Series to Control Stimulus-Secretion Coupling in Mast Cells: Use of Neomycin to Distinguish between G-Proteins Controlling Polyphosphoinositide Phosphodiesterase and Exocytosis

Shamshad Cockcroft, Tim W. Howell, and Bastien D. Gomperts

Department of Experimental Pathology, University College London, London WC1E 6JJ, United Kingdom

Abstract. Provision of GTP (or other nucleotides capable of acting as ligands for activation of G-proteins) together with Ca^{2+} (at micromolar concentrations) is both necessary and sufficient to stimulate exocytotic secretion from mast cells permeabilized with streptolysin-O. GTP and its analogues, through their interactions with G_p , also activate polyphosphoinositide-phosphodiesterase (PPI-pde generating inositol 1,4,5-trisphosphate and diglyceride [DG]). We have used mast cells labeled with [^3H]inositol to test whether the requirement for GTP in exocytosis is an expression of G_p activity through the generation of DG and consequent activation of protein kinase C, or whether

GTP is required at a later stage in the stimulus secretion sequence. Neomycin (0.3 mM) inhibits activation of PPI-pde, but maximal secretion due to optimal concentrations of guanosine 5'-O-(3-thiotriphosphate) (GTP- γ -S) can still be evoked in its presence. When ATP is also provided the concentration requirement for GTP- γ -S in support of exocytosis is reduced. This sparing effect of ATP is nullified when the PPI-pde reaction is inhibited by neomycin. We argue that the sparing effect of ATP occurs as a result of enhancement of DG production and through its action as a phosphoryl donor in the reactions catalyzed by protein kinase C.

WE have shown that rat mast cells permeabilized with streptolysin-O undergo exocytotic secretion when provided with micromolar concentrations of Ca^{2+} together with GTP, xanthosine triphosphate, or inosine triphosphate (18, 19). These nucleotides are capable of acting as ligands for activation of G-proteins (5, 33). The combination of Ca^{2+} + GTP is both necessary and sufficient for secretion to occur. There is no absolute requirement for the presence of any other water soluble metabolite though ATP has a sparing effect on the concentration requirements for both the Ca^{2+} and the GTP. Since polyphosphoinositide-phosphodiesterase (PPI-pde),¹ the enzyme that generates the second messengers inositol 1,4,5-trisphosphate and diglyceride (DG), is subject to control by a GTP-binding protein, G_p (9, 24, 26, 31), this represents one possible site of action whereby the effect of guanine nucleotides might act to control secretion in the permeabilized cells. It is now well understood that the water soluble product of PPI-pde activation, inositol 1,4,5-trisphosphate, mobilizes Ca^{2+} from intracellular stores. DG is the activator of protein kinase C (4, 27). However, the existence of G_p , and a defined role for GTP in receptor activation mechanisms does not preclude

the possibility that GTP might have other roles in the complete sequence of events which leads to exocytotic release of secretory materials.

In the experiments reported here we have used [^3H]inositol-labeled mast cells permeabilized with streptolysin-O to follow the effects of Ca^{2+} and guanosine 5'-O-(3-thiotriphosphate) (GTP- γ -S) on both PPI-pde activation (by measurement of released [^3H]inositol phosphates [IPs]) and on secretion (release of histamine). We have used neomycin, an aminoglycoside antibiotic that is known to inhibit PPI-pde (11, 30) to distinguish the effects of guanine nucleotides on PPI-pde activation and exocytosis. We find that guanosine 5'-O-(3-thiotriphosphate) (GTP- γ -S, a non-hydrolysable analogue of GTP) can still stimulate exocytosis under conditions where PPI-pde is fully inhibited by neomycin. This suggests that GTP (and hence a G-protein, G_E [14, 15]) is involved at a late stage in the stimulus-secretion process.

Materials and Methods

Labeling of Mast Cells with [^3H]Inositol

Peritoneal washings of eight rats were filtered through nylon gauze to remove debris. The mast cells were then isolated by centrifugation through four 2-ml cushions of 35% (wt/vol) BSA (Path-O-Cyte 4; Miles Laboratories, UK) as previously described (19) and then combined, resuspended in 50 ml of sterile RPMI (1640) and washed by centrifugation. They were

1. *Abbreviations used in this paper:* DG, diglyceride; GTP- γ -S, guanosine 5'-O-(3-thiotriphosphate); IP, inositol phosphate; pde, phosphodiesterase; PIP, phosphatidylinositol monophosphate; PPI, polyphosphoinositide.

resuspended in 8-ml of Medium 199 (containing penicillin [50 IU/ml] and streptomycin [50 µg/ml]; in some experiments gentamycin [50 µg/ml] was also included). 200 µCi of [³H]inositol was added and the cells plated out in four 35-mm plastic petri dishes (Falcon Plasticware; Becton-Dickinson, Oxford, UK) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 18 h the cells were examined: their appearance was normal; spontaneous degranulation was never observed and compound 48/80 evoked a normal secretory response. The cells were resuspended by agitation with a buffer (for details of composition see below: LiCl was omitted at this stage) supplemented with 0.5 mM EDTA. External isotope and EDTA were removed by washing twice by centrifugation and the cells were finally resuspended at $\sim 5 \times 10^6$ ml⁻¹. LiCl (10 mM) which inhibits inositol phosphatase (1, 16) was added and the cells incubated for 30 min at 37°C.

Calcium Buffers and Nucleotides

Ca²⁺ was buffered at concentrations between 10⁻⁷ and 10⁻⁵ M (pCa7 to pCa5), and Mg²⁺ was set at 2 mM by the use of appropriate EGTA buffers which were prepared as previously described (3). The maximum error due to varying the concentration of Mg-ATP in the range 0–5 mM was <0.02 pCa. Nucleotides were prepared as 50 or 100 mM stocks at pH 6.8 and stored frozen at -20°C.

Cell Permeabilization and Stimulation

For measurement of secretion and generation of IPs cells were suspended in a buffered salt solution (pH 6.8) which comprised 137 mM-NaCl, 2.7 mM-KCl, 10 mM-LiCl, 20 mM piperazine-N,N'-bis(2-ethanesulphonate), 5.6 mM-glucose, 1 mg/ml BSA, and 25 µM EGTA. Typically 50 µl of the labeled cells were added to 4 vol of a mixture containing streptolysin-O (to give a final concentration of 0.4 IU/ml), CaEGTA (3 mM EGTA final concentration), and nucleotides and neomycin as detailed in the text and figure legends. After incubation for 10 min at 37°C the cells were sedimented by centrifugation (5 min, 300 g at 4°C). A sample (typically 25 µl) of supernatant was removed and diluted to 1 ml with PBS (pH 7) for determination of secreted histamine as previously described (18).

IP Determinations

To the remaining supernatant sufficient chloroform, methanol and water were added such that the final proportions were 0.5:1.0:0.4 in a single-phase mixture. A two-phase system was then produced by addition of a further 0.5 ml each of water and chloroform. The IPs, present in the aqueous phase, were separated from free inositol and glycerophosphoinositol by passage through Dowex 1 × 8 anion exchange resin. Typically, 1.4 ml were loaded on to short columns contained within Pasteur pipettes. Free inositol was washed off with 6 ml of water and glycerophosphoinositol was removed with 4 ml of 60 mM sodium tetraborate/5 mM sodium formate. Individual IPs were then sequentially eluted by adding increasing concentrations of ammonium formate in 0.1 M formic acid: 0.2 M for inositol monophosphates (IP₁), 0.4 M for inositol bisphosphates (IP₂), and 1 M for inositol trisphosphates (IP₃). Measurement of total IPs was established as a sufficient indicator of PPI-pde activation, so that in most experiments it was only necessary to elute with 1 M ammonium formate/0.1 M formic acid (after removal of free inositol and glycerophosphoinositol).

For the time-course experiment (Fig. 2), 100-µl samples were withdrawn from bulk incubations at specified times and quenched by adding to 2 ml of ice-cold PBS (pH 7). After centrifugation as described above, 200 µl of the supernatant were removed for measurement of secreted histamine. The remainder was loaded on to the Dowex columns for analysis of IPs. It was established that >95% of the IPs were released from the permeabilized cells and were present in the supernatant.

Although neomycin interferes with the method of histamine determination when present at high concentrations, it has no effect when present below 30 µM in the final histamine assay. Since we routinely used an aliquot of 25 µl diluted to 1 ml, the concentration of neomycin never exceeded 10 µM at this stage. To exclude the possibility that neomycin interferes with the binding of IPs to the Dowex columns, we established that addition of neomycin at the end of an experiment was without effect on GTP-γ-S stimulated IP production (results not shown).

Analysis of Inositol Lipids

In those experiments where the inositol lipids were analyzed, the chloroform layer and the protein interface were re-extracted under acid conditions as described previously (10). Carrier PPI was also added at this stage. The

lipids were analyzed by TLC as described previously (9, 10) except that they were identified by brief staining with I₂ vapor.

Results

Fig. 1 illustrates the dependence on Ca²⁺ of secretion and IP production due to introduction of GTP-γ-S (a non-hydrolyzable analogue of GTP) into the permeabilized mast cells. In confirmation of previous results, secretion due to GTP-γ-S is strictly dependent on the presence of Ca²⁺ (in the range pCa 7–5). By comparison, activation of PPI-pde due to GTP-γ-S is well established at pCa 7 and is only slightly increased when Ca²⁺ is raised to 10 µM. This result is very similar to that reported for human neutrophils in

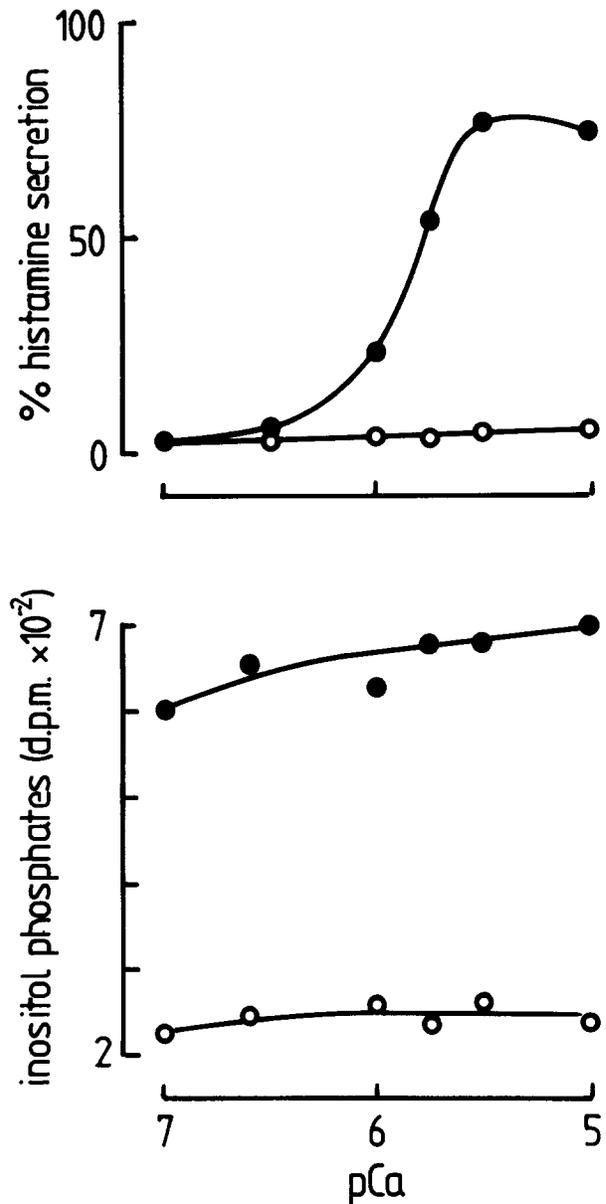


Figure 1. Ca²⁺ dependence of histamine secretion and PPI-pde activation. Mast cells were permeabilized with streptolysin-O (0.4 IU/ml) in the presence of 3 mM CaEGTA buffer (to maintain pCa 5) in either the presence (closed symbols) or absence (open symbols) of 20 µM-GTP-γ-S.

which maximal activation (also measured in the absence of ATP) of PPI-pde by GTP- γ -S occurs at pCa 7 (7).

The time course of secretion and IP production due to GTP- γ -S is shown in Fig. 2. Secretion commences after a lag of 30 s and takes 2–3 min for completion. (This delay is due to the time required for permeabilization; we have since shown that secretion occurs promptly when 10 μ M GTP- γ -S is added to trigger cells pretreated with streptolysin-O [2 min] in the presence of Ca²⁺ [our unpublished results].) Release of IP₃ is also apparent at 30 s. This increases for 2 min after which the levels slowly begin to decline such that at 5 min the IP₃ is \sim 80% of the maximal level. An increase in IP₂ occurs with a similar time-course but the extent of the increase is much less than that of IP₃. Changes in IP₁ were small and not always observed (data not shown). In the absence of GTP- γ -S there was no time-dependent increase in the level of IP₂ and IP₃.

Although PPI-pde activation is not always accompanied by secretion (see Fig. 1), the experiments so far presented indicate that secretion might always be accompanied by activation of PPI-pde. For this reason it remains possible that the activation of G_p is essential for secretion to occur. We addressed this question by using neomycin to inhibit PPI-pde activation. The experiment illustrated in Fig. 3 indicates that neomycin can effectively block PPI-pde activation at concentrations above 10⁻⁴ M whilst secretion due to an optimal concentration of GTP- γ -S is only marginally affected. Fig. 4 compares the dependence on GTP- γ -S of secretion and

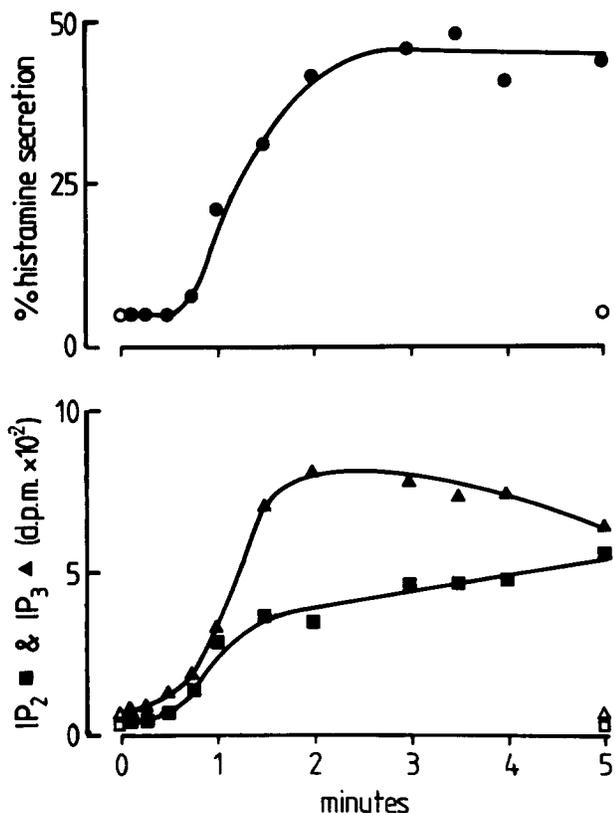


Figure 2. Time-course of histamine secretion and IP generation. Mast cells were permeabilized in the presence of GTP- γ -S (20 μ M) and Ca²⁺ (pCa5). Samples were removed at the times indicated. (\blacktriangle) IP₃; (\blacksquare) IP₂. Open symbols indicate absence of GTP- γ -S.

PPI-pde activation in the presence and absence of neomycin (300 μ M). While neomycin modulates exocytosis by shifting the requirement for GTP- γ -S to concentrations higher than normal, it totally suppresses IP production at all concentrations of GTP- γ -S.

We previously showed that the effective affinity for GTP- γ -S in the secretory reaction is enhanced in the presence of ATP (19). Since ATP is also a substrate for the inositol lipid kinases (10), we tested the effect of ATP on the GTP- γ -S requirement for PPI-pde activation. In the presence of ATP there is an increase in the cellular content of PIP and phosphatidylinositol bisphosphate (PIP₂); this occurs at the expense of PI. In cells permeabilized in the absence of ATP the ratio of PI/PIP/PIP₂ was 92.6:5.7:1.6; in its presence the ratio changed to 82:14:4. This increase in the PPIs (>2 \times fold) causes an increment in both the basal and the stimulated IP formation. The concentration of GTP- γ -S causing half maximal production of IPs is 1–2 μ M ($n = 4$) in the absence of ATP and 0.3–0.5 μ M ($n = 2$) in its presence.

While these experiments establish that secretion induced by GTP- γ -S can occur without detectable activation of PPI-pde when neomycin is provided, it is clear that neomycin is

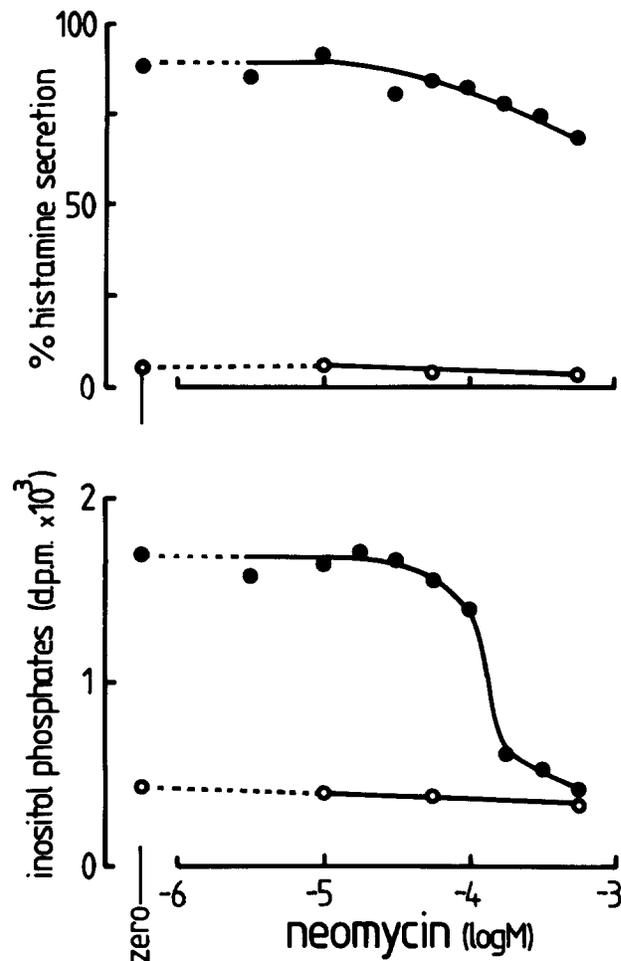


Figure 3. Effect of neomycin on histamine secretion and PPI-pde activation. Mast cells were permeabilized in the presence of Ca²⁺ (pCa5) and various concentrations of neomycin, either in the presence (closed symbols) or absence (open symbols) of 20 μ M GTP- γ -S.

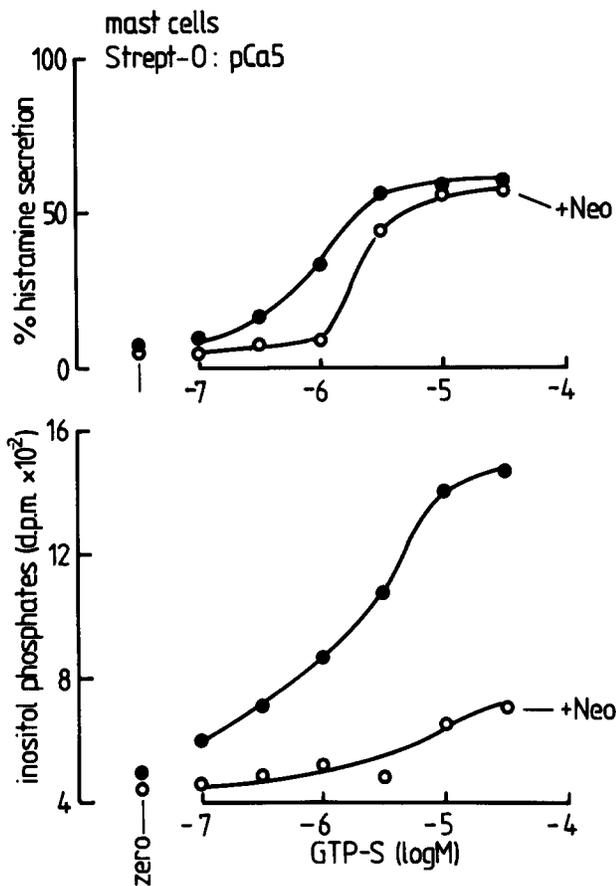


Figure 4. Effect of neomycin on the dependence of GTP- γ -S of histamine secretion and PPI-pde activation. Mast cells were permeabilized in the presence of Ca^{2+} (pCa5) and various concentrations of GTP- γ -S in either the absence (closed symbols) or presence (open symbols) of 0.3 mM-neomycin.

nonetheless capable of modulating the secretory response (Fig. 4). If the effect of ATP in sparing the concentration of GTP- γ -S required to cause secretion is due to enhanced generation of the products of the PPI-pde reaction, then we could expect that neomycin would reverse this. Fig. 5 illustrates the effects of neomycin on the dependence on GTP- γ -S of both secretion and IP production in the presence of ATP. When neomycin is present the dependence on GTP- γ -S for secretion shifts to higher concentrations (Fig. 5). In contrast, the effect of neomycin on PPI-pde activation remains simply that of an inhibitor preventing the formation of IPs.

Discussion

We have shown that GTP and its analogues are able to cause secretion from permeabilized mast cells and neutrophils (2, 19). Agonist-induced secretion from both these cell types is closely linked to the reactions which lead to Ca^{2+} mobilization and activation of protein kinase C through activation of PPI-pde. We and others have shown that GTP and its analogues can activate this enzyme when applied to both isolated membranes and permeabilized cells by interaction at a G-protein which we have called G_P (9, 24, 26, 31). The purpose of the experiments presented in this paper has been to find out whether the effects of GTP analogues on secretion are

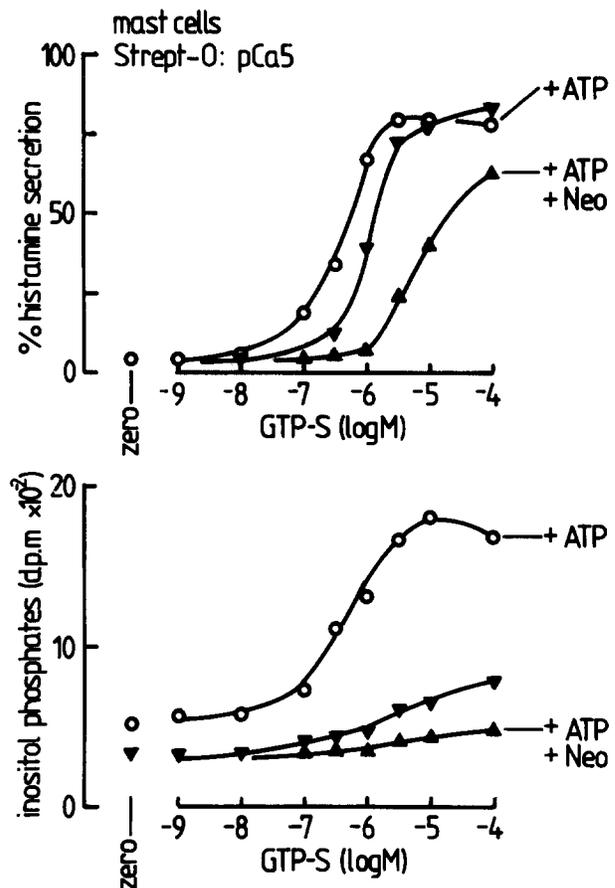


Figure 5. Effect of ATP and neomycin on the dependence of GTP- γ -S of histamine secretion and PPI-pde activation. Mast cells were permeabilized in the presence of Ca^{2+} (pCa5) and various concentrations of GTP- γ -S. Control (\blacktriangledown); plus ATP (1 mM) (\circ); plus ATP (1 mM) and neomycin (0.3 mM) (\blacktriangle).

due exclusively to the activity of G_P linked events, or whether two distinct G-proteins (G_P and G_E) acting in series, are involved (14, 15).

Secretion and IP production can both be activated by GTP- γ -S in the absence of added phosphorylating nucleotide. For both functions, Ca^{2+} must be present but the concentrations of Ca^{2+} which are required are very different. Secretion typically requires Ca^{2+} at \approx pCa6 (though this shifts to \approx pCa6.4 when ATP is present [19]). Activation of PPI-pde is almost fully satisfied by Ca^{2+} at pCa 7 (7) and at this concentration histamine secretion is scarcely detectable. This result suggests that under normal conditions (i.e., in intact cells) with Ca^{2+} at its resting level of pCa 7, activation of PPI-pde is essential to raise the cytosol Ca^{2+} to levels commensurate with stimulation of exocytosis.

The strongest separation between the two GTP- γ -S induced functions, amounting to a clear demonstration that they are mediated by distinct G-proteins, comes from the use of neomycin to block the PPI-pde activity (11, 30). Neomycin is understood to prevent the action of PPI-pde by binding to the polar head groups of the PPIs (29). We have found that secretion can be fully maintained in the presence of neomycin at concentrations which inhibit PPI-pde. The only effect of neomycin that we have been able to discern on the secre-

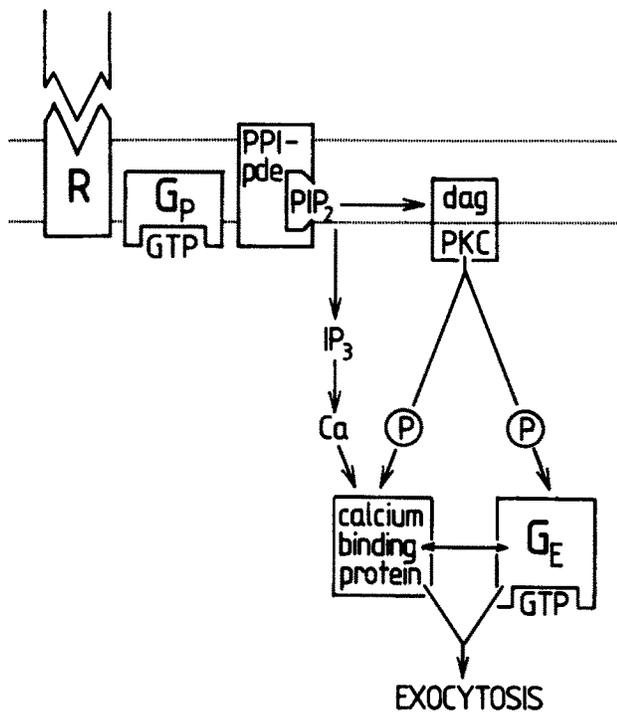


Figure 6 Schematic representation of the interrelationships between G_p and G_E in the stimulus secretion sequence.

tory reaction is that higher concentrations of GTP- γ -S are required to induce an equivalent extent of secretion.

These results do not conflict with our previous demonstration that neomycin inhibits secretion when it is included alongside GTP- γ -S trapped in mast cells which have been transiently permeabilized (by the use of ATP⁴⁻) (9). The two experiments are totally different. In permeabilized-resealed cells (which are intact and remain responsive to stimulation through the IgE receptor system [13]), it is necessary to activate PPI-pde to generate IP₃ to mobilize intracellular Ca²⁺ while in the present experimental system the requirement for IP₃ is obviated by the use of Ca⁺ buffers. Here, we are only concerned with the generation of DG and consequent events mediated by protein kinase C.

We previously reported that ATP spares the concentration requirement for guanine nucleotides in the secretory reaction. It also substantially increases the production of IPs after PPI-pde activation by GTP- γ -S and it is possible that these two observations are linked; i.e., the effect of ATP in reducing the concentration of GTP- γ -S required for histamine secretion may occur as a consequence of PPI-pde activation. If this is the case then activation of protein kinase C is implicated. Support for this idea comes from the observation that the sparing effect of ATP on GTP- γ -S concentration for secretion is not manifest when neomycin is present. Indeed, neomycin shifts the dependence of GTP- γ -S to slightly higher concentrations in both the presence and the absence of ATP.

A schematic representation of possible inter-relationships between the two sites of action of GTP- γ -S is shown in Fig. 6. G_p and G_E act in series in the regulation of the reactions which culminate in exocytosis. Early events involving the binding of agonists to receptors, leading to production of DG

and IP₃ through the mediation of G_p are now widely acknowledged (8). In the intact cell IP₃ (and its subsequent metabolites such as IP₄) mediate the rise in cytosol Ca²⁺. In the experiments described here the role of IP₃ (which leaks from the permeabilized cells) in mobilizing Ca²⁺ from intracellular stores is substituted by provision of Ca/EGTA buffers. The other product of PPI-pde activation, DG, will only be able to stimulate protein kinase C-dependent phosphorylation when ATP is provided.

Here we are concerned with the late events involving GTP (and its analogues), Ca²⁺, the effects of ATP, and the role of protein kinase C activation on mast cell exocytosis. Guanine nucleotides, by their interaction with G_E , act synergistically with Ca²⁺ to cause histamine secretion in a manner not requiring generation of DG. However, under conditions in which DG is formed, and in the presence of ATP, then the effective affinities for both Ca²⁺ and GTP- γ -S are enhanced (19). The inference is that while phosphorylation is not an essential event in the exocytotic process, phosphorylation probably by protein kinase C does modulate the affinity for GTP- γ -S and Ca²⁺.

Is it pertinent to ask whether G_E controls exocytosis in intact mast cells stimulated by physiological agonists? There is just one hint that it might do so. Mast cells cultured for prolonged periods in the presence of ribavirin, a guanosine analogue which selectively suppresses intracellular GTP levels (20), lose the ability to respond to stimulation by Ca²⁺ ionophores (25).

For secretion of serotonin from platelets (permeabilized by high voltage discharge) (17, 22, 23) GTP appears to act simply through G_p since its effects closely parallel those of thrombin or phorbol ester (an activator of protein kinase C). In rabbit neutrophils (permeabilized with Sendai virus) secretion of β -glucuronidase (a marker of azurophilic granules) can be stimulated by guanine nucleotides at <10⁻¹⁰ M Ca²⁺ (2). Under these conditions, activation of PPI-pde by guanine nucleotides does not occur (7) and so GTP must act at a site other than G_p . GTP- (analogue) induced Ca²⁺ independent secretion also occurs from RINm5F cells (release of insulin) (32) and bovine adrenal medullary chromaffin cells (6) (but see reference 21 for an alternative view). In parathyroid cells GTP analogues also induce secretion but no data have been presented which would allow the effect to be ascribed to any particular G-protein (12, 28). In these cells, Ca²⁺ is inhibitory to guanine nucleotide-induced secretion.

Ca²⁺, DG, and GTP probably act as intracellular signals to a catalytic process which results in specific fusions between secretory granules and the plasma membrane. The fusogenic entity is likely to be an integral component of either one membrane or the other (or both), since exocytosis can occur in cells extensively depleted of cytosol proteins (18, 19). The exocytotic systems that we have considered here differ amongst themselves; these differences can be regarded as reflections of a wider repertoire of secretory control mechanisms, the nature of which will only become fully apparent when more cells and tissues have been examined by permeabilization techniques.

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