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Nucleotides and Divalent Cations as Effectors and Modulators of Exocytosis in Permeabilized Rat Mast Cells [and Discussion]

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Nucleotides and divalent cations as effectors and modulators of exocytosis in permeabilized rat mast cells

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SUMMARY

The idea that the universal trigger to exocytosis (the terminal step in the secretory process) is an elevation of the cytosol concentration of Ca^{2+}, and that it is dependent on ATP, is no longer tenable. Working with streptolysin-O-permeabilized mast cells (and other myeloid cells) we have shown that non-hydrolysable analogues of GTP can stimulate exocytosis after depletion of Ca^{2+} (i.e. at concentrations below 10^{-9} M) and ATP. Such Ca^{2+}- and ATP-independent exocytosis is strongly dependent on the presence of Mg^{2+}, and the requirement for Mg^{2+} declines as the concentration of Ca^{2+} is brought up to 10^{-7} M. We argue that Ca^{2+} serves to regulate the binding of guanine nucleotides to G_E, a GTP-binding protein that regulates exocytosis through its interaction with C_E, a calcium-binding protein which serves as an intracellular pseudo-receptor. The onset of exocytosis, following provision of Ca^{2+} and guanine nucleotides to the permeabilized cells, is preceded by delays which are sensitive to the order of provision of the two effectors (i.e. Ca^{2+} and guanine nucleotides), the presence or absence of Mg^{2+}, and the identity of the activating guanine nucleotide. In view of the similarity of these features with the activation kinetics of adenylyl cyclase, we argue that G_E behaves as a member of the heterotrimeric class of signal transducing G-proteins such as G_S.

1. INTRODUCTION

If conventional wisdom and the text books are to be believed, then Ca^{2+} must be regarded as the key component in the regulation of exocytotic secretion. Although there are a number of striking exceptions (e.g. the secretion of parathyroid hormone (Sherwood *et al.* 1966; Brown *et al.* 1987; Brown 1991) and renin (Fray *et al.* 1987; Park *et al.* 1986)), it remains true to say that most regulated secretory events are associated with an increase in the cytosol concentration of free calcium; most secretory cells can be stimulated by applying procedures designed to elevate the level of cytosol Ca^{2+} (e.g. Ca^{2+}-carrying ionophores (Gomperts 1984)) and most secretory systems are inhibited by procedures designed to prevent the elevation in cytosol Ca^{2+}. Thus, a place for Ca^{2+} in the regulation of secretion seems assured even if there are very few clues available to indicate what it is that elevation of Ca^{2+} might actually do.

One reason why the Ca^{2+} ion has achieved such prominence in discussions concerning the mechanism of secretion is that it is simply the most visible and the most easily manipulated of the possible intracellular effectors of cell activation processes. Prior to the advent of techniques of selective plasma membrane permeabilization, it was almost impossible to ask questions concerning other messengers which might regulate secretion, either by alterations in their concentration, or by variation in their affinity for specific binding proteins. It is now evident that Ca^{2+} is but one of a number of regulatory effectors.

Certainly, the idea of a unique and pivotal role for Ca^{2+} was never in doubt at an earlier Discussion Meeting of the Royal Society devoted to the Control of Secretion (Gregory *et al.* 1982). This was held just 10 years ago and predated the developments of fluorescence techniques for the on-line measurement of cytosol Ca^{2+} (Tsien *et al.* 1982, 1984*a*, *b*) which have revealed many instances of secretion not accompanied by elevation of cytosol Ca^{2+} (Bruzzone *et al.* 1986; Di Virgilio *et al.* 1984; Pozzan *et al.* 1984). Only a single contribution was devoted to a consideration of the exocytotic event (membrane fusion, the terminal step in the stimulus-secretion pathway). Here it was shown that Ca^{2+}, at concentrations buffered in the micromolar range, could induce the release of catecholamines from permeabilized adrenal chromaffin cells (Baker & Knight 1981). In agreement with the accumulated experience based on the investigation of intact cells, this paper also gave support to the idea that ATP might be an essential component in the exocytotic mechanism.

2. MEASURING EXOCYTOSIS FROM PERMEABILIZED MAST CELLS

Our work concerns the mechanism of secretion in myeloid cells, in particular the mast cells which, following stimulation, release a large number of so-

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called 'mediators' of the acute allergic response. In the resting cells, these are contained within about 1000 membrane-bound granules which dominate the appearance of the cells even at the level of resolution offered by the light microscope. Indeed, in these cells it is a simple matter to assess the stimulation of secretion as the extent of visible degranulation. Most work on secretion has however concentrated on measurement of secreted histamine; latterly we have preferred to measure the release of the lysosomal enzyme hexosaminidase.

To regulate the composition of the cytosol with precision it is necessary to render the plasma membrane permeant, while maintaining the integrity of internal structures. There is a wide range of techniques by which this can be achieved (Gomperts 1990), and we have stressed the importance of understanding the nature of the permability lesions, in terms of their filtrability and duration (Koffer & Gomperts 1989; Tatham & Gomperts 1991). In the work to be described, we have applied streptolysin-O (SL-O), a cholesterol-directed cytolytic protein derived from culture filtrates of *Streptococcus* (Bhakdi & Tranum-Jensen 1985, 1987) which generates lesions in plasma membranes of dimensions exceeding 12 nm in diameter (Buckingham & Duncan 1983). When applied to mast cells, SL-O allows the rapid (less than 5 min) efflux of cytosol proteins (Howell & Gomperts 1987; Koffer & Gomperts 1989), and almost immediate exchange (less than 20 s) of low molecular mass (less than 1000) metabolites and other aqueous solutes (Lillie *et al.* 1991).

In our work we have measured the extent and kinetics of secretion from the permeabilized cells under a wide variety of conditions, concentrating on: (i) Ca^{2+} (buffered in the μM range with EGTA or HEDTA); (ii) Mg^{2+}; (iii) ATP and other possible phosphorylating nucleotides; (iv) GTP and GTP analogues (GTP-γ-S, GppNHp etc); (v) nucleoside diphosphates (ADP, GDP); and (vi) electrolyte composition (NaCl, Na.glutamate).

3. Ca^{2+} AND GUANINE NUCLEOTIDE: ESSENTIAL EFFECTORS

For mast cells permeabilised by SL-O in a simple buffered electrolyte solution (NaCl), it is evident that elevation of Ca^{2+} (to pCa5, 10^{-5} M) is insufficient to induce secretion. Nor is the combination Ca^{2+} plus ATP capable of activating the exocytotic mechanism†. However, the combination of Ca^{2+} together with other nucleoside triphosphates such as XTP, ITP or GTP (figure 1*a*) is capable of inducing release of histamine or hexosaminidase (Howell & Gomperts 1987; Howell *et al.* 1987): these nucleoside triphosphates are all known to be activating ligands for adenylyl cyclase, and this observation provided an early clue that a GTP-binding protein might be involved in the regulation of exocytosis. The argument was strengthened with the finding that non-hydrolysable analogues of GTP (figure 1*b*) (GTP-γ-S > GppNHp > $GppCH_2p$) could induce extensive secretion when provided to the permeabilized cells together with Ca^{2+} in the absence of ATP (Howell *et al.* 1987).

Because neither ATP (Howell *et al.* 1987; Koopmann & Jackson 1990) nor Mg^{2+} (Lillie *et al.* 1991) are required for exocytosis from the permeabilized cells, a reaction involving phosphorylation cannot be an essential or integral step in the late stages of the exocytotic pathway. However, when ATP is provided (as the Mg^{2+} salt) the sensitivity for both of the

† The act of permeabilization must deplete the intracellular ATP within seconds (Lillie *et al.* 1991) (and in our work the cells are routinely pretreated prior to permeabilization with metabolic inhibitors to the point that they are refractory to stimulation by normal receptor-directed agonists, or Ca^{2+} ionophores (Howell *et al.* 1987; Bennett *et al.* 1980)).

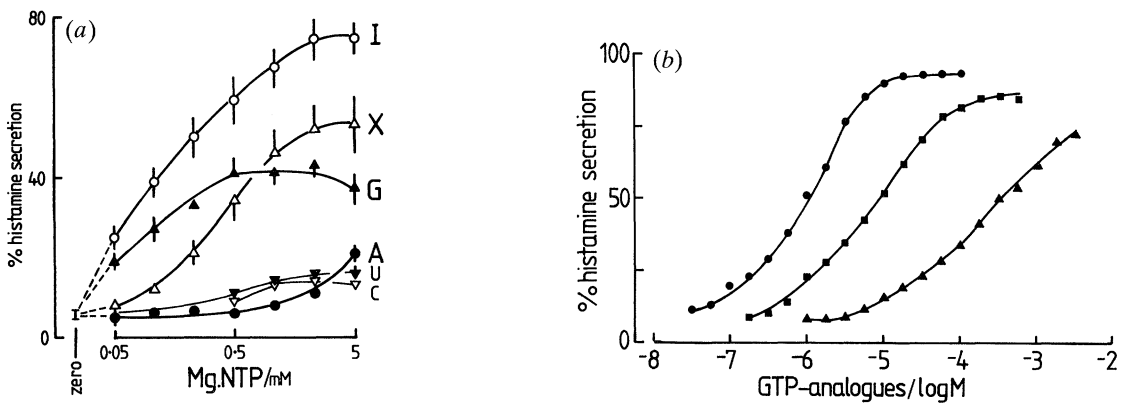

Figure 1. The dual-effector system for exocytosis: guanine and related nucleoside triphosphates synergize with Ca^{2+} to induce secretion from permeabilized mast cells. Exocytosis from permeabilized mast cells is triggered by provision of a dual effector system comprising Ca^{2+} and any nucleotide capable of activating G-proteins. The cells were pretreated with metabolic inhibitors to deplete intracellular ATP, and then permeabilized by SL-O in the presence of 10^{-5} M Ca^{2+} (pCa5, buffered with EGTA) and nucleotides as indicated. The cells were then incubated for 10 min at 37°, sedimented by centrifugation, and the supernatant was analysed for the presence of secreted histamine (this figure) or hexosaminidase. To establish a calibration scale, further sets of samples from cells lysed with Triton X100 (100%) and reagent blanks (0%) were prepared. Symbols: (*a*) I, ITP; X, XTP; G, GTP; A, ATP: U, UTP; C, CTP. (*b*) circles, GTP-γ-S; squares GppNHp; triangles, $GppCH_2p$ (from Howell *et al.* 1987).

essential effectors is considerably enhanced (Howell *et al.* 1987; Koopmann & Jackson 1990). The sensitivity for Ca^{2+} increases approximately 3-fold and approaches a high affinity situation similar to that achieved following stimulation of RBL cells by IgE-directed crosslinking ligands (Beaven *et al.* 1984); in some experiments the sensitivity for the guanine nucleotide increases by more than 50-fold. ATP must be regarded as a modulator, not an effector of exocytosis. The enhancement in the affinity of the essential effectors due to ATP is mediated by protein kinase C (Cockcroft *et al.* 1987; Howell *et al.* 1989; Koopmann & Jackson 1990; Lillie *et al.* 1991).

Exocytosis can be triggered by guanine nucleotides (together with Ca^{2+}) under conditions in which the phospholipase-C reaction is inhibited by neomycin (Cockcroft *et al.* 1987; Aridor & Sagi-Eisenberg 1991). The guanine nucleotide thus activates exocytosis at a late stage in the pathway through interaction with a GTP-binding protein distinct from G_P (the activator of polyphosphoinositide specific phospholipase-C (Cockcroft & Gomperts 1985)) and we have called this G_E (Gomperts *et al.* 1986).

(*a*) *Glutamate enhances affinity for guanine nucleotides and inhibits GTPase*

Permeabilisation of the cells in solutions of zwitterionic electrolytes (such as glutamate, glycine or GABA) instead of simple uni/univalent salt solutions (such as NaCl) allows one to perceive different aspects of the control processes leading to exocytosis (figure 2). Using isotonic glutamate, it is possible to achieve substantial secretion by application of either Ca^{2+} (i.e. without added guanine nucleotide), or a guanine nucleotide at very low levels of Ca^{2+} (pCa9). For the Ca^{2+}-dependent response provision of ATP is mandatory, and we originally thought this was true also for the GTP-γ-S dependent response (Churcher & Gomperts 1990). In the absence of ATP, GTP-γ-S stimulates only a very low level of secretion (*ca.*, 10%) and although initially disregarded by us (Churcher & Gomperts 1990), it is now apparent that this is significant and dependent on provision of a non-hydrolysable analogue of GTP.

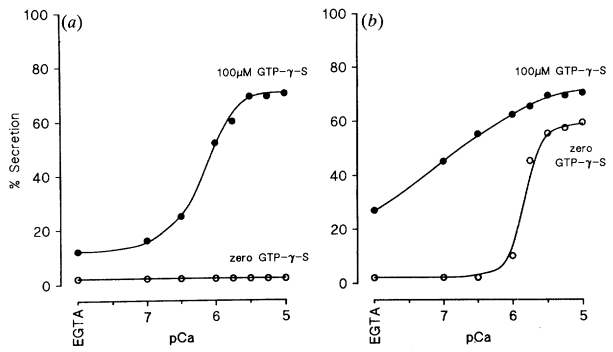

Figure 2. In glutamate buffer, ATP enhances GTP-γ-S induced secretion, and reveals Ca^{2+}-independent secretion. (*a*) Mast cells pretreated with metabolic inhibitors were permeabilized in buffers composed of isotonic glutamate in the absence of ATP. They respond to the dual effector system of Ca^{2+} plus GTP-γ-S. There is a significant amount of secretion (≈10%) in the effective absence of Ca^{2+}. (*b*) When 1 mM ATP is also provided, the GTP-γ-S-dependent component is enhanced and a Ca^{2+}-dependent response is also revealed.

4. Ca^{2+} AND GUANINE NUCLEOTIDE ACT IN SERIES

We wish to propose that Ca^{2+} and guanine nucleotides, through their interactions with appropriate binding proteins C_E and G_E, act sequentially to enable exocytosis in myeloid cells. Neither C_E nor G_E have been identified. We envisage the role of Ca^{2+} as a ligand for an intracellular pseudo-receptor C_E, which regulates the affinity of the GTP-binding protein G_E for guanine nucleotides and Mg^{2+}. These conclusions are presented schematically in figure 3 and are based on the following observations.

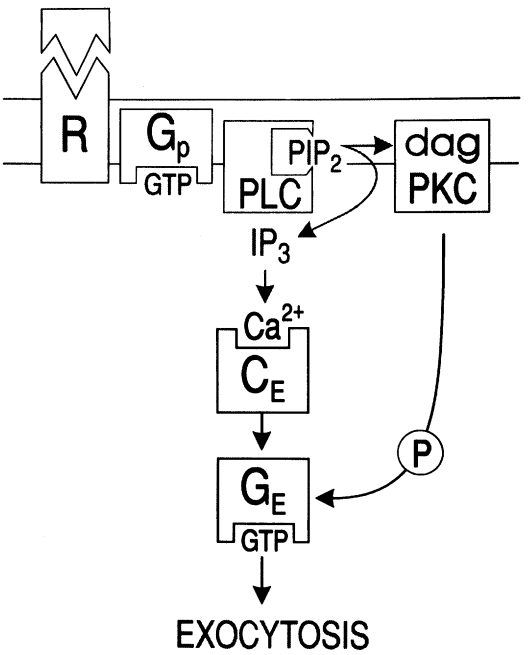

Figure 3. Schematic representation of the main regulatory components of secretion in myeloid cells. Two GTP-binding proteins act in series to control stimulus-secretion coupling; G_P transduces external signals emanating from cell surface receptors, and controls the activity of phospholipase C. Two products are generated. Inositol 1,4,5-trisphosphate (IP_3, which normally causes the release of Ca^{2+} in intact cells) leaks rapidly from permeabilized cells, but diglyceride (dag), the activator of protein kinase C is retained. Exocytosis occurs from mast cells following pretreatment with metabolic inhibitors and permeabilization in the presence of Ca^{2+} and GTP-γ-S: the affinity for both is enhanced, due to a protein kinase C-catalysed phosphorylation, when ATP is provided. Neither of the binding proteins for Ca^{2+} (C_E) nor guanine nucleotide (G_E) has been identified, but we know that G_E is distinct from G_P. Mast cells permeabilized in zwitterionic electrolytes (glutamate) undergo secretion in response to GTP-γ-S in the absence of Ca^{2+}; Ca^{2+} by itself is unable to elicit secretion, but spares the requirement for Mg^{2+} in GTP-γ-S-induced secretion. These observations underlie the proposal that Ca^{2+} and guanine nucleotide act in series in the regulatory sequence leading to exocytosis.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

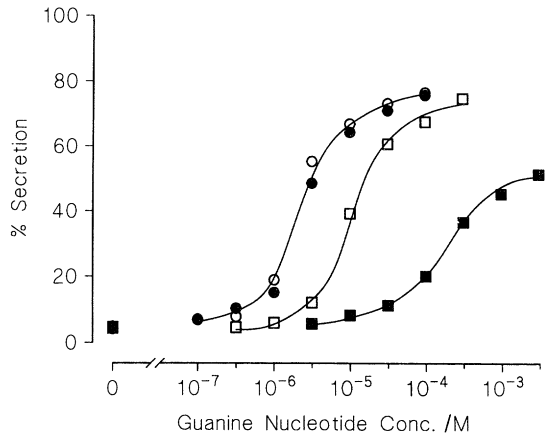

Figure 4. Deprivation of Mg^{2+} enhances GTP-induced exocytosis from permeabilized rat mast cells. Mast cells, pretreated with metabolic inhibitors and suspended in buffered NaCl, were permeabilized in the presence of Ca^{2+} (buffered at pCa5) and guanine nucleotides as indicated, in presence and absence of Mg^{2+}. At the end of 10 min the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase. Closed circles, GTP-γ-S + Mg^{2+}; open circles, GTP-γ-S − Mg^{2+}; closed squares, GTP + Mg^{2+}; open squares, GTP − Mg^{2+}.

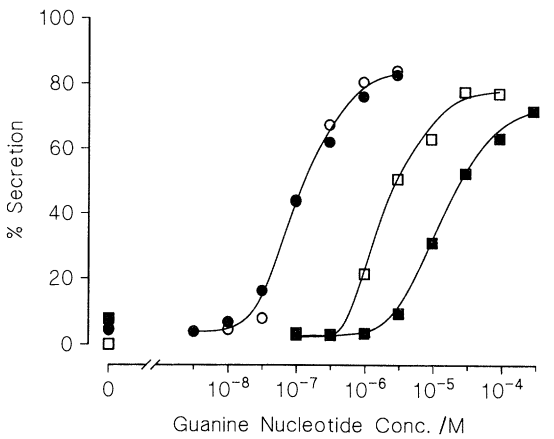

Figure 6. Low concentrations of GTP and GTP-γ-S support secretion in glutamate buffer at pCa5 in the absence of ATP. Mast cells, suspended in isotonic glutamate and pretreated with metabolic inhibitors, were permeabilized in the presence of 10^{-5} M Ca^{2+} together with GTP-γ-S or GTP at the concentrations indicated, in the presence or absence of 2 mM Mg^{2+}. At the end of 10 min the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase. Closed circles, GTP-γ-S + Mg^{2+}; open circles, GTP-γ-S − Mg^{2+}; closed squares, GTP + Mg; open squares, GTP − Mg^{2+}.

1. Mg^{2+} selectively diminishes the sensitivity for GTP (but not for GTP-γ-S) (figures 4 and 6). Late addition of Mg^{2+} halts GTP-induced secretion abruptly (figure 5): from these observations we conclude that G_E operates as a GTPase.

2. For cells permeabilised in isotonic glutamate, Ca^{2+}-induced secretion has an absolute requirement for ATP (figure 2): secretion is then enhanced by low concentrations (less than 100 μM) and inhibited by high concentrations of GDP (figure 7). This indicates that a GTP-binding protein is involved and we conclude that activation of a G-protein is always necessary to achieve secretion.

3. Again, for cells permeabilized in isotonic glutamate, secretion can be stimulated by GTP-γ-S in the effective absence of Ca^{2+} (i.e. pCa9) (figure 2). This leads us to believe that the G-protein G_E acts at a later stage in the secretory pathway than Ca^{2+}, since Ca^{2+}-induced secretion always requires the presence of a guanine nucleotide (paragraph 2 above, and figure 7) but guanine nucleotides can induce secretion at pCa9 (figures 2 and 8).

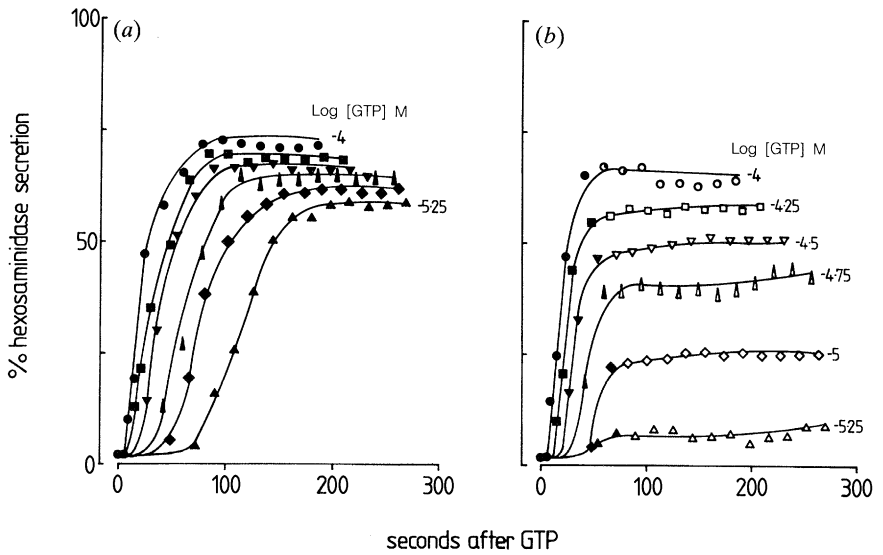

Figure 5. Late addition of Mg^{2+} causes abrupt termination of secretion from cells undergoing exocytosis in response to GTP. (*a*) Mast cells, suspended in buffered NaCl and pretreated with metabolic inhibitors were permeabilized in the presence of calcium buffer (EGTA, 5 mM, pCa5) and triggered one minute later by addition of GTP at concentrations indicated. Timed samples were withdrawn and processed for analysis of secreted hexosaminidase. In (*b*) $MgCl_2$ (5 mM) was then added to the secreting cells after about 1 min (open symbols) and sampling was continued.

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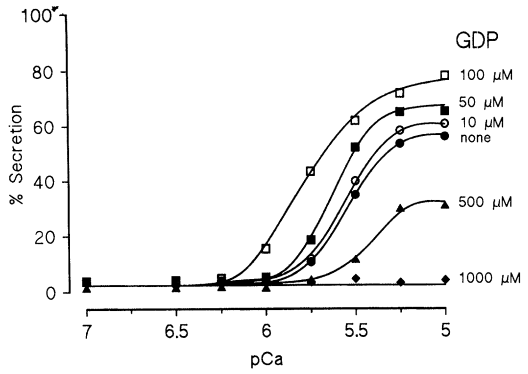

Figure 7. ATP and Ca^{2+} dependent secretion in glutamate based buffer can be enhanced by low concentrations and inhibited by high concentrations of GDP. Mast cells, suspended in isotonic glutamate based buffers and pretreated with metabolic inhibitors, were incubated with SL-O and 1 mM Mg.ATP in the presence of Ca^{2+} (buffered as indicated) and in the presence of GDP at various concentrations (as indicated). At the end of 10 min the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase.

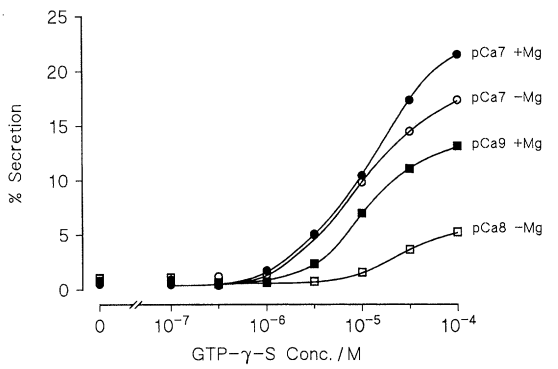

Figure 8. GTP-γ-S stimulates secretion at vanishingly low concentrations of Ca^{2+} in the absence of ATP. Mast cells, suspended in glutamate based buffer and pretreated with metabolic inhibitors, were incubated with SL-O at low (pCa7) or vanishingly low (5 mM EGTA, 5 mM HEDTA) concentrations of Ca^{2+} and in the presence of various concentrations of GTP-γ-S (as indicated). Closed symbols indicate Mg^{2+} (1 mM) and EGTA buffering, open symbols indicate suppression of Mg^{2+} to less than 10^3 M with HEDTA buffering. At the end of 10 min the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase.

Only non-hydrolysable guanine nucleotides are capable of inducing Ca^{2+}-independent secretion, and there is a strong requirement for Mg^{2+}. This is probably the reason why GTP fails to simulate since presence of Mg^{2+} will ensure its hydrolysis.

The role of Ca^{2+} appears to be in the regulation of G_E function. Elevation of Ca^{2+} from pCa9 to pCa7 (i.e. normal intracellular resting levels) spares the requirement for Mg^{2+} (figure 8) and at higher concentrations (pCa6) there is no Mg^{2+} dependence. Also, elevation of Ca^{2+} increases the magnitude of the secretory response to any given concentration of guanine nucleotide. In this way the role of Ca^{2+} appears to be similar to that of glucagon on the

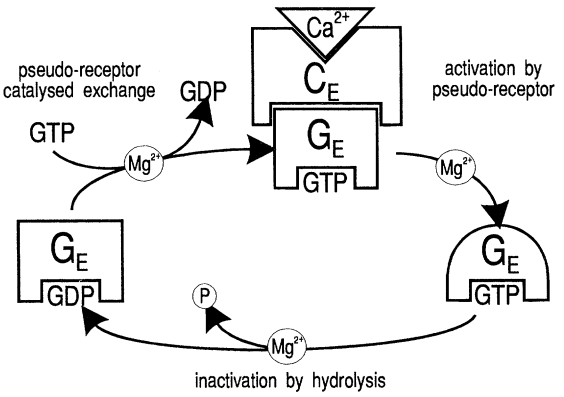

Figure 9. Schematic representation of the Mg^{2+}-dependent steps in the activation and deactivation of G_E and its regulation by the pseudo-receptor C_E. Kinetic analysis reveals delays in the onset of exocytosis when permeabilized cells are deprived of Mg^{2+}. Their duration is dependent on the concentration and the identity of the stimulating guanine nucleotide. These characteristics, together with the finding that deprivation of Mg^{2+} enhances secretion due to GTP (but not GTP-γ-S), lead to the proposal of three discrete sites of action of Mg^{2+} in the regulation of G_E. In this respect, the guanine nucleotide-binding protein controlling exocytosis appears to have a strong resemblence to G_S, the GTP-binding protein which regulates adenylyl cyclase.

adenylyl cyclase system of hepatocyte membranes, sparing the requirement for Mg^{2+} in cyclase activation and increasing the extent of activation attainable at any given concentration of guanine nucleotide.

From these similarities we suggest that the Ca^{2+}-binding protein, C_E, has the characteristics of an intracellular pseudo-receptor catalysing guanine nucleotide exchange on G_E. Not only does the stimulated pseudo-receptor (i.e. stimulated by Ca^{2+}) preclude the requirement for Mg^{2+}, but it also increases the extent of the secretory response by at least tenfold.

5. KINETICS OF EXOCYTOSIS REVEAL ONSET DELAYS

We have also investigated the onset kinetics of exocytosis from permeabilized cells†. These experiments provide a view of the activation process which also points to the suggestion that the guanine nucleotide binding protein G_E shares mechanistic features with the heterotrimeric G-proteins characterised by G_S, the activator of adenylyl cyclase. These conclusions are presented schematically in figure 9 and are based on the following observations.

Depending on the conditions applied, exocytosis from permeabilized cells may be preceded by extended time delays. These are sensitive to the order of addition of the two essential effectors and also to the presence or absence of Mg^{2+} (figures 10 and 11). In these experiments the cells were permeabilized either in the presence of a range of concentrations of GTP-γ-

† The kinetic experiments were all done on cells permeabilized in NaCl solutions. Because both Ca^{2+} and guanine nucleotide are required under these conditions, this allows the cells to be equilibrated first with one effector and then triggered (after one minute) by addition of the second effector.

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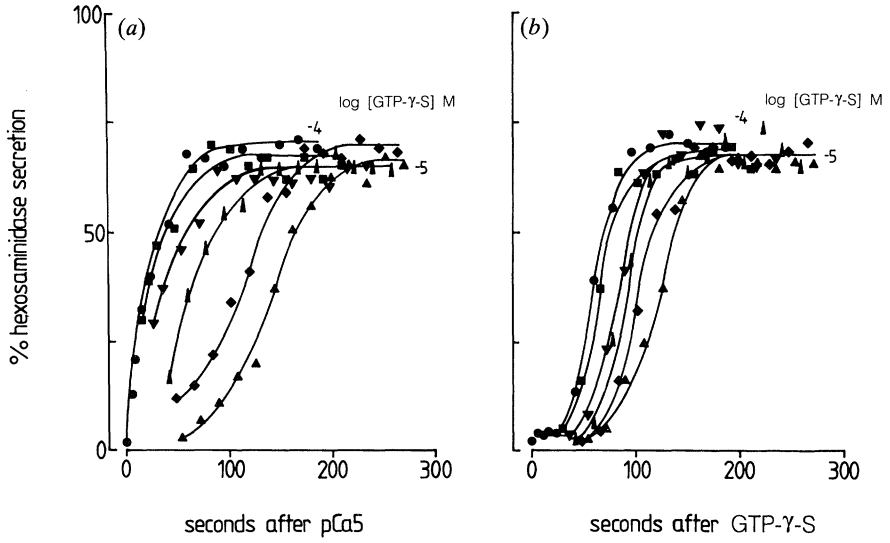

Figure 10. Timecourse of Ca^{2+}-triggered and GTP-γ-S-triggered exocytosis from mast cells permeabilized by SL-O in the absence of Mg^{2+}. Mast cells, suspended in buffered NaCl and pretreated with metabolic inhibitors were permeabilized in the presence of (*a*) GTP-γ-S at concentrations indicated or (*b*) calcium buffer (in this experiment, Mg^{2+} was omitted and Ca^{+} was buffered using HEDTA 5 mM, pCa5). One minute later exocytosis was triggered by addition of the complementary effector ((*a*) Ca^{2+}, pCa5; or (*b*) GTP-γ-S at concentrations indicated) and timed samples were withdrawn and processed for measurement of secreted hexosaminidase. GTP-γ-S concentration: circles, 10^{-4} M; squares, $10^{-4.2}$ M; inverted triangle, $10^{-4.4}$ M; elongated triangle, $10^{-4.6}$ M; diamonds, $10^{-4.8}$ M; upright triangles, 10^{-5} M.

S and then triggered one minute later by addition of Ca^{2+} (pCa5) (a), or (b) triggered with a range of GTP-γ-S after permeabilisation in the presence of Ca^{2+} (pCa5).

When GTP-γ-S is used to trigger exocytosis from cells previously equilibrated with Ca^{2+} in the absence of Mg^{2+}, delays preceding secretion are clearly manifest (figure 10*b*). These become shorter as the concentration of the guanine nucleotide is increased, tending to minimum of about 30 s. The delay due to stimulation by 10^{-2} M GTP-γ-S (data not shown) is not significantly shorter than for 10^{-3} M. In contrast (figure 10*a*), when the order of addition of the two effectors is reversed, the onset of exocytosis is accelerated at all concentrations of GTP-γ-S, and becomes abrupt if it is sufficiently high. In the presence of

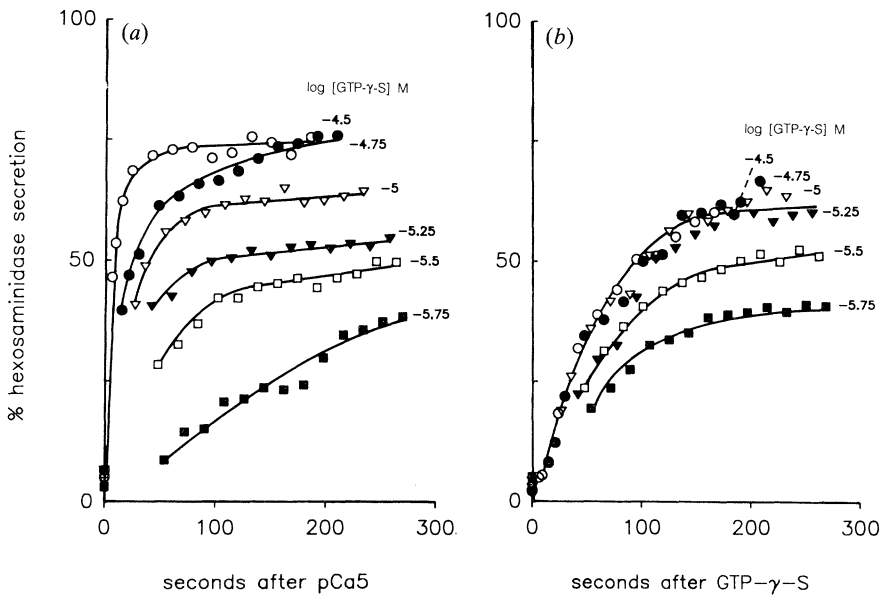

Figure 11. Timecourse of Ca^{2+}-triggered and GTP-γ-S-triggered exocytosis from mast cells permeabilized by SL-O in the presence of Mg^{2+}. Mast cells, suspended in buffered NaCl and pretreated with metabolic inhibitors were permeabilized in the presence of (*a*) GTP-γ-S at concentrations indicated or (*b*) calcium buffer (EGTA, 5 mM, pCa5). One minute later exocytosis was triggered by addition of the complementary effector ((*a*) Ca^{2+}, pCa5; or (*b*) GTP-γ-S at concentrations indicated) and timed samples were withdrawn and processed for measurement of secreted hexosaminidase. GTP-γ-S concentration: closed circles, $10^{-4.5}$ M; open circles, $10^{-4.25}$ M; open triangles, 10^{-5} M; closed triangles, $10^{-5.25}$ M; open squares, $10^{-5.5}$ M; closed squares, $10^{-5.75}$ M.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

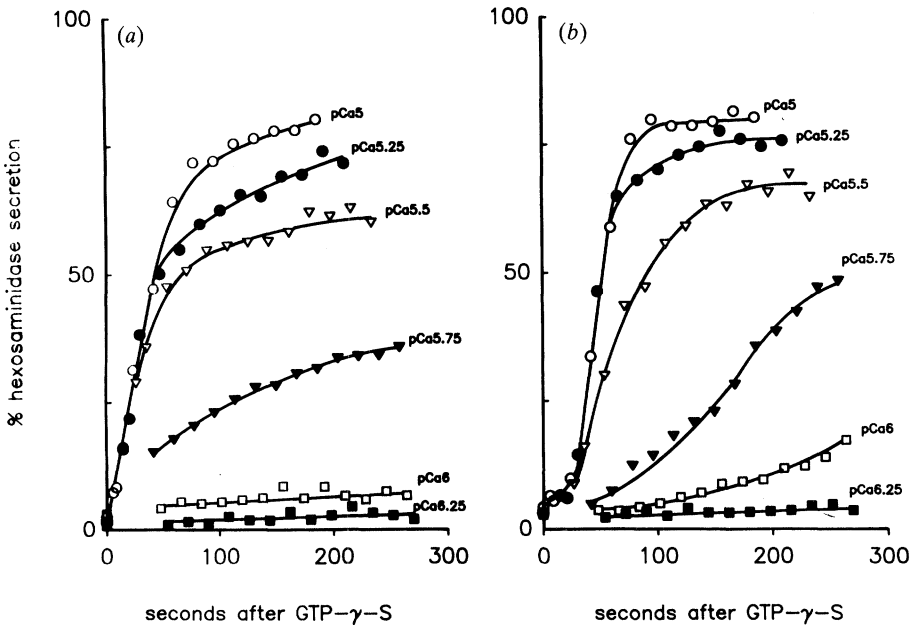

Figure 12. The effect of Ca^{2+} on the time course of GTP-γ-S-triggered exocytosis from mast cells permeabilized by SL-O in the presence or absence of Mg^{2+}. Mast cells, pretreated with metabolic inhibitors were suspended in buffered NaCl and in the presence (*a*) or absence (*b*) of Mg^{2+} (2 mM) were permeabilized in the presence of calcium buffers set to regulate pCa as indicated. One minute later, exocytosis was triggered by addition of GTP-γ-S (100 μM) and timed samples were withdrawn and processed for measurement of secreted hexosaminidase. Calcium concentration; open circles, pCa5; closed circles, pCa5.25; open triangles, pCa5.5; closed triangles, pCa5.75; open squares, pCa6; closed squares, pCa6.25.

Mg^{2+}, the onset of exocytosis is accelerated though there remains just a hint of a short onset delay when Ca^{2+}-loaded cells are triggered with GTP-γ-S (figure 11*b*). When the order of addition of the two effectors is reversed (i.e. GTP-γ-S, then Ca^{2+}), exocytosis commences abruptly at all concentrations of the guanine nucleotide (figure 11*a*).

It is most unlikely that these delays reflect diffusion artefacts due to the time taken for GTP-γ-S to attain its intracellular site of action. If this were the case then one would not expect that they would be so sensitive to the presence or absence of Mg^{2+}. Nor would one expect the delays to be substantially different depending on the identity of the guanine nucleotide (compare figures 10*b* and 6*b*). Much more likely is the possibility that they indicate the time taken for GTP-γ-S to bind to G_E (reflected in the sensitivity to the order of addition of the two effectors) and then to induce activation (e.g. by conformational change or by subunit dissociation, reflected by the limiting minimal delay, and its sensitivity to the identity of the activating guanine nucleotide).

The primary determinant of the duration of the onset delays is the identity and the concentration of the guanine nucleotide. Figure 12 shows the kinetics of secretion from cells triggered by GTP-γ-S (100 μM) after equilibration with a range of Ca^{2+} concentrations. For cells loaded with Ca^{2+} (range pCa5–pCa5.75) in the presence of Mg^{2+}, exocytosis commences abruptly following provision of GTP-γ-S. When Mg^{2+} was excluded, delays due to the higher concentrations of Ca^{2+} were of the order of 30 s and apparently invariant. At lower concentrations of GTP-γ-S the delay due to high loading concentrations of Ca^{2+} becomes more extended (typically 50 s at 30 μM GTP-γ-S: data not shown). For cells triggered by addition of GTP the limiting delays are shorter (see figure 6) and for cells stimulated by high concentrations of GppNHp the delays extend to beyond 150 s (not shown).

We believe that the results we have obtained, especially the modulatory effects of Mg^{2+} on the onset kinetics of exocytosis, can best be explained in terms of an interaction with a GTP-binding protein having features in common with G_S (Birnbaumer *et al.* 1985; Premont & Iyengar 1990). In the following paragraphs we summarize the salient features of the G_S system with respect to activation of adenylyl cyclase and the effects of Mg^{2+}. We also summarize the effects of Mg^{2+} on p21ras to indicate how the regulation of G_E by guanine nucleotides and Mg^{2+} differs from the regulation of low molecular mass monomeric GTP-binding proteins of this class.

(*a*) *Effects of Mg^{2+} on the function of G_S*

There appear to be at least three discrete sites of Mg^{2+} action on the regulation of adenylyl cyclase by G_S.

1. Activation of adenylyl cyclase G_S by guanine nucleotides is preceded by delays sensitive to the presence of Mg^{2+} and the identity of the guanine nucleotide (Birnbaumer *et al.* 1985). Mg^{2+} curtails the delays preceding cyclicAMP generation (Iyengar & Birnbaumer 1981; Iyengar 1981). For any given concentration of Mg^{2+}, the delays are more extended

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

if GppNHp, rather than GTP, is used as the activating ligand, indicating that they register a step other than the off-rate of the GDP which must be common to all ligands (Iyengar 1981; Iyengar & Birnbaumer 1981; Birnbaumer *et al.* 1980). The effects of Mg^{2+} on delays in activation of cyclase and activation of isolated G-proteins by GTP-γ-S (measured by changes in tryptophan fluorescence) have been attributed to the Mg^{2+}-induced conversion of an inactive GTP-bound form to an active form of the GTP-binding protein (Iyengar & Birnbaumer 1981; Iyengar 1981; Higashijima *et al.* 1987*b*), which is catalysed by a ligand-bound receptor at much lower (10^3-fold) concentrations of Mg^{2+} (Iyengar & Birnbaumer 1982).

2. The hormone sensitive GTPase function of G_S is also dependent on Mg^{2+} (Brandt & Ross 1986; Higashijima *et al.* 1987*a–c*).

3. In the absence of Mg^{2+} the binding of GTP-γ-S becomes reversible (monitored by changes in tryptophan fluorescence or binding of radioactive GTP-γ-S) (Higashijima *et al.* 1987*a–c*; Bokoch *et al.* 1984; Northup *et al.* 1982).

(*b*) *Effects of Mg^{2+} on the function of proteins of the ras family*

Mg^{2+} also has profound effects on the binding of nucleotides to GTP-binding proteins of the ras family, but these are quite distinct from those associated with G_S, G_i, G_O, etc. Reduction in the concentration of Mg^{2+} leads to a marked increase in the exchange rate of GDP for GTP (p21N-ras^{GDP} lifetime reduced from 20 min to less than 30 s), with GTP assuming a tenfold higher relative affinity than GDP (Hall & Self 1986) and so, contrary to the signal transducing G-proteins, and contrary to our observations on exocytosis, the absence of Mg^{2+} increases the affinity for the activating guanine nucleotide. Similar results have been obtained for rhoB p20 (Kuroda *et al.* 1989). More detailed knowledge of the effects of Mg^{2+} on the nucleotide binding and activation of this family of proteins awaits the identification of the target effector(s) and other modulatory proteins (e.g. GAP) and studies of their interaction with ras.

It appears that the effects of Mg^{2+} on G_E share remarkable similarity to the effects of Mg^{2+} on the heterotrimeric G-protein G_S. One point of difference is that in the exocytotic system of myeloid cells, the GTP-binding protein G_E is probably stimulated by an intracellular pseudo-receptor which is the Ca^{2+}-binding protein, C_E. As when glucagon is provided to activate adenylyl cyclase in hepatocyte membranes, provision of Ca^{2+} in the exocytotic system ensures a very low requirement for Mg^{2+}. Despite this, the effects of Mg^{2+} deprivation that we observe are compatible with the effects of Mg^{2+} deprivation on G_S stimulation of cyclase in the absence of glucagon, when the Mg^{2+} requirement is higher.

As outlined above, Mg^{2+} affects G_S in three ways: (i) it renders the binding of GTP-γ-S effectively irreversible; (ii) it allows activation of the G-protein after nucleotide binding, and (iii) it allows the GTPase to function. Our results are well explained in terms of the same three processes and their sensitivity to the presence or absence of Mg^{2+}. (i) In the absence of Mg^{2+}, GTP-γ-S binding becomes reversible and so there is a concentration dependent component of the delays in the onset of secretion. (ii) Absence of Mg^{2+} also affects activation of the nucleotide bound G-protein and we see this expressed as the limiting delay in onset that cannot be abolished by increasing GTP-γ-S concentration. (iii) In the absence of Mg^{2+} there is an increase in the sensitivity and magnitude of secretion induced by GTP. Conversely addition of Mg^{2+} to cells, initially triggered by GTP in its absence, terminates secretion abruptly. Both these observations are compatible with the notion that G_E has a Mg^{2+} dependent GTPase function.

From data derived from experiments carried out on cells permeabilized in both glutamate and chloride based buffers it would seem that there is a remarkable similarity in the regulation of the secretory mechanism of rat mast cells and the adenylyl cyclase system. Both can be stimulated by guanine nucleotides alone if Mg^{2+} is present, and both show Mg^{2+} dependence in nucleotide binding, hydrolysis and activation of the G-protein. When a suitable ligand is provided (Ca^{2+} for the exycytotic system of mast cells and glucagon for the cyclase system of hepatocyte membranes) guanine nucleotides are able to cause stimulation at much lower concentrations and the requirement for Mg^{2+} is obviated.

We anticipate that this analysis of the regulation of secretion will provide a rational basis for the identification and isolation of the proteins G_E and C_E which appear to mediate this process.

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Discussion

F. McCormick (*Department of Molecular Biology, Cetus Corporation, California, U.S.A.*). All the evidence on which the authors base their conclusions supporting the idea of a signal-transducing type G-protein as being the mediator of regulated exocytosis is indirect. In view of the wealth of information pointing to defined low molecular mass GTPases regulating accuracy and direction of vesicular traffic in the early stages of the secretory pathway, and in the budding reaction of *Saccaromyces cerevesiae*, do they really believe that their G-protein is something different?

B. D. Gomperts. Yes. First of all, I should stress once again that we are investigating regulated exocytosis. The examples Dr McCormick cites are for unregulated steps in the secretory pathway, or for a constitutive secretory reaction. Thus, at the start, the biology is different. Next, just to reiterate, the effects of Mg^{2+} deprivation on regulated exocytosis (G_E-mediated) and on adenylyl cyclase (G_S-mediated) are rather similar. Mg^{2+} deprivation inhibits the GTPase reaction and retards GDP and GTP exchange and activation. For the low molecular mass monomeric GTPases, Mg^{2+} deprivation actually enhances the rate of GTP binding. Next, note that in regulated exocytosis, it is the non-hydrolysable analogues of GTP, or conditions in which the GTPase reaction is inhibited (i.e. Mg^{2+} deprivation) which provide the best stimulus. For constitutive membrane trafficking it is essential that the GTP is hydrolysed simply for the GTP-binding protein to be able to recycle repetitively between GDP and GTP bound states.

This experiment will help to convince Dr McCormick (see figure D1). It was done by Tim Howell in

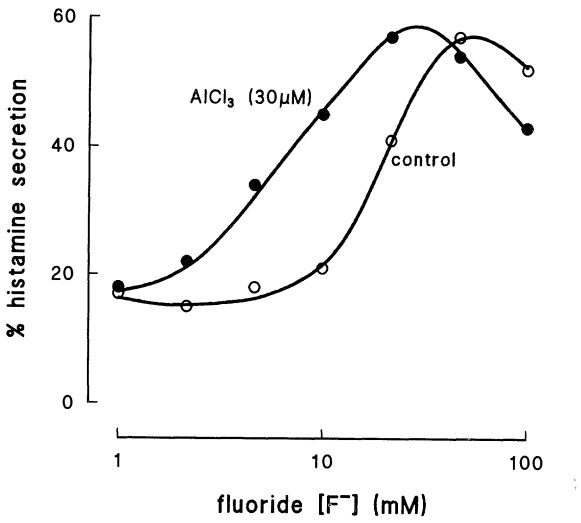

Figure D1.

the early days of permeabilization with SL-O but we never published it and similar results have since been reported by others (Sorimachi *et al.* 1988). We tested fluoride, and fluoraluminate as intracellular effectors for histamine from mast cells. Necessarily in this experiment we were unable to use a buffering system to regulate pCa (as this would have depleted the concentration of the Al^{3+}); the concentration of Ca^{2+} (always present as a contaminant in unbuffered solutions) was probably around 10^{-5} M as application of GTP-γ-S (10 μM) induced 80% secretion in the same experiment. F^-, when applied at concentrations between 10–100 mM induces secretion in the absence of added guanine nucleotide. The effect of added Al^{3+} is then to enhance the apparent affinity by about three fold. I think that you will agree with me that this looks very like the behaviour of the αβγ signal transducing G-proteins (G_S, G_i, G_O, transducin, etc). As far as is known, none of the low molecular mass, monomeric GTPases can be stimulated by $[AlF_4]^-$ (Kahn 1991).

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