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Guanine nucleotide is essential and Ca^{2+} is a modulator in the exocytotic reaction of permeabilized rat mast cells

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Exocytosis from metabolically depleted permeabilized rat mast cells was measured in response to provision of Ca^{2+} and guanine nucleotide [GTP or guanosine 5′-[γ-thio]triphosphate (GTP[S])]. For cells permeabilized in simple salt solutions (NaCl), both of these effectors were required to induce secretion. Exclusion of Mg^{2+} caused an increase in both the sensitivity of the system to GTP and the extent of secretion elicited, while having no such effects on secretion induced by GTP[S]. The effect of Mg^{2+} depletion on the ability of GTP to stimulate secretion is probably due to the dependence on Mg^{2+} of the GTPase activity of G_E (a postulated GTP-binding protein which mediates exocytosis). This argues that a persistent stimulus to the G-protein is required to support secretion. Affinity for both GTP[S] and GTP is enhanced when the cells are permeabilized in zwitterionic electrolytes (glutamate, γ-aminobutyric acid, glycine) instead of NaCl. Under these conditions, secretion occurs in response to provision of either GTP[S] [in the effective absence of Ca^{2+} (pCa 9)] or Ca^{2+} (in the absence of guanine nucleotide). Secretion induced by GTP[S] is strongly promoted by the presence of Mg^{2+} at concentrations in the millimolar range; this promotion by Mg^{2+} declines as the concentration of Ca^{2+} is elevated towards pCa 7. At pCa 6, Mg^{2+} is without effect. Ca^{2+}-induced secretion requires the provision of MgATP. Since this is further enhanced by low concentrations ($< 100\ \mu M$) and then inhibited by high concentrations of GDP, the essential role of ATP is likely to be in the maintenance of GTP via transphosphorylation by a nucleoside diphosphate kinase reaction. Thus, under conditions of high affinity (glutamate environment), GTP[S] alone is capable of inducing exocytosis. Ca^{2+} acts in concert with guanine nucleotides: it enhances the rate and extent of secretion and increases the affinity for Mg^{2+} and guanine nucleotides in the activation of the GTP-binding protein (G_E) which regulates exocytosis.

INTRODUCTION

Mast cells permeabilized by streptolysin-O (SL-O) in solutions of NaCl or other simple uni/univalent electrolytes can secrete their contained histamine and lysosomal enzymes when provided with both Ca^{2+} and any nucleotide capable of activating a GTP-binding protein (the dual-effector system) (Howell *et al.*, 1987; Koopmann & Jackson, 1990). The presence of both Ca^{2+} and an activating guanine nucleotide are necessary, and together they are sufficient to induce 100% release. The rank order of effectiveness for stimulation of exocytosis by the synthetic non-hydrolysable guanine nucleotides is guanosine 5-[γ-thio]-triphosphate (GTP[S]) > guanosine 5-[βγ-imido]triphosphate (Gpp[NH]p) > guanosine 5′-[αβ-methylene]triphosphate (Gpp[CH_2]p) (Howell *et al.*, 1987). There is no requirement for ATP and so phosphorylation does not comprise a step in the late events of the exocytotic pathway (Howell *et al.*, 1987). While the three non-hydrolysable analogues of GTP are capable of delivering a maximal stimulus to exocytosis when applied at micromolar concentrations, the nucleoside triphosphates ITP, XTP and GTP only induce secretion when presented at concentrations in the millimolar range, in the rank order ITP > XTP > GTP (Howell *et al.*, 1987). It thus appears that the stimulus is best provided by those nucleotides which can maintain the activation of a GTP-binding protein. Similar rank orders of nucleotide effectiveness for support of exocytosis have been discerned in other myeloid cells such as neutrophils (Stutchfield & Cockcroft, 1988) and eosinophils (Cromwell *et al.*, 1991).

Although not a requirement, ATP (as the Mg^{2+} salt) modulates the exocytotic reaction by enhancing the affinity for both Ca^{2+} and the guanine nucleotide. In common with other secretory-cell types (Knight and Baker, 1983; Haslam & Davidson, 1984) this effect is mediated by phosphorylation reactions catalysed by protein kinase C (Howell *et al.*, 1987; Lillie *et al.*, 1991). In permeabilized mast cells, other nucleoside triphosphates including ITP, XTP, CTP and UTP are also capable of enhancing effector affinity, but do so as a consequence of transphosphorylation of residual ADP to form ATP. Significantly, GTP is incapable of enhancing effector affinity, either directly or indirectly (Lillie *et al.*, 1991).

In contrast to the situation which pertains when the cells are permeabilized in simple salt solutions, the use of zwitterionic electrolytes (such as glutamate) allows secretion to be elicited by either Ca^{2+} or a guanine nucleotide acting alone. However, for secretion to proceed following stimulation by a single effector, the presence of ATP now appears to be mandatory (Churcher & Gomperts, 1990). In a previous report (Churcher & Gomperts, 1990) the action of ATP in enabling exocytosis by a single effector (from cells permeabilized in iso-osmotic glutamate) was interpreted as evidence of phosphorylations mediated by protein kinase C to permit the operation of two parallel pathways (i.e. one mediated by Ca^{2+} and the other by guanine nucleotide), leading to fusion of the secretory granules with the plasma membrane. In the experiments described here we have re-examined the function of ATP in the light of recent observations which indicate that an alternative role for ATP in the induction of secretion from permeabilized cells is in the transphosphorylation of other nucleoside diphosphates (Lillie *et al.*, 1991). Our results indicate that such secretion induced by single effectors results from an enhancement by glutamate of the affinity for GTP and inhibition of the GTPase activity of a GTP-binding protein (G_E) which regulates exocytotic membrane fusion. For

Abbreviations used: GTP[S], guanosine 5′-[γ-thio]triphosphate; Gpp[NH]p, guanosine 5-[βγ-imido]triphosphate; Gpp[CH_2]p, guanosine 5′-[αβ-methylene]triphosphate; SL-O, streptolysin-O; HEDTA, *N*-hydroxyethylethylenediaminetriacetic acid, pCa, $-\log_{10}[Ca^{2+}]$; hexosaminidase, *N*-acetyl-β-D-glucosaminidase; GABA, γ-aminobutyric acid.

mast cells stimulated immediately upon permeabilization, the guanine nucleotide is the *sine qua non* for exocytosis; on the other hand, Ca^{2+}, which regulates the sensitivity of the system for GTP, is, in the limit, dispensible.

MATERIALS AND METHODS

Cells were obtained by peritoneal lavage of large (> 500 g) male Sprague–Dawley rats. The mast cells were isolated from contaminating types by centrifugation through a cushion of Percoll (Pharmacia, Milton Keynes, Bucks., U.K.) as previously described (Tatham & Gomperts, 1990), washed twice by resuspension and centrifugation and finally resuspended in appropriate iso-osmotic electrolyte solutions [i.e. formulated with NaCl, sodium glutamate, glycine or γ-aminobutyric acid (GABA)]. These solutions were initially prepared as 1.36 M stocks containing 0.2 M-Pipes (nominal 10 × final concentration). The pH was adjusted to pH 6.8 by further addition of Pipes or NaOH. Before use these concentrated stocks were diluted with water to concentrations equivalent to 290 mOsm measured on a WESCOR model 5000 vapour pressure osmometer (Wescor Inc., Logan, UT, U.S.A.). BSA (1 mg·ml^{-1}) was added and the pH readjusted to 6.8. When required, Mg^{2+} was added from a 0.3 M stock solution prepared by titrating a suspension of analytical reagent grade MgO with Pipes.

An important feature of this work has been to compare the dependence of the extent of secretion on Ca^{2+} and guanine nucleotides in the presence and absence (i.e. under conditions of severe depletion) of Mg^{2+}. The specification for Aristar NaCl (BDH) states an upper limit of 0.05 p.p.m. for magnesium, which would give 19 nM-Mg^{2+} in a solution of 0.15 M-NaCl. Under the conditions of calcium buffering (5 mM-EGTA at pH 6.8) this would not be significantly reduced. In order to reduce the concentration of Mg^{2+} still further, we have used HEDTA (*N*-hydroxyethylethylenediaminetriacetic acid) as an alternative calcium buffer. This chelator has the virtue of binding Mg^{2+} with an affinity approaching that with which it binds Ca^{2+}. Atomic absorption analysis of the electrolyte solutions revealed the presence of contamination by Mg^{2+} (1 μM) and Ca^{2+} (5 μM). Calculations (using the affinity constants listed in Table 1) indicate suppression of the free ionized concentrations of both bivalent cations to less than 10^{-9} M by EGTA (5 mM) and to $< 10^{-9}$ M (Mg^{2+}) and $< 10^{-8}$ M (Ca^{2+}) by HEDTA.

The calcium buffers were prepared by mixing equimolar solutions [100 mM nominal concentration (Tatham & Gomperts, 1990; Gomperts & Tatham, 1992)] of EGTA or HEDTA with their respective calcium salts in proportions calculated with a computer program based on the algorithm of Perrin & Sayce (1967) and using the binding constants listed in Table 1. The calcium buffers were diluted in appropriate electrolyte solutions so that the final chelator concentration was 5 mM. We have checked the concentrations of free Ca^{2+} in the EGTA and HEDTA buffers against each other, using a Ca^{2+}-sensitive electrode. A linear scale of potential (slope 31 mV/decade, at 20 °C) was obtained for both sets of buffers, with the HEDTA-buffered solutions registering a potential equivalent to a constant decrement of 0.125 pCa unit compared with the equivalent EGTA-buffered solution. In drawing our graphs, this constant error has been disregarded. Note that since the Ca^{2+} was provided as the Cl^- salt, the contribution of the calcium buffers to the final concentration of Cl^- varied from 0.62 mM to 1.24 mM or from 0.13 mM to 5.78 mM as the pCa varied in the range 7–5 for EGTA and HEDTA buffers respectively.

For the work described in this paper, we have adapted the previous methods for incubations and analysis of secreted hexosaminidase (which employed plastic test tubes) to the use of 96-well microtitre plates. The methods have been described in detail elsewhere (Tatham & Gomperts, 1990; Gomperts & Tatham, 1992). Cells were incubated for 10 min at 37 °C with calcium buffers, guanine nucleotides and SL-O (0.4 unit·ml^{-1}) as indicated, in a total volume of 90 μl. The reactions were quenched by addition of 200 μl of ice-cold NaCl (0.15 mM) buffered at pH 7 with potassium phosphate. After centrifugation, 50 μl samples of supernatant were transferred to corresponding wells in black plastic 96-well microtitre plates. To establish a calibration scale, further sets of samples from reagent blanks and from cells lysed with Triton X-100 (0.1 %) were prepared. The reactions were initiated by the addition of 50 μl of substrate (1 mM-4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide dissolved in 0.2 M-citrate buffer, pH 4.5), and the plates were maintained at 37 °C for about 90 min. Reactions were terminated by the addition of 150 μl of Tris (1 M) and the fluorescence (365–450 nm) was measured using an automatic fluorescence plate reader (Fluoroskan; Flow Laboratories) equipped with facilities for direct transfer of data for processing on a computer spread sheet.

To measure the time course of secretion (see Figs. 4 and 5), incubations were carried out in a Teflon block drilled with eight wells (3 ml) spaced identically with the wells of a microtitre plate. Reactions were triggered by addition of SL-O, and samples (100 μl) were withdrawn using an 8-channel multipipette at timed intervals and quenched into 200 μl of ice-cold phosphate-buffered saline (pH 7) contained in a 96-well microtitre plate. Further processing and analysis was as above.

Important reagents were purchased from the following sources: SL-O from Wellcome Diagnostics, Dartford, Kent, U.K.; NaCl (Aristar Grade), MgO and $CaCl_2$ (1 M volumetric solution) from BDH Chemicals, Poole, Dorset, U.K.; guanine nucleotides (as 100 mM solutions) from Boehringer-Mannheim, Lewes, E. Sussex, U.K.; EGTA and HEDTA from Fluka Chemie AG, CH-9470 Buchs, Switzerland; glutamic acid and Pipes from Sigma.

Table 1. Thermodynamic and stability constants used in the formulation of calcium buffers

Binding constants at 37 °C were derived from the values of ΔH and ΔS (Anderegg, 1964; Wright *et al.*, 1965; Moeller & Chu, 1966; Martell & Smith, 1974) listed below. Values of pK_a have been converted (by addition of 0.11) to mixed constants for compatibility with the standard buffers used for calibration of glass electrodes (Martell & Smith, 1974).

	EGTA	HEDTA
$\Delta H(H_1)$	−5.84	−9
$\Delta S(H_1)$	23.3	14
$\Delta H(H_2)$	−5.76	−2.4
$\Delta S(H_2)$	20.8	16
ΔH(Ca)	−8.38	−6.5
ΔS(Ca)	21.6	15
ΔH(Mg)	5.18	3.4
ΔS(Mg)	41.5	43
log K(H,L)	9.32	9.515
log K(H,HL)	8.72	5.23
log K(H,HHL)	2.76	2.62
log K(H,HHHL)	2.11	
log K(Ca,L)	10.63	7.86
log K(Mg,L)	5.42	7.00
log K(Ca,HL)	5.3	
log K(Mg,HL)	3.4	
log $K_{app.}$ (pH 6.8, 37 °C)		
Ca^{2+}	6.19	5.13
Mg^{2+}	1.6	4.27

RESULTS

When mast cells permeabilized in simple NaCl-based buffers are deprived of Mg^{2+}, the effective affinity of GTP in the stimulation of exocytosis is considerably increased. Fig. 1(*a*) demonstrates the dependence of hexosaminidase secretion on the concentration of either GTP-[S] or GTP in the presence of a fixed concentration of Ca^{2+} (pCa 5) and in the presence or absence of Mg^{2+}. Prior to permeabilization, the cells were treated with metabolic inhibitors for 5 min to the point at which no secretion occurred in response to ionophores or agonistic ligands (Bennett *et al.*, 1980; Howell *et al.*, 1987). To ensure thorough depletion of Mg^{2+}, the chelator HEDTA was used to regulate pCa instead of the more conventional EGTA, and under these conditions we calculate the concentration of free Mg^{2+} to be less than 10^{-9} M (see Table 1). The EC_{50} (GTP) shifts from approx. 7.8×10^{-4} M in the presence of Mg^{2+} to 10^{-5} M in its absence. Under these conditions the sensitivity to GTP becomes fully comparable with that to Gpp[NH]p and exceeds that to Gpp[CH_2]p in similar experiments (Howell *et al.*, 1987, carried out in the presence of Mg^{2+}). Exclusion of Mg^{2+} also increases the maximal level of secretion which can be elicited by GTP. In contrast, exclusion of Mg^{2+} from the system has no apparent effect on the sensivity or the magnitude of the response to the non-hydrolysable analogue. A plausible explanation of this result is that the omission of Mg^{2+} prevents the hydrolysis of GTP by GTP-binding proteins and thus ensures that the GTP-binding protein, G_E, which mediates exocytosis, becomes persistently activated.

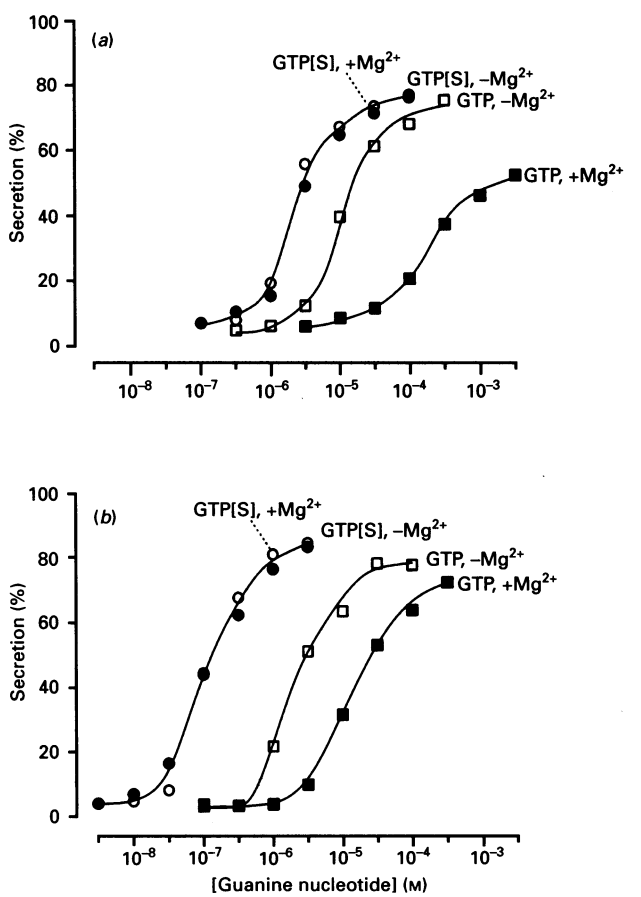

Fig. 1. Exclusion of Mg^{2+} enhances the ability of GTP to induce exocytosis from permeabilized rat mast cells

Mast cells, suspended in buffered NaCl (*a*) or glutamate (*b*) and pretreated with metabolic inhibitors, were incubated with SL-O and Ca^{2+} (buffered at pCa 5) and guanine nucleotides as indicated, in the 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. ■, □, GTP; ●, ○, GTP[S]. ■, ●, Presence of Mg^{2+} (1 mM, Ca^{2+} buffered by 5 mM-EGTA); □, ○, Mg^{2+} absent (Ca^{2+} buffered by 5 mM-HEDTA [Mg^{2+}] < 10^{-9} M).

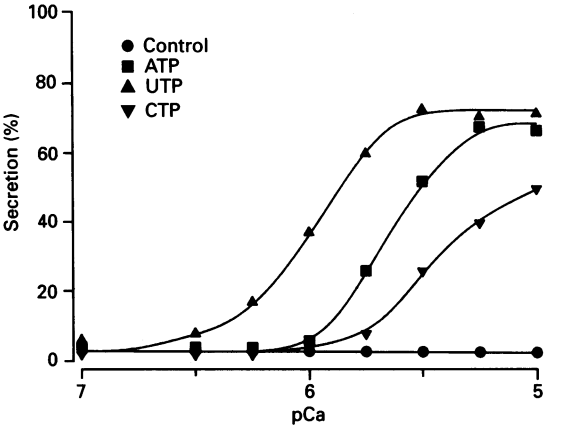

Fig. 2. Dependence on nucleoside triphosphates of the exocytosis stimulated by Ca^{2+} from mast cells permeabilized in glutamate

Mast cells, suspended in buffered iso-osmotic sodium glutamate containing Mg^{2+} (2 mM) and pretreated with metabolic inhibitors, were incubated with SL-O, Ca^{2+} (buffered at the concentrations indicated) and nucleoside triphosphates [●, control; ■, ATP (1 mM); ▲, UTP (1 mM); ▼, CTP (1 mM)]. At the end of 10 min the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase.

If, instead of using simple uni/univalent salt solutions, the cells are permeabilized in iso-osmotic solutions of D- or L-glutamate or L-aspartate, then it is possible to induce secretion in response to either Ca^{2+} (in the high range, pCa 6–pCa 5, in the absence of added guanine nucleotide) or GTP[S] (100 μM, at vanishingly low levels of Ca^{2+}) (Churcher & Gomperts, 1990). Such single-effector-induced secretion requires the presence of MgATP. We have obtained similar results following permeabilization of the cells in iso-osmotic glycine or GABA (not shown). Other nucleoside triphosphates such as UTP and CTP (see Fig. 2) can substitute for ATP in the support of secretion mediated by Ca^{2+} in the glutamate environment. The finding that secretion is supported by a range of nucleosides suggests the involvement of nucleoside diphosphate kinase, since the well-known protein kinases all appear to require ATP (Lillie *et al.*, 1991).

The data presented in Fig. 1(*b*) support the idea that the ability of GTP to sustain secretion in zwitterionic buffers reflects an increase in the sensitivity to GTP over that which pertains in Cl^--based buffers. Firstly, there is a marked enhancement in the sensitivity to both GTP[S] (EC_{50} shifted from 1.8×10^{-6} M to $\sim 10^{-7}$ M) and GTP (in the absence of Mg^{2+}; EC_{50} shifted from $\sim 10^{-5}$ M to $\sim 1.8 \times 10^{-6}$ M) when glutamate is the main electrolyte anion. Second, when glutamate is the major anion, low concentrations of GTP provide a strong stimulus to secretion even in the presence of Mg^{2+} ($EC_{50} \sim 1.8 \times 10^{-5}$ M) (cf. Fig. 1*a*). In chloride (with 2 mM-Mg^{2+}), very high concentrations of GTP (i.e. in the millimolar range) are required to induce secretion and even at the highest Ca^{2+} concentration (pCa 5) the maximum extent of release does not exceed 60% (Howell *et al.*, 1987). The simplest explanation of this second observation is that, in addition to enhancing the affinity for guanine nucleotides, glutamate suppresses the Mg^{2+}-dependent GTPase activity of G_E compared with Cl^-.

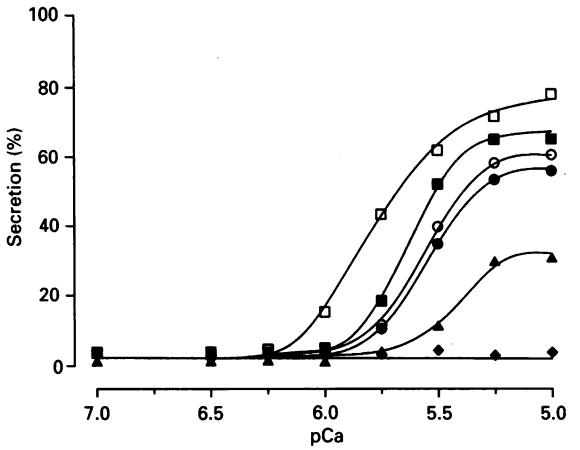

Fig. 3. Ca^{2+}-induced (ATP-dependent) secretion in glutamate-based buffer is enhanced by low concentrations and inhibited by high concentrations of GDP

Mast cells, suspended in iso-osmotic glutamate-based buffers and pretreated with metabolic inhibitors, were incubated with SL-O, 1 mM-MgATP, Ca^{2+} (buffered at the concentrations indicated), Mg^{2+} (1 mM) and GDP at various concentrations. At the end of 10 min the cells were sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase. GDP: ●, none; ○, 10 μM; ■, 50 μM; □, 100 μM; ▲, 500 μM; ◆, 1000 μM.

The key role of G_E in the regulation of exocytosis from permeabilized mast cells is made clear by the results illustrated in Fig. 3. The experiment tests the susceptibility of Ca^{2+}-only-induced responses to modulation by GDP. Perhaps somewhat surprisingly, both the extent of secretion and the $EC_{50}(Ca^{2+})$ are suppressed by concentrations of GDP in excess of 100 μM. At concentrations below this the effect of GDP is actually to enhance the extent of Ca^{2+}-induced (ATP-dependent) secretion. It appears that it is GDP that limits the extent of secretion. Obviously a high proportion of GDP is rapidly dissipated from the cells along with all other free aqueous solutes following permeabilization. We conclude that GTP remains an absolute requirement and that the addition of exogenous GDP at low concentrations allows more GTP to be formed by trans-phosphorylation, and thus enhances secretion above the 60% level. The ability of Ca^{2+} to stimulate secretion in the absence of added guanine nucleotide becomes apparent only because of the increased sensitivity to formed GTP in the zwitterionic electrolyte environment (even in the presence of Mg^{2+}). The inhibition of secretion at the higher concentrations of GDP then probably occurs as a result of competition at the GTP-binding site (though in a previous investigation the non-metabolic-guanosine 5′-[β-thio]diphosphate conspicuously failed to cause inhibition at 1 mM (Churcher & Gomperts, 1990)].

In contrast with Ca^{2+}, GTP[S] can stimulate a true *single effector* secretion. This occurs in the absence of ATP and in the effective absence (10^{-9} M) of Ca^{2+}. Fig. 4 shows time courses of secretion due to GTP[S] (100 μM) over a range of Ca^{2+} concentrations (pCa 9–5) in the presence and absence of Mg^{2+} (2 mM). Note that in these experiments the trigger was applied simultaneously with the permeabilizing agent (SL-O), and so any delays preceding the onset of secretion must also register a time artefact (< 20 s) of permeabilization. The reason for adopting this procedure was that in the glutamate system (especially in the absence of Mg^{2+}) the permeabilized cells lose secretory competence much more rapidly than they do in Cl^- media, and so it is not possible to allow a period (1 min) for equilibration with one effector before applying the second as a trigger. For this reason more detailed kinetic studies have been performed in Cl^- media (Lillie & Gomperts, 1992; T. H. W. Lillie & B. D. Gomperts, unpublished work). Using HEDTA (5 mM) to suppress the concentrations of both bivalent cations (approx. pCa 8), a small amount of secretion commenced after an extended delay of about 150 s, eventually achieving about 3%. In other more prolonged incubations (10 min) we found that such Ca^{2+}- and Mg^{2+}-deprived cells could achieve no more than about 5% secretion. When Mg^{2+} was supplied (1 mM), and using EGTA as a buffer to achieve an even lower concentration of Ca^{2+} (5 mM-

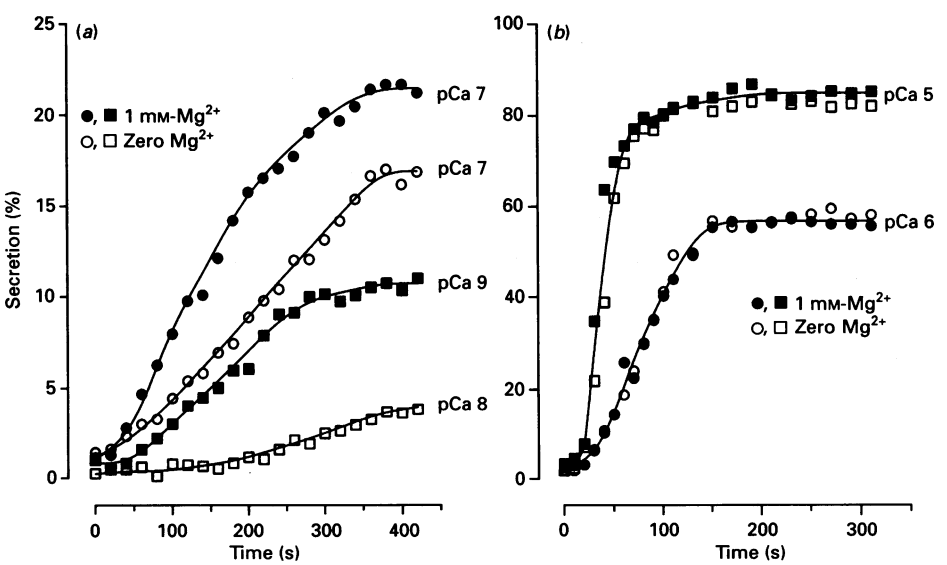

Fig. 4. Time course of exocytosis showing the effect of Ca^{2+} concentration on the dependence of secretion on Mg^{2+}

Mast cells, suspended in glutamate-based buffers and pretreated with metabolic inhibitors, were triggered to secrete by addition of SL-O together with 100 μM-GTP[S] and Ca^{2+} buffers (pCa 5, 6, 7 or EGTA/HEDTA alone) in the presence (5 mM-EGTA-buffered; ●, ■) or absence (5 mM-HEDTA-buffered, ○, □) of Mg^{2+} (1 mM). Samples were removed at 10 s (pCa 5 and 6) or 20 s (pCa 7 and EGTA) intervals and quenched in ice-cold iso-osmotic NaCl. The cells were then sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase.

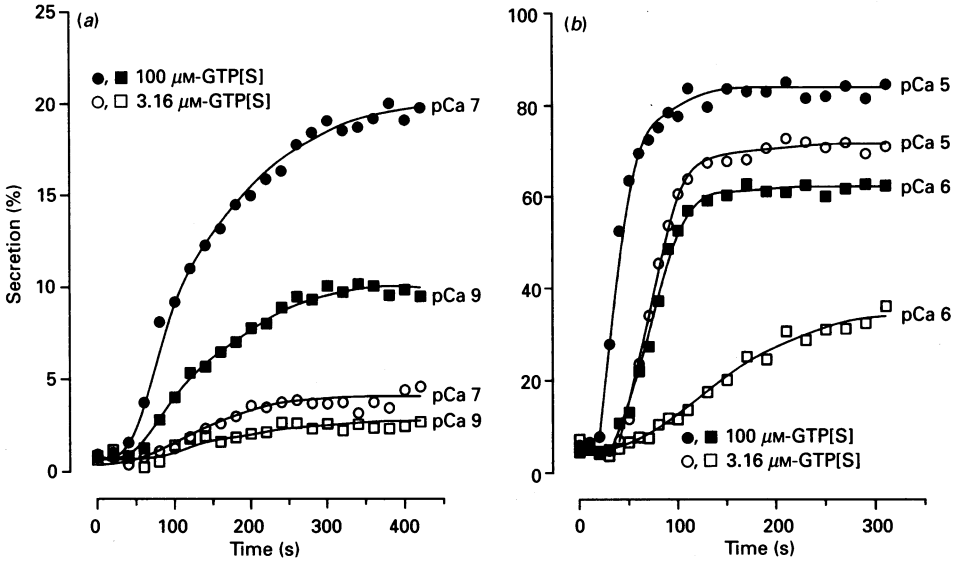

Fig. 5. Time course of exocytosis showing the effect of Ca^{2+} concentration on the dependence of secretion on GTP[S]

Mast cells, suspended in glutamate-based buffers and pretreated with metabolic inhibitors, were triggered to secrete by addition of SL-O together with EGTA Ca^{2+} buffer (5 mM; pCa 5, 6, 7 or EGTA alone) and 1 mM-Mg^{2+} in the presence of 100 μM (●, ■) or 3.16 μM (○, □) GPS[S]. Samples were removed at 10 s (pCa 5 and 6) or 20 s (pCa 7 and EGTA) intervals and quenched in ice-cold iso-osmotic NaCl. The cells were then sedimented by centrifugation and the supernatant sampled for analysis of secreted hexosaminidase (an identical result was obtained with 2 mM-Mg^{2+}).

EGTA, ~ pCa 9), the secretion commenced earlier (within 50 s), and in this experiment terminated after about 300 s at 10%. As the Ca^{2+} concentration was raised, secretion commenced earlier and the enhancing effect of Mg^{2+} became progressively diminished, so that it was without effect at pCa 6 as shown in Fig. 4(*b*). In addition, with the elevation of Ca^{2+} concentration the rate of secretion became faster and the delays preceding commencement were further decreased.

We have been unable to determine whether the requirement for Mg^{2+} (in the absence of Ca^{2+}) should be regarded as being absolute. It is possible that the low-level secretion (~ 5%) that occurs in the absence of Mg^{2+} (suppressed by HEDTA to $< 10^{-9}$ M) is a reflection of the higher free concentration of Ca^{2+} (pCa 8) that remains when HEDTA rather than EGTA is used as the chelator. This small amount of Ca^{2+} might relieve the dependence on Mg^{2+} just sufficiently to allow the very low level of exocytosis that we observe. What is clear is that, in the absence of Ca^{2+}, Mg^{2+} strongly promotes GTP[S]-induced secretion. Furthermore, the two activating cations interact at separate binding sites. Evidence for this comes from the observation that secretion is inhibited when the concentration of Mg^{2+} is raised above 10 mM; under these conditions, Ca^{2+} (pCa 6) can still initiate GTP[S]-induced secretion (results not shown).

The absolute dependence of secretion on GTP[S] was maintained at all concentrations of Ca^{2+}, although as the concentration of Ca^{2+} was increased from pCa 9 to pCa 7 (Fig. 5*a*) and from pCa 6 and pCa 5 (Fig. 5*b*) the extent of secretion at any given concentration of GTP[S] became greater and the time taken for completion was shorter. In this experiment the concentration of Mg^{2+} was fixed at 1 mM. As in the experiment of Fig. 4, raising the concentration of Ca^{2+} systematically decreased the delay before the onset of secretion. The effect of Ca^{2+} appears to be primarily in the control of the sensitivity to the guanine nucleotide.

Unlike the non-hydrolysable GTP[S], when GTP was used as the stimulating ligand, high concentrations of Mg^{2+} were unable to substitute for Ca^{2+} even for cells permeabilized in glutamate (results not shown). The reason for this is most probably due to the activity of a GTPase. Once again, a very low concentration (pCa 7) sufficed to induce exocytosis.

DISCUSSION

From the results presented here, it is apparent that regulated exocytosis from rat mast cells permeabilized with SL-O has an absolute requirement for a guanine nucleotide. This holds true regardless of whether the cells are permeabilized in simple uni/univalent (NaCl) or in zwitterionic electrolytes (glutamate, glycine, GABA etc.). In glutamate, however, we have found that a hydrolysis-resistant guanine nucleotide (GTP[S], but not GTP) can stimulate a true single-effector secretion of low magnitude in the effective absence of Ca^{2+} (pCa 9) (Figs. 4*a* and 5*a*). This is strongly promoted by Mg^{2+}, but we have been unable to demonstrate that this is an absolute requirement.

Previous investigations on single mast cells using the patch–clamp technique to monitor the increase in capacitance caused by fusion of secretory granules have repeatedly shown that exocytosis can be achieved by infusion of GTP[S] together with EGTA (i.e. at a vanishingly low cytosol concentration of Ca^{2+}) (Fernandez *et al.*, 1984; Penner *et al.*, 1987; Neher, 1988) or by Ca^{2+} (at concentrations greater than pCa 6) in the absence of added guanine nucleotide (Penner & Neher, 1988). These results were all achieved under circumstances in which we would now predict conditions of high affinity for the guanine nucleotide (due both to the presence of ATP and to the use of an intracellular dialysing solution formulated with glutamate). Reconstitution experiments using nucleoside diphosphate kinase have indicated the possibility of direct phosphorylation of GDP bound to monomeric GTP-binding proteins (ADP-ribosylating factor; Randazzo *et al.*, 1991), and, with less certainty (Kikkawa *et al.*, 1991), to heterotrimeric GTP-binding proteins (G_s and G_o; Kikkawa *et al.*, 1990). There is some evidence that in rat liver membranes this enzyme and G_s are closely associated (Kimura & Shimada, 1990). These observations, taken together with the results described here, suggest that it is unlikely that ATP-dependent Ca^{2+}-induced exocytosis from either permeabilized or

patch-clamped cells is ever truly independent of guanine nucleotide.

Further support for an important role for GTP in Ca^{2+}-induced secretion from intact cells has previously been indicated by manipulations designed to cause selective depletion of guanine nucleotides. Mast cells treated with mycophenolic acid or ribavirin [inhibitors of inosine monophosphate dehydrogenase, and hence of the purine nucleoside salvage pathway (Yamada *et al.*, 1988)] failed to respond not only to IgE-directed ligands, but also to Ca^{2+} ionophores (Marquardt *et al.*, 1987; Wilson *et al.*, 1988).

Our results also extend previous findings concerning the central role of guanine nucleotides in the stimulation of exocytosis from other myeloid granulocytes. We previously reported that, whereas GDP inhibits azurophilic granule exocytosis stimulated by Ca^{2+} from rabbit neutrophils permeabilized by treatment with *Sendai* virus, GTP[S] can induce exocytosis at concentrations of Ca^{2+} as low as 10^{-10} M (Barrowman *et al.*, 1986). From this we inferred an essential role for a G-protein, G_E (Gomperts *et al.*, 1986). GTP[S] also induces extensive Ca^{2+}-independent secretion of hexosaminidase from guinea pig eosinophils permeabilized by SL-O in glutamate; in these cells, Ca^{2+} by itself is unable to induce secretion even when ATP is provided (Nusse *et al.*, 1990).

Our results stand in contrast to those concerning the role of GTP in the constitutive interactions of the various pathways of vesicular traffic in endocytosis and exocytosis (Melançon *et al.*, 1987; Salminen & Novick, 1987; Miller & Moore, 1991). The GTP-binding proteins determining directionality and accuracy in the constitutive pathway of secretion of mammalian cells appear to be members of the rab family (Goud *et al.*, 1990; Chavrier *et al.*, 1990; Balch, 1990; Fischer von Mollard *et al.*, 1990; Südhof & Jahn, 1991). It is widely accepted that the hydrolysis of GTP, by allowing GTP-binding proteins (e.g. SEC4, rab3a) to cycle repeatedly between membrane-bound and free states, enables a continuous delivery of products along a succession of compartments and finally to the eventual point of release (Bourne, 1988; Novick *et al.*, 1988; Bacon *et al.*, 1989; Walworth *et al.*, 1989; Bourne *et al.*, 1990; Fischer von Mollard *et al.*, 1991; Miller & Moore, 1991).

At a functional level at least, our observations, especially the various effects of Mg^{2+}, recall the activation of adenylate cyclase. As with the stimulation of secretion from permeabilized mast cells, a low-level stimulation of adenylate cyclase can be induced by non-hydrolysable analogues of GTP in the absence of an activated hormone receptor but in the presence of Mg^{2+} (Iyengar, 1981; Iyengar & Birnbaumer, 1981, 1982). When an activating ligand for the receptor is supplied, the extent and rate of cyclic AMP production are markedly increased and the dependence on Mg^{2+} is diminished 1000-fold (Iyengar & Birnbaumer, 1982). Similarly, in the activation of exocytosis, Ca^{2+} increases the rate and extent of secretion, and more importantly brings about a systematic enhancement in the affinity for GTP[S] and Mg^{2+}. In these respects at least, G_E appears to operate in a similar manner to G_s in that it is able to stimulate its target (the fusion apparatus) on provision of a non-hydrolysable guanine nucleotide in the absence of a receptor-directed ligand (i.e. Ca^{2+}) so long as Mg^{2+} is present. The kinetic pattern of activation of G_E (as measured by secretion in Cl^--based buffers), which is characterized by a Mg^{2+}-dependent onset delay, also has some similarity with activation of G_s (Lillie & Gomperts, 1992; T. H. W. Lillie & B. D. Gomperts, unpublished work).

The receptor-linked GTP-binding proteins have also been shown to be sensitive to the anionic environment (Higashijima *et al.*, 1987). However, for those that have been examined, the effects are the opposite to that which we have inferred regarding the interaction of Cl^- with G_E. In the case of G_o, Cl^- inhibits the GTPase function while enhancing the affinity for both GTP and GTP[S] via an effect on the rate of dissociation. Similar mechanisms may underlie the enhancing effect of chloride and azide on the stimulation of adenylate cyclase by hormones, fluoride and GTP analogues (Johnson *et al.*, 1975; Svoboda & Christophe, 1978). Either way, the effect of Cl^- in all of these systems [including exocytosis (Churcher & Gomperts, 1990)] is expressed at low concentrations (< 20 mM), and on the basis of investigations on G_o, a binding site for the Cl^- ion has been suggested (Higashijima *et al.*, 1987). So far as we are aware, there are no published data concerning the effect of glutamate (or other zwitterions) on the activation of adenylate cyclase or isolated GTP-binding proteins.

We would like to propose that guanine nucleotide exchange on G_E is catalysed by Ca^{2+} following binding to a calcium binding protein (C_E) which has the characteristics of an intracellular pseudo-receptor. When this is stimulated (by Ca^{2+}) then the requirement for Mg^{2+} is obviated. Not only does the stimulated pseudo-receptor preclude the requirement for Mg^{2+} but it also increases the extent of the secretory response by at least 10-fold, enhancing the response to any given concentration of guanine nucleotide. Although the low-molecular-mass GTP-binding proteins related to ras are widely understood to be regulated by soluble exchange proteins (Bourne *et al.*, 1990) located in the cytosol, the signal-transducing G-proteins of the heterotrimeric class are, in general, subject to regulation by cell surface receptors. An exception to this rule may be the growth-associated protein (GAP-43) of neurite growth cones, which can regulate the binding affinity for GTP[S] of G_o when these are reconstituted together *in vitro* (Strittmatter *et al.*, 1990). If the interpretation of our data is correct, and C_E can eventually be shown to have the properties of a pseudo-receptor acting in series with G_E, then this will represent another example of an intracellular protein controlling the affinity of a signal-transducing G-protein.

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