ATP-dependent and ATP-independent pathways of exocytosis revealed by interchanging glutamate and chloride as the major anion in permeabilized mast cells

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Most investigations of the mechanism of regulated exocytosis have involved the use of secretory cells permeabilized in glutamate-based electrolyte solutions. In our previous work we have used NaCl-based electrolyte solutions. For secretion to occur from rat mast cells under these latter conditions, a dual effector system comprising Ca^{2+} and a guanine nucleotide are required; together they are sufficient. Here we compare the secretion from mast cells permeabilized in solutions of different electrolytes. Replacement of Na^+ by K^+ had little effect. Replacement of Cl^- by Br^- , SO_4^{2-} , gluconate, isethionate, acetate, tartrate, succinate, etc. affected the maximal extent of secretion elicited by the dual effectors Ca^{2+} and guanosine-5'-O-(3-thiotriphosphate) (Ca^{2+} -plus-GTP- γ -S) but had little influence on the effective affinity for Ca^{2+} . The dicarboxylic amino acids (L- and D-glutamate, and L-aspartate) permitted exocytosis to be elicited by Ca^{2+} or GTP- γ -S alone. Secretion stimulated by GTP- γ -S is strongly inhibited by Cl^- (50% inhibition by 20 mM Cl^-), whereas the extent of Ca^{2+} -induced secretion is proportional to the concentration of glutamate in mixed electrolyte buffers. Unlike dual-effector stimulation, secretion due to the single effectors requires adenosine triphosphate (ATP) and is prevented by inhibitors of protein kinase C. These results point to the existence of two parallel pathways for control of exocytosis in permeabilized cells, one ATP dependent, the other ATP independent.

Introduction

Techniques of selective plasma membrane permeabilization have been instrumental in advancing the understanding of the mechanism of regulated exocytotic secretion (Gomperts and Fernandez, 1985; Knight and Scrutton, 1986).

By permeabilizing cells it is possible to regulate the concentration of otherwise impermeant effector molecules in the cytosol with precision and thus to test their propensity to stimulate or to modulate secretion. In this respect, most attention has been devoted to the effects of Ca^{2+} , guanine nucleotides, cyclic nucleotides, and adenosine triphosphate (ATP) as plausible effectors and modulators of the exocytotic mechanism in a wide range of different cell types (Gomperts, 1990). However, there are many other soluble components in the cytosol, ranging from electrolytes to proteins, the effect of which may be far from negligible. Because the mobile cations and anions that constitute the electrolyte in the cytosol are rapidly and infinitely diluted on permeabilization, it is necessary to give consideration to the composition of the permeabilizing medium. After initial studies of exocytosis from permeabilized adrenal chromaffin cells (Knight and Baker, 1982), in which it was found that the Cl^- anion inhibits Ca^{2+} -induced catecholamine secretion, most investigators in this field have employed glutamate (as the potassium salt) as the main electrolyte anion.

In our work on permeabilized rat mast cells, we have habitually used NaCl as the main electrolyte (Howell and Gomperts, 1987) for the reason that the presence of glutamate prevents the subsequent determination of secreted histamine by the o-phthalaldehyde coupling technique. The measurement of lysosomal enzyme release is an equally valid way of assessing mast cell secretion (Schwartz *et al.*, 1979) and has the advantage that it is not subject to this form of interference. For mast cells permeabilized in NaCl-based buffer solutions, activation of binding proteins for both Ca^{2+} and guanosine triphosphate (GTP) (G_E , a GTP-binding protein mediating exocytosis: Gomperts, 1990) is necessary to induce release of histamine and β -N-acetylglucosaminidase (hexosaminidase) (Howell *et al.*, 1987). Together, Ca^{2+} and GTP

are sufficient (Howell *et al.*, 1987), and ATP serves only as a modulator by regulating the effective affinity for these two effectors, probably by means of phosphorylation reactions mediated by protein kinase C (Cockcroft *et al.*, 1987; Howell *et al.*, 1989). It also retards the onset of secretion (Gomperts and Tatham, 1988; Tatham and Gomperts, 1989). In this paper we examine the effects of alternative electrolyte systems on the secretory process of mast cells: whereas most of these have little effect other than altering the maximal extent of lysosomal enzyme secretion (compared with NaCl), glutamate and aspartate stand out in allowing secretion to occur in the presence of either Ca^{2+} or guanosine-5'-O-(3-thiotriphosphate) (GTP- γ -S)¹ alone. Under these conditions provision of ATP becomes mandatory.

Results

Figure 1 illustrates the dependence on Ca^{2+} of secretion of hexosaminidase from mast cells permeabilized by streptolysin-O (SL-O) in the presence of GTP- γ -S (100 μM), ATP (1 mM), and a series of different electrolyte solutions. Of the electrolytes tested, only KSCN was unable to support the secretory process. The effect of substituting K^+ for Na^+ was slight, but substitution of other anions for chloride caused a diminution in the maximal extent of secretion in the order chloride > sulphate > L-glutamate > isethionate > bromide > acetate. In this series, with the exception of glutamate, the effective affinity (EC_{50}) for Ca^{2+} in the secretory process fell within 0.25 of a $-\log_{10}[\text{Ca}^{2+}]$ (pCa) unit, and secretion declined to zero as the concentration of Ca^{2+} was reduced to pCa 7. In a separate experiment devised to test the effects of further anions, we found that secretion remained dependent on Ca^{2+} (EC_{50} again similar to Cl^-) and maximal release in the order tartrate > succinate > Cl^- > gluconate (data not shown).

When the cells were permeabilized in the presence of Na-L-glutamate, a modest extent of secretion (20% in the experiment illustrated in Figure 1) was maintained even when the concentration of Ca^{2+} was reduced to $\sim\text{pCa} 8.5$ (3 mM ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid [EGTA]; no added

Figure 1. Effect of various electrolyte solutions on Ca^{2+} -plus-GTP- γ -S-induced secretion from rat mast cells permeabilized by streptolysin-O. Mast cells prepared in appropriate isotonic buffer solutions, and pretreated with metabolic inhibitors, were incubated for 10 min together with streptolysin-O (0.4 iu $\cdot\text{ml}^{-1}$), ATP (1 mM), GTP- γ -S (100 μM), and 3 mM Ca^{2+} -buffer to regulate pCa as indicated. The cells were sedimented and the supernatants assayed for secreted hexosaminidase.

calcium). Similar responses were obtained when the cells were permeabilized in an isotonic solution of D-glutamate or L-aspartate (not shown) and so it is most unlikely that the effect of the acidic amino acids might be mediated through a specific receptor system. The following experiments were devised to characterize in greater detail the phenomenon of the GTP- γ -S-induced (Ca^{2+} -independent) or Ca^{2+} -induced (GTP-independent) secretion that occurs in the glutamate-based electrolyte system.

Figure 2 presents results of an experiment in which we compared the Ca^{2+} and GTP- γ -S dependence for secretion in the glutamate- and chloride-based electrolyte systems for cells permeabilized in the presence of ATP (1 mM). The characteristics of secretion in the chloride system are similar to those we have reported previously (Howell *et al.*, 1987; Gomperts and Tatham, 1988). Secretion requires the presence of both effectors, and elevation of the concentration of either Ca^{2+} or GTP- γ -S spares the concentration requirement for the other effector. Thus it can be seen that, as the concentration of GTP- γ -S is elevated from 10^{-7} to 10^{-5} M, the apparent affinity for Ca^{2+} increases from $\sim\text{pCa} 5.75$ to pCa 6.3. For cells permeabilized in a glutamate-based electrolyte solution and stimulated by a high concentration (100 μM) of

¹ Abbreviations: DMSO, dimethyl sulfoxide; EGTA, ethylene glycol-bis(β -aminoethylether)- N,N,N',N' -tetraacetic acid; GTP- γ -S, guanosine-5'-O-(3-thiotriphosphate); hexosaminidase, β -N-acetylglucosaminidase; pCa, $-\log_{10}[\text{Ca}^{2+}]$; PIPES, piperazine- N,N' -bis(2-ethanesulphonic acid); SL-O, streptolysin-O.

Figure 2. Dependence of exocytosis on Ca^{2+} and GTP- γ -S from mast cells permeabilized in NaCl-based buffer and Na-L-glutamate-based buffer. Mast cells, treated as described in the legend to Figure 1, were incubated for 10 min with streptolysin-O ($0.4 \text{ iu} \cdot \text{ml}^{-1}$), ATP (1 mM), and various pCa and GTP- γ -S as indicated in (A) NaCl (137 mM)- or (B) Na-L-glutamate (125 mM)-based buffers. The cells were sedimented and the supernatants assayed for secreted hexosaminidase.

GTP- γ -S, secretion still occurs at concentrations of Ca^{2+} well below pCa 7. High concentrations of Ca^{2+} induce an extent of secretion that is then enhanced by the presence of GTP- γ -S, although (consistent with the data of Figure 1) the maximum extent of secretion at the optimal concentration of GTP- γ -S is somewhat less than for cells permeabilized in NaCl.

The experiment illustrated in Figure 3 was designed to test the role of ATP under conditions in which secretion was stimulated by a single effector. Here we examined the Ca^{2+} -dependence of secretion in the presence and absence of GTP- γ -S (100 μM) and ATP (1 mM) in both electrolyte systems. For the chloride-based buffer, in agreement with previous results (Howell *et al.*, 1987), secretion is markedly dependent on provision of both Ca^{2+} and the guanine nucleotide. When ATP is provided there is an enhancement in the effective affinity for Ca^{2+} , and, in this particular experiment, we observed also a small extent of secretion (10%) at pCa 5 without GTP- γ -S. In the glutamate system, a substantial degree of secretion could be induced by either Ca^{2+} or GTP- γ -S alone, but such single-effector stimulation has an absolute requirement for ATP. Ca^{2+} -induced secretion is clearly discernible within 10 s and complete within 2 min of elevating Ca^{2+} to pCa 5 (data not shown). GTP- γ -S-induced secretion occurs during a much more prolonged timecourse and

is 95% complete by 10 min (i.e., the standard period of incubation in these experiments).

In the absence of ATP, Ca^{2+} alone is without effect, and the extent of GTP- γ -S-induced secretion in the absence of Ca^{2+} (3 mM EGTA), although just significant, is small (10%). Guanosine-5'-O-(2-thiodiphosphate) (GDP- β -S) (1 mM) inhibits secretion due to GTP- γ -S alone (100 μM) and together with low levels of Ca^{2+} , but has no effect on secretion induced by any concentration of Ca^{2+} when this is used as the sole effector (Figure 4). This indicates that Ca^{2+} -induced (ATP-dependent) secretion occurs by a mechanism that obviates involvement of the GTP-binding protein, G_E .

The experiment illustrated in Figure 5 shows that ATP-dependent secretion stimulated by Ca^{2+} acting alone can be fully suppressed by treating the permeabilized cells with a peptide (RFARKGALRQKNV) pseudosubstrate (PKC-I) for protein kinase C (House and Kemp, 1987; Alexander *et al.*, 1989). Very similar results were obtained by treating the cells before permeabilization with palmityl methyl glycerol (AMG.C₁₆, a diether analogue of diglyceride), which inhibits protein kinase C (van Blitterswijk *et al.*, 1987; Howell *et al.*, 1989; Kramer *et al.*, 1989). In contrast, as illustrated in Figure 6, ATP-independent secretion induced by the dual effector system is hardly affected by the inhibitors AMG.C₁₆ and H-7 (we were unable to test

Figure 3. Ca^{2+} -dependence of secretion in the presence and absence of GTP- γ -S and ATP from cells permeabilized in isotonic Na-L-glutamate- and NaCl-based buffers. Mast cells, treated as described, were incubated for 10 min with streptolysin-O ($0.4 \text{ iu} \cdot \text{ml}^{-1}$), various pCa, GTP- γ -S, and ATP (1 mM) as indicated in Na-L-glutamate- or NaCl-based buffers. The cells were sedimented and the supernatants assayed for secreted hexosaminidase. Δ , $\blacktriangle$, GTP- γ -S absent; $\circ$, $\bullet$, GTP- γ -S, 100 μM . Filled symbols indicate Cl^- -based buffer, open symbols indicate glutamate-based buffer.

the peptide inhibitor under conditions of zero ATP, since ATP is required to maintain responsiveness in the permeabilized cells during the time needed to introduce the membrane impermeant inhibitor peptide into the cytosol; Howell *et al.*, 1989). Note that these two compounds (also PKC-I; not shown) also have the effect of reducing the extent of GTP- γ -S-induced (ATP-dependent, Ca^{2+} -independent) secretion. To the extent that these compounds are specific inhibitors of protein kinase C, it can be concluded that ATP-dependent secretion is mediated by protein kinase C.

Previous work has shown that there is no requirement for ATP for stimulation of secretion by the dual effector system (Ca^{2+} -plus-GTP- γ -S) (Howell *et al.*, 1987). For this reason it is pertinent to ask whether the presence of ATP is required at the time of exocytosis for stimulation by a single effector, or whether secretion can persist in cells pretreated with phorbol ester (and therefore presumed to be hyperphosphorylated), and then permeabilized and stimulated in absence of ATP. The experiment illustrated in Figure 7 shows that secretion induced by a single effector requires the presence of ATP (as in Figure 3) but that it must be present at the time of stimulation. Cells pretreated with phorbol myristate acetate and then permeabilized

and stimulated in the absence of ATP failed to secrete. We conclude that, under these conditions of stimulation with a single effector, protein phosphorylation is likely to comprise an essential step in the pathway leading to exocytosis.

The buffer systems used for the experiments described above were based on isoosmotic electrolyte solutions formulated with either 137 mM NaCl or 125 mM Na.glutamate. The only other significant anions in these buffers are the sulfonic acid residue of the pH buffering compound (20 mM piperazine-*N,N'*-bis(2-ethanesulfonic acid) [PIPES]) and Cl^- itself, which is present in the CaEGTA buffers. This rises from 4 to 9.6 mM as the concentration of free Ca^{2+} increases through the range from zero (EGTA) to pCa 5. In the experiment illustrated in Figure 8, we used a series of mixed buffers containing both NaCl and Na.glutamate in varying proportions to examine the concentration dependence of the two individual anions on Ca^{2+} -induced and GTP- γ -S-induced secretion. We tested the dependence on Ca^{2+} for secretion in the presence of ATP (1 mM) and in the presence (solid symbols) or absence (open symbols) of GTP- γ -S (100 μM). In confirmation of results presented above, much higher concentrations of Ca^{2+} were required to induce secretion when GTP- γ -S was absent. Under these conditions there

Figure 4. Selective inhibition of the GTP- γ -S-dependent component of exocytosis by GDP- β -S. Mast cells, treated as described, were incubated for 10 min with streptolysin-O in Na-L-glutamate with ATP (1 mM) and various pCa, GTP- γ -S (100 μM), and GDP- β -S (1 mM) as indicated. $\circ$, $\bullet$, GTP- γ -S 100 μM ; Δ , $\blacktriangle$, GTP- γ -S absent. Closed symbols indicate GDP- β -S, 1 mM.

Figure 5. Inhibition of Ca^{2+} -induced, ATP-dependent secretion by inhibitors of protein kinase C. After metabolic inhibition for 5 min, mast cells in 125 mM Na.glutamate were permeabilized with SL-O in the presence of 0.2 mM calcium buffer, pCa 7, and then after 1 min treated with AMG.C₁₆ (100 μM) or DMSO (0.3%). After a further 4 min they were transferred to mixtures of ATP (100 μM) and 3 mM calcium buffer to regulate Ca^{2+} in the range pCa 7–pCa 5. Alternatively, the cells were pretreated with metabolic inhibitors for 5 min then permeabilized for 3 min and then treated with PKC-I (0.5 mM) for 2 min before stimulation. The reactions were quenched after 10 min incubation and released hexosaminidase was measured as described. $\circ$, control; Δ , PKC-I; $\square$, AMG.C₁₆.

was a progressive enhancement in the extent of Ca^{2+} -induced secretion as the proportion of glutamate in the buffer was increased. When GTP- γ -S was provided, the effect of low concentrations of Ca^{2+} (e.g., pCa 6.5) increased with the proportion of glutamate in the buffer, but it was only when Cl^- was almost totally excluded that we observed an element of Ca^{2+} -independent secretion. In this sense Cl^- may be regarded as an inhibitor of GTP- γ -S-induced (Ca^{2+} -independent) secretion. The experiment illustrated in Figure 9 stresses the effect of Cl^- as an inhibitor of GTP- γ -S-induced (Ca^{2+} -independent) secretion and shows that half-maximal inhibition is achieved at ~ 20 mM Cl^- . If Cl^- were totally excluded, it is likely that the extent of secretion induced by GTP- γ -S alone would approach the level that we have routinely achieved with Ca^{2+} .

Discussion

Nearly all investigations of the exocytotic mechanism in permeabilized cells have involved

experimental procedures using glutamate as the main electrolyte anion. There are a few reports (particularly concerning pituitary cells) in which acetate (Yamamoto *et al.*, 1987), propionate (Rønning and Martin, 1985), and gluconate (Mason *et al.*, 1988) have been used. Apart from our own work (Barrowman *et al.*, 1986, 1987), that of others on neutrophils (Smolen and Stoehr, 1985; 1986) and HL60 cells (Stutchfield and Cockcroft, 1988), and our work on mast cells (Howell and Gomperts, 1987), the use of the chloride anion has generally been eschewed. The reason for this derives from the initial work on permeabilized bovine adrenal medullary (chromaffin) cells (Baker and Knight, 1981; Knight and Baker, 1982), in which it was shown that in comparison with glutamate, Cl^- inhibits Ca^{2+} -induced catecholamine release.

Figure 6. Effect of protein kinase C inhibitors on ATP-dependent and ATP-independent secretion from mast cells permeabilized in a glutamate-based buffer. In this experiment cells were treated according to two separate schemes. The open symbols indicate cells that were pretreated with metabolic inhibitors for 5 min before permeabilization with SL-O (0.4 IU $\cdot$ ml $^{-1}$) in the presence of a low concentration (0.2 mM) of calcium buffer to maintain pCa 7. Inhibitors of protein kinase C (AMG.C₁₆ or H-7, both 100 μM) were added after 1 min and then, after a further 4 min, the cells were transferred to solutions containing GTP- γ -S (100 μM), ATP (100 μM), and calcium buffer (3 mM) to regulate pCa as indicated. Under conditions of metabolic inhibition and prepermeabilization for 5 min, the secretory response becomes dependent on provision of ATP (Howell *et al.*, 1989). The closed symbols indicate cells that were first treated for 4 min with inhibitors of protein kinase C and then directly permeabilized (without metabolic inhibition) and stimulated to secrete in the presence of GTP- γ -S and Ca^{2+} . Under these conditions, there is no requirement for ATP.

Figure 7. Effect of phorbol ester pretreatment and ATP depletion on exocytosis induced by Ca^{2+} from mast cells permeabilized in Na-L-glutamate-based buffer. Mast cells were treated with phorbol myristate acetate (100 nM) (or DMSO control) for 3 min, and then with metabolic inhibitors for a further 2 min before permeabilization in the presence or absence of ATP (1 mM) and a range of pCa (GTP- γ -S absent). Closed symbols indicate presence of ATP: ● ○, PMA present; ▲ △, PMA absent.

Physiological justification for this choice lies in the fact that for most cell types the Cl^- anion is a relatively minor component of the cytosol, although in resting human myeloid cells (neutrophils and monocytes) rather high concentrations of Cl^- (~100 mM) have been recorded (Ince *et al.*, 1987). Although it has not been measured directly in mast cells, a high Cl^- concentration would be consistent with the low value of the resting membrane potential [~ -20 mV (Bronner *et al.*, 1989)].

As with the mast cells (this paper), substitution of Na^+ for K^+ in bovine chromaffin cells (Knight and Baker, 1982) had little effect. Likewise, high concentrations of thiocyanate were found to cause total suppression of secretion, whereas Br^- was intermediate. However, the effect of acetate was only marginal in the chromaffin cells. It appeared that inhibition of catecholamine release by anions follows the lyotropic series and most likely arises from disordering (chaotropism), which could be expected to affect protein-protein or protein-membrane interactions (Adam, 1941; Glasstone, 1946). A similar inference was drawn to explain the observation that the affinity for Ca^{2+} in exocytosis from isolated sea urchin egg cortices is high in solutions of glycine, acetate, gluconate, ϵ -aminocaproate, or glutamate but is much lower in solutions of Cl^- , Br^- , I^- , or NO_3^- (Sasaki, 1984). High affinity for Ca^{2+} could be restored to inhibited egg cortices by provision of proteins released by treatment with KCl.

To elicit secretion from mast cells permeabilized in NaCl-based electrolyte solutions, a dual effector system comprising both Ca^{2+} and a guanine nucleotide is required (Howell *et al.*, 1987; Gomperts and Tatham, 1988). Given stimulation by the dual effectors, substitution of isotonic Cl^- by thiocyanate causes total suppression of secretion. However, most of the other anionic substitutions had little effect beyond altering the maximal extent of secretion (tartrate > succinate > Cl^- > SO_4^{2-} > Br^- > isethionate > acetate). Although the available data are too sparse to be able to make a meaningful comparison of the effects of different anions on mast cells and other systems in general, it is already apparent that the rank orders for suppression of secretion from mast cells on the one hand, and chromaffin cells and sea urchin cortices on the other, are quite different.

In comparison with the simple anions, the effect of glutamate on the exocytotic reaction from mast cells falls into a different category. Although the maximal extent of secretion inducible by optimal concentrations of the dual effectors (Ca^{2+} -plus-GTP- γ -S) is somewhat less

Figure 8. Characteristics of Ca^{2+} -induced, and GTP- γ -S-induced secretion from mast cells permeabilized in buffers formulated from mixtures of NaCl and Na-L-glutamate. Mast cells, prepared in mixtures of NaCl- and Na-L-glutamate-based buffers, were incubated for 10 min with streptolysin-O, ATP (1 mM), GTP- γ -S (100 μM), and 3 mM Ca^{2+} buffers to regulate pCa as indicated. Electrolyte concentrations (mM), (glutamate: Cl^-): ● ○, 125:0; ■ □, 94:35; ▲ △, 63:69; ◆ ◇, 32:103; ▼ ▽, 0:137. Solid symbols indicate secretion in the presence of GTP- γ -S (100 μM); open symbols, GTP- γ -S absent.

Figure 9. Inhibition of GTP- γ -S-induced (Ca^{2+} -independent) secretion by low concentrations of Cl^- . Mast cells, prepared in mixtures of NaCl- and Na-L-glutamate-based buffers, were incubated for 10 min with streptolysin-O, ATP (1 mM), EGTA (3 mM, to suppress Ca^{2+} to below pCa8), and GTP- γ -S (100 μM).

than can be elicited in the presence of NaCl, secretion can also be induced by Ca^{2+} or GTP- γ -S acting alone. For this to occur, ATP must be present at the time of stimulation. Experiments with three structurally unrelated inhibitors indicate that a phosphorylation reaction catalyzed by protein kinase C is likely to be an integral step when exocytosis is stimulated by Ca^{2+} alone. This is quite unlike the situation that pertains when the cells are stimulated by the dual effector system. In this case there is no requirement for ATP and hence no possibility of an obligatory phosphorylation; indeed, on the basis of kinetic experiments, we have suggested that a dephosphorylation reaction might comprise one of the enabling steps (Gomperts and Tatham, 1988; Tatham and Gomperts, 1989). When the dual stimulus (Ca^{2+} -plus-GTP- γ -S) is applied, phorbol ester pretreatment enhances the effective affinity for both effectors, even when the cells are then deprived of ATP at the time they are permeabilized (Howell *et al.*, 1989). To elicit secretion by either Ca^{2+} or GTP- γ -S alone, phorbol ester pretreatment is without effect unless ATP is present so that phosphorylation can occur at the time of stimulation.

When exocytosis is triggered by single effectors, the contributions of Cl^- and glutamate are different, depending on whether stimulation is by Ca^{2+} or by GTP- γ -S. When a Ca^{2+} stimulus

is provided (i.e., no guanine nucleotide) the extent of secretion decreases progressively as the proportion of chloride is increased. Secretion is strictly dependent on the presence of glutamate because there is no reaction when it is totally excluded. For GTP- γ -S-induced secretion, the effect of chloride is better regarded as that of an inhibitor of secretion; 40 mM Cl^- , a concentration probably well below the physiological level (Ince *et al.*, 1987), is sufficient to cause full suppression of secretion. For these reasons it appears that there are at least two different effects of the anions on secretion, which become individually apparent depending on the nature of the stimulating effector.

We are not aware of any systematic investigation of the effects of anions on the binding of Ca^{2+} by calcium-binding proteins. However, specific effects of anions on G-protein functions have been described (Higashijima *et al.*, 1987). Thus, both Cl^- and Br^- increase the affinity of G_{α} for GTP- γ -S and GTP, whereas SO_4^{2-} decreases affinity and F^- , I^- , and NO_3^- have little effect. The enhancing effect of Cl^- is expressed at concentrations in the range 3–20 mM (i.e., probably below the normal range of intracellular Cl^- concentrations in myeloid cells [Ince *et al.*, 1987]), and a specific binding site on the G-protein has been inferred. These phenomena are not confined to G_{α} and may well explain the enhancing effect of chloride and azide on the stimulation of adenylyl cyclase by hormones, fluoride, and GTP-analogues (Johnson *et al.*, 1975; Svoboda and Christophe, 1978). How this relates to our observations is far from clear, especially because Cl^- inhibits GTP- γ -S-induced (Ca^{2+} -independent) secretion, but—bearing in mind the strong sequence homologies that exist between all G-protein α -subunits and also the monomeric GTP-binding proteins—a direct anion effect at the level of G_E remains a possibility. The categorization of G_E as a member of either of these general classes of GTP-binding proteins so far has not been properly established (Gomperts *et al.*, 1990).

The results presented here lend further complexity to an already complex picture of control in the exocytotic pathway. For the moment they relate only to the mast cell, and it is not possible at this stage to perceive their general or particular relevance to other secretory systems. In one important respect, our results allow a resolution of a controversy concerning the role, essential or not, of Ca^{2+} as an effector of exocytosis in mast cells. In all reports involving the application of patch-clamp techniques to monitor exocytosis in mast cells, it has been found

that exocytosis can be induced by GTP- γ -S alone. Ca^{2+} is not required, and the membrane capacitance increases (registering membrane expansion due to fusion of secretory granules) even when EGTA is included as a component in the pipette filling solution (Fernandez *et al.*, 1984; Neher, 1987; Neher and Penner, 1988; Penner *et al.*, 1987). In the light of the present experiments, this can now be understood since these investigations have universally involved the use of pipette buffers formulated with glutamate. Agreement is also achieved concerning the efficacy of the Ca^{2+} -alone stimulus, previously shown to induce degranulation at high (in the range pCa6–pCa5) concentrations in patch-clamped cells (Penner and Neher, 1988).

However, further complication arises from the finding that secretion stimulated by single effectors is dependent on the presence of ATP. Because inhibition of protein kinase C prevents secretion, we understand that a protein phosphorylation reaction is likely to comprise an integral step in this route to exocytosis. This contrasts completely with the situation that pertains when a dual stimulus is applied. Here, cells previously treated with metabolic inhibitors to the point of being refractory to stimulation by receptor-directed agonists (Howell *et al.*, 1987) or Ca^{2+} -ionophores (unpublished results) remain responsive to late provision of effectors for several minutes after permeabilization with SL-O (Howell *et al.*, 1989). Under these conditions protein phosphorylation cannot occur, and therefore it does not comprise a step in the terminal pathway.

The present investigation offers no guidance concerning the identity of the substrate(s) of the protein kinase C reaction after stimulation by single effectors. After antigen stimulation of RBL-2H3 cells, both the light and heavy chains of myosin become phosphorylated by protein kinase C to an extent that correlates closely with the extent of secretion (Ludowyke *et al.*, 1989). This could have the effect of reducing the affinity for myosin light chain kinase with a consequent reduction in filament stability, thus permitting the approach of granules to the plasma membrane.

We conclude that at least two parallel pathways for the control of exocytosis coexist in the permeabilized mast cell. One of these appears to involve phosphorylation; the other is phosphorylation-independent and may even involve protein dephosphorylation (Gomperts and Tatham, 1988; Tatham and Gomperts, 1989). Support for the idea of two regulatory pathways comes from the finding that, whereas secretion

stimulated by crosslinking of IgE-receptors occurs independently of a late-acting G-protein (G_E) (Ishizaka, 1989; Saito *et al.*, 1989), the receptors for ligands such as compound 48/80 appear to act late in the exocytotic pathway, directly with G_E (Aridor and Sagi-Eisenberg, manuscript submitted).

Methods

Mast cells were obtained by peritoneal lavage of male Sprague-Dawley rats. They were purified to near homogeneity by centrifugation through a 2-ml cushion of PERCOLL (Pharmacia Ltd., Milton Keynes, Bucks, UK) as previously described (Tatham and Gomperts, 1990) and washed twice by centrifugation in appropriate isotonic electrolyte solutions. These were initially prepared as 1.36-M stocks containing 0.2 M PIPES (nominal 10 $\times$ final concentrations). The pH was adjusted to pH 6.8 by further addition of PIPES or NaOH. For use with cells these stocks were diluted in water to concentrations equivalent to 290mOsm measured on a WESCOR model 5500 vapor pressure osmometer (Wescor Inc., Logan, UT, USA). Bovine serum albumin (1 mg $\cdot$ ml $^{-1}$) was added and the pH readjusted to 6.80.

Secretion was initiated after pretreating the cells with metabolic inhibitors (6 mM 2-deoxyglucose and 5 μ M antimycin A) for 5 min to reduce intracellular ATP. They were then added to solutions at 37 $^\circ$ containing SL-O (permeabilizing agent, 0.4 iu $\cdot$ ml $^{-1}$ final), CaEGTA buffers (EGTA final concentration 3 mM), GTP- γ -S, and ATP as indicated in the figures and text. These were prepared from concentrated stocks by dilution in appropriate electrolyte solutions. Reactions were terminated after 10 min by addition of ice-cold NaCl (0.15 M) buffered at pH 7 with K.phosphate (10 mM). The cells were sedimented by centrifugation and the supernates sampled for measurement of β -N-acetyl-D-glucosaminidase as previously described (Gomperts *et al.*, 1983).

Ca^{2+} was buffered at concentrations between 10 $^{-7}$ M and 10 $^{-5}$ M (pCa 7–pCa 5) and Mg^{2+} was set at 2 mM by the use of EGTA buffers. These were prepared by mixing equimolar solutions (100 mM nominal concentration) of EGTA and Ca.EGTA in proportions calculated with a computer program (Tatham and Gomperts, 1990). The calcium buffers were diluted in appropriate electrolyte solutions so that the final concentration of EGTA was 3 mM. Under these conditions the maximum error due to varying the concentration of ATP in the range 0–5 mM was <0.02 pCa. Note that as Mg^{2+} and Ca^{2+} were provided as chloride salts, the contribution of the CaEGTA buffers to the final concentration of Cl^- varies from 4 to 9.6 mM as pCa increases in the range 7–5. In some experiments the cells were treated with 3 mM EGTA containing no added Ca^{2+} . If we assume, as a worst estimate, the presence of calcium as a contaminant in our buffers at 25 μ M, then the concentration of free [Ca^{2+}] (at pH 6.8) will be $\sim$ 9 nM (pCa 8.23). Secretion occurring under these conditions is described as being Ca^{2+} -independent.

Protein kinase C inhibitors (AMG.C16 (Bachem, Buben-dorf, Switzerland) and Compound H-7 (Seikagaku, Kogyo Co., Tokyo, Japan) were made up as stock solutions (at 30 and 10 mM, respectively) in dimethyl sulfoxide (DMSO). Additions of DMSO were made as appropriate. PKC-I peptide was dissolved at 10 mM in phosphate-buffered saline.

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