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Evidence for protein dephosphorylation as a permissive step in GTP-γ-S–induced exocytosis from permeabilized mast cells

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Mast cells permeabilized by streptolysin O secrete histamine and lysosomal enzymes in response to provision of a dual effector system comprising Ca^{2+} and a guanine nucleotide (e.g., GTP-γ-S[2]) at concentrations in the micromolar range. These are both necessary and together they are sufficient. There is no requirement for adenosine triphosphate (ATP) and hence no obligatory phosphorylation reaction in the terminal stages of the exocytotic pathway. When exocytosis is induced by Ca^{2+}-plus-GTP-γ-S (i.e., no ATP) added at times after permeabilization (the permeabilization interval), cellular responsiveness declines so that there is no response to provision of the two effectors (both at 10^{-5} M) if they are initially withheld and then added after 5 min. Here we show that this decline in responsiveness is characterized by a time-dependent reduction in the effective affinity for Ca^{2+}. Affinity for Ca^{2+} and hence secretory competence can then be restored if ATP is added alongside the stimulus. Unlike cells stimulated to secrete at the time of permeabilization, exocytosis from cells that have undergone the cycle of permeabilization-induced refractoriness followed by ATP-induced restoration can be triggered by Ca^{2+} alone: after such conditioning there is no requirement for guanine nucleotide. In contrast, dependence on guanine nucleotide remains mandatory in cells that have been pretreated (i.e., before permeabilization) with okadaic acid (understood to be an inhibitor of protein phosphatases 1 and 2A) or phorbol myristate acetate (an activator of protein kinase C). These results indicate that obligatory dependence on guanine nucleotide is retained when the cells are treated under conditions conducive to maintained phosphorylation. It is concluded that the exocytotic mechanism of permeabilized mast cells is enabled by a dephosphorylation reaction and that the effector of the guanosine triphosphate (GTP)-binding protein (G_E) that mediates exocytosis is likely to be a protein phosphatase.

Introduction

Stimulation of the exocytotic reaction of mast cells permeabilized in simple NaCl-based buffers requires only the presence of Ca^{2+} and a guanine nucleotide, which interact at an unidentified G-protein designated G_E (Howell *et al.*, 1987; Churcher and Gomperts, 1990). These are both necessary and, under favorable conditions, they are sufficient to release 100% of the histamine and acid hydrolase content of the secretory granules. Exocytosis proceeds in the absence of adenosine triphosphate (ATP) and so it follows that a protein phosphorylation reaction does not constitute a necessary step in the exocytotic sequence. However, if the two essential effectors are initially withheld from the permeabilized cells and then provided after 5 min, no release of secretory products is elicited (Howell *et al.*, 1989). We refer to the period between permeabilization and addition of effectors as the permeabilization interval. Provision of ATP after a 5-min permeabilization interval can restore full responsiveness and, because cells treated with AMG.C_{16} (an inhibitory analogue of diglyceride [van Blitterswijk *et al.*, 1987; Kramer *et al.*, 1989]) remain refractory to stimulation (Howell *et al.*, 1989), a reaction mediated by protein kinase C is implicated in this process of recovery.

In addition, ATP has a quite separate modulatory role, acting at a control point downstream from the above events. This has been revealed by kinetic studies, where a delay in the onset of exocytosis from permeabilized cells is apparent only in the presence of ATP (Gomperts

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[2] Abbreviations: SL-O, streptolysin O; GTP-γ-S, guanosine-5′-O-(3-thiotriphosphate); hexosaminidase, β-*N*-acetylglucosaminidase; pCa, $-\log_{10}[Ca^{2+}]$; PKC-I, pseudosubstrate inhibitor peptide (RFARKGALRQKNV) of protein kinase C; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

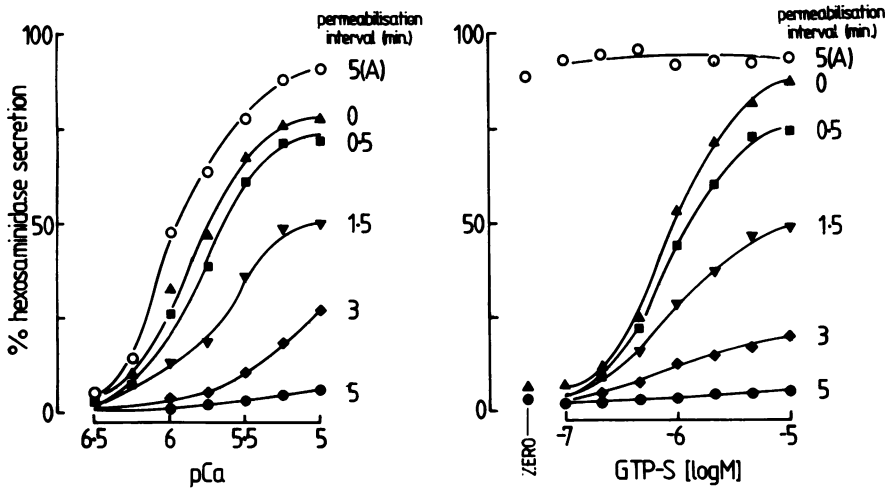

Figure 1. **Dependence on Ca^{2+} and GTP-γ-S of secretion from permeabilized mast cells stimulated by addition of Ca^{2+}-plus-GTP-γ-S at set times after permeabilization.** Mast cells, pretreated for 5 min with metabolic inhibitors and suspended in buffered salt solution containing 0.2 mM calcium buffer (pCa7) were permeabilized by addition of SL-O (0.4 iu ml^{-1}) and then triggered by transfer at set times (the permeabilization interval) to tubes containing either (A) GTP-γ-S (10 μM) and calcium buffers (3 mM) to give a range of Ca^{2+} or (B) a range of GTP-γ-S and calcium buffer (3 mM) to regulate pCa5.25. At 5 min, cells (open symbols) were also transferred to solutions of the two effectors supplemented with ATP (100 μM). The incubations were terminated after a further 10 min, and the hexosaminidase released into the supernatant was determined as described.

and Tatham, 1988; Tatham and Gomperts, 1989). In this sense ATP may be regarded as an inhibitor of an enabling reaction that controls the progress towards membrane fusion and release. Because cell activation mechanisms so often depend on a balance between phosphorylation and dephosphorylation (Cohen and Cohen, 1989), we have proposed that a dephosphorylation may be required for exocytosis to proceed.

In this paper we examine cells that have been conditioned to the point of unresponsiveness by permeabilization in the absence of ATP and the subsequent restoration of their competence by its re-addition. We show that exocytosis after recovery induced by ATP requires only the single effector, Ca^{2+}, but that if the cells are pretreated with okadaic acid (an inhibitor of protein phosphatases 1 and 2A [Bialojan and Takai, 1988; Haystead *et al.*, 1989; Cohen *et al.*, 1990]) or phorbol myristate acetate (PMA, an activator of protein kinase C), then provision of the guanine nucleotide remains mandatory. These results provide evidence for a Ca^{2+}-dependent step subsequent to that mediated by G_E. G_E may control one or more effectors, and these are likely to possess protein phosphatase activity.

Results

After permeabilization by streptolysin O (SL-O), the ability of mast cells to undergo exocytosis in response to provision of Ca^{2+}-plus-GTP-γ-S decays over a period of ~5 min (Howell *et al.*, 1989). The results illustrated in Figure 1 show that this decay is characterized by a progressive shift in the dependence of secretion on the concentration of Ca^{2+}. In this experiment, the cells were permeabilized (after pretreatment with metabolic inhibitors to deplete ATP) with SL-O and stimulated to secrete by provision of guanosine-5′-O-(3-thiotriphosphate) (GTP-γ-S, 10 μM) and a range of $-\log_{10}[Ca^{2+}]$ (pCa) either immediately or after a series of defined permeabilization intervals. When ATP (100 μM) was provided together with the stimulus after a permeabilization interval of 5 min, then the affinity for Ca^{2+} was restored and exocytosis was once again elicited.

Because ATP is not required for exocytosis, and a phosphorylation reaction therefore does not comprise an obligatory step in the exocytotic sequence, we understand that the role of ATP in causing restoration of responsiveness is likely to be that of allowing the exocytotic mechanism to recognize the Ca^{2+}, through regulation of the affinity of a Ca^{2+}-binding protein (Gomperts *et al.*, 1987; Howell *et al.*, 1987). As shown in Figure 2, secretory competence can be restored after a 5-min permeabilization interval by ATP at concentrations in the range 10–100 μM. The experiment also shows that restoration can be inhibited by provision of the

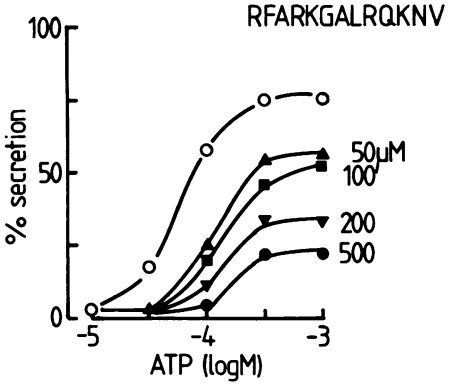

Figure 2. **Dependence on ATP concentration for restoration of secretory competence in cells rendered refractory by permeabilization at pCa7: effect of PKC-I peptide.** Mast cells were permeabilized at pCa7 and incubated at 37°C. After 3 min, PKC-I peptide was added and the incubations continued for a further 2 min, when the cells were transferred to tubes containing 3 mM calcium buffer (pCa5), GTP-γ-S (10 μM), and MgATP at the concentrations indicated. Incubations were terminated after a further 10 min. ○, control; ▲, PKC-I 50 μM; ■, 100 μM; ▼, 200 μM; ●, 500 μM.

protein kinase C pseudosubstrate PKC-I (at concentrations of 50 μM and above, added to the cells 3 min after permeabilization). It follows that the action of ATP is probably that of a phosphoryl donor in a reaction catalyzed by protein kinase C. This is in accordance with previous results (Howell *et al.*, 1989) based on the use of the diglyceride analogue AMG.C_{16}, which similarly prevents the restoration of secretory competence by ATP.

We have also measured the dependence of secretion on GTP-γ-S (with Ca^{2+} constant at pCa5.25) during the period after permeabilization (Figures 1B and 3A). The progressive decline in responsiveness is once again apparent, but there does not appear to be any systematic shift in the effective affinity for the guanine nucleotide; the extent of secretion elicited by all concentrations simply decreases with time. When the stimulus was supplemented with ATP at 5 min to restore responsiveness, Ca^{2+} alone, in the range pCa6–pCa5 (Figure 3B) was sufficient to stimulate exocytosis. It is as if the GTP-dependent step in the exocytotic sequence has been obviated or bypassed by the simple expedient of incubating the permeabilized cells at pCa7 for a few minutes in the absence of ATP. The possibility thus arises that the reaction catalyzed by the target enzyme of G_E occurs spontaneously as a consequence of such conditioning.

We have obtained results similar to those illustrated in Figure 3A on at least 25 occasions over a period of 1 yr; however, it must be said that in other experiments, after add-back to ATP, a complete independence of guanine nucleotide was not achieved and instead the secretion was characterized by a considerable enhancement in the apparent affinity for GTP-γ-S (i.e., commencing at concentrations as low as 10^{-9} M: see Figure 3C in Howell *et al.*, 1989). The observed guanine nucleotide independence and the increased sensitivity toward GTP-γ-S are likely to be manifestations of the same phenomenon, but the reason underlying this variability is not clear.

Figure 4 illustrates the time dependence of the onset of GTP-independent secretion. In this

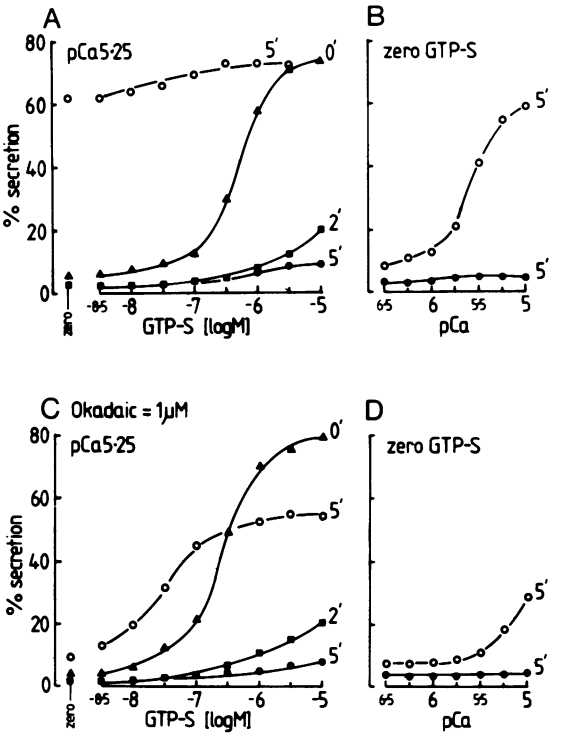

Figure 3. **GTP-γ-S-dependence of secretion from permeabilized mast cells stimulated by addition of Ca^{2+}-plus-GTP-γ-S at set times after permeabilization: okadaic acid ensures retention of GTP-dependence for secretion from cells rendered refractory by prepermeabilization and restored by ATP.** Mast cells were treated with DMSO (A and B) or 1 μM okadaic acid (C and D) for 5 min, followed by metabolic inhibitors and 0.2 mM calcium buffer (pCa7) for a further 2 min, and then permeabilized by addition of SL-O. At set times (the permeabilization interval) the cells were stimulated to secrete by transfer to solutions containing 3 mM calcium buffer (pCa5.25) and GTP-γ-S as indicated (A and C) and also to solutions containing a range of Ca^{2+} (0 GTP-γ-S: B and D). At 5 min (open symbols), cells were also transferred to solutions supplemented with 100 μM ATP. The incubations were terminated after a further 10 min, and the hexosaminidase released into the supernatant was determined as described.

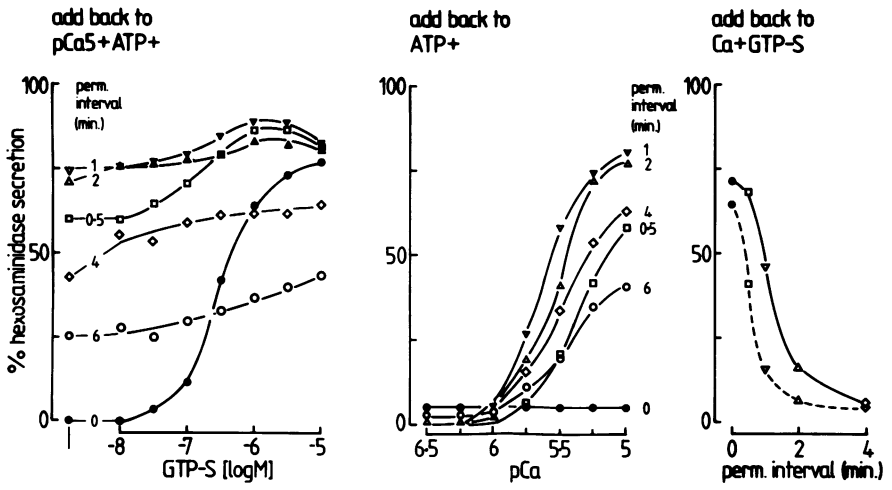

***Figure 4.* Development of GTP-independence.** Mast cells permeabilized at pCa7 were transferred at set times to solutions containing (A) 100 μM ATP, 3 mM calcium buffer (pCa5.25) and a range of GTP-γ-S; (B) 100 μM ATP and a range of pCa (i.e., 0 GTP-γ-S); (C) GTP-γ-S (10 μM) and calcium buffers at pCa5.25 (broken line) or pCa5 (upper line) (i.e., 0 ATP).

experiment, the cells were permeabilized and incubated at pCa7 for various intervals before transfer to solutions containing 100 μM ATP and (A) 10 μM Ca^{2+} with a range of concentrations of GTP-γ-S or (B) a range of Ca^{2+} (and no GTP-γ-S). Figure 4C illustrates the time-dependent decline in responsiveness to the combination of Ca^{2+}-plus-GTP-γ-S (at pCa5 and pCa5.25) in the absence of ATP. In confirmation of previous work, the data of Figure 4, A and B, demonstrate that Ca^{2+}, even in the presence of ATP, is insufficient to trigger secretion when provided at the time of permeabilization. However, after a permeabilization interval of 0.5 min in this particular experiment, the addition of ATP and Ca^{2+} already caused almost 60% secretion. This became even greater when the permeabilization interval was extended to 1 and 2 min. In parallel with these effects (as shown in Figure 3C), the permeabilized cells deprived of ATP became rapidly unresponsive to Ca^{2+}-plus-GTP-γ-S (Figure 4C). Beyond 2 min, the extent of the ATP-dependent, Ca^{2+}-induced secretion also declined slowly.

In view of the possibility (indicated by kinetic experiments [Gomperts and Tatham, 1988; Tatham and Gomperts, 1989]) that dephosphorylation may be a key event in activation, we have tested the ability of okadaic acid, a specific inhibitor of protein phosphatases types 1 and 2A (Bialojan and Takai, 1988; Haystead *et al.*, 1989; Cohen *et al.*, 1990), to modulate the characteristics of decline during the permeabilization interval and the ability of ATP to restore responsiveness. The results of such an experiment are shown in Figure 3C. Here it can be seen that, although the curves describing dependence of secretion on GTP-γ-S at 0, 2, and 5 min are almost superimposable with those of the control experiment (Figure 3A), when responsiveness is restored by ATP the dependence on the guanine nucleotide remains almost absolute. In accordance with this, we observed that Ca^{2+} alone is sufficient to cause exocytosis (Figure 3B), although the concentration required is somewhat greater (~0.5 pCa unit) than when the guanine nucleotide is provided (compare Figure 1A).

A similar retention of dependence on guanine nucleotide occurs in cells that are pretreated with PMA and that are thus presumed to be hyperphosphorylated. In the experiment illustrated in Figure 5, mast cells were treated with PMA (100 nM) for 3 min, and then subjected to 40-fold dilution in a solution containing meta-

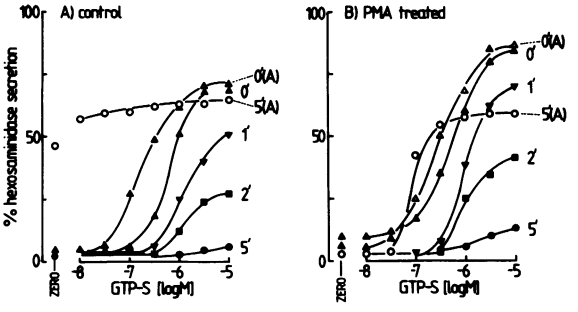

***Figure 5.* Phorbol ester pretreatment ensures GTP dependence for secretion from cells rendered refractory by prepermeabilization and restored by ATP.** Mast cells were treated with (A) DMSO or (B) PMA (100 nM) for 3 min, followed by metabolic inhibitors for a further 2 min and then permeabilized at pCa7. At set times the cells were transferred to solutions containing calcium buffer (pCa5.25) and GTP-γ-S as indicated. At 0 and 5 min (open symbols), cells were also transferred to solutions supplemented with ATP. The incubations were terminated after a further 10 min, and the hexosaminidase released into the supernatant was determined as described.

bolic inhibitors for 2 min before permeabilization and delayed stimulation with Ca^{2+} and with ATP-plus-Ca^{2+} at 5 min. After add-back to ATP, the PMA-treated cells remained refractory to stimulation unless the guanine nucleotide was also provided. The results provided in this experiment again underline the fact that both effectors are absolutely necessary even when ATP is provided when the cells are stimulated immediately at the time of permeabilization (curves 0′(A) in both panels).

Discussion

GTP-binding proteins have been implicated as mediators of vesicle fusion at a number of stages in the pathway leading to both constitutive and regulated secretion. In reconstitution experiments, GTP is required to enable trafficking to occur between the endoplasmic reticulum and the Golgi stacks (Bacon *et al.*, 1989; Beckers and Balch, 1989), and then through the Golgi (Melançon *et al.*, 1987; Segev *et al.*, 1988) and again in the terminal stages of the constitutive secretory pathway in cells as diverse as yeast (Salminen and Novick, 1987) and mammalian liver (Melançon *et al.*, 1987). In general, nonhydrolyzable analogues of GTP inhibit vesicle traffic (Melançon *et al.*, 1987), and so it is understood that the GTP-binding proteins must cycle repeatedly between the GTP- and GDP-bound states (Novick *et al.*, 1988; Bourne, 1988; Bacon *et al.*, 1989). However, an alternative view, based on reconstitution of endosome fusion, does allow for a supportive role for GTP-γ-S so long as the system is depleted of cytosol factors (Mayorga *et al.*, 1989).

A role for GTP-binding proteins (G_E) in the terminal stages of regulated exocytosis has also been discerned in several mammalian cells (Gomperts, 1990a,b), but in these systems the effective affinity of the nonhydrolyzable analogues of GTP (GTP-γ-S, GppNHp, and Gpp-CH_2p) in support of the secretory process can be as much as 10^3 times higher than that of the parent nucleotide (Howell *et al.*, 1987). These findings are based on investigations of semi-intact (i.e., permeabilized) cells, and in mast cells the effect of cytosol proteins is to suppress the effective affinities of both essential effectors (Koffer and Gomperts, 1989). Although the argument is far from being settled, we take the view that the role and characteristics of G_E are distinct from those of the GTP-binding proteins that ensure accuracy and directionality of membrane interaction in the constitutive pathway (Bourne, 1988). A more appropriate paradigm for G_E function is possibly provided by the familiar G-proteins such as G_S and transducin. Persistent binding of GTP (or analogues) results in dissociation and release of α-subunits from such heterotrimeric G-proteins with consequent activation of downstream effectors (e.g., adenylyl cyclase and cyclic GMP phosphodiesterase).

The experiments described here raise the possibility that the introduction of GTP-γ-S into permeabilized mast cells and the consequent activation of G_E serves to activate a dephosphorylation reaction that is necessary to enable progression through the terminal stages of the exocytotic pathway. The implication is that an effector controlled by the (as yet undefined) G-protein G_E might be a phosphoprotein phosphatase.

This conclusion relies on the generation of conditions in which secretion from mast cells permeabilized in simple NaCl-based buffers, normally dependent on provision of a dual effector system comprising both Ca^{2+} and a guanine nucleotide (Howell *et al.*, 1987; Churcher and Gomperts, 1990), can be triggered by Ca^{2+} alone. This is achieved by permeabilizing the cells for a few minutes in an isoosmotic solution comprising NaCl, a pH-buffering anion (piperazine-*N,N′*-bis(2-ethanesulfonic acid), PIPES), and a low concentration of calcium buffer (0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid, EGTA) which maintains the level of Ca^{2+} at 100 nM (i.e., at about the level that normally pertains in resting cells). Under this regime, in which no steps are taken to ensure maintenance of a phosphorylation state, the effective affinity for Ca^{2+} in the stimulation of exocytosis declines systematically with time.

Affinity for Ca^{2+} in the refractory cells can be restored by provision of ATP in a reaction that is prevented when the cells are treated with PKC-I peptide (House and Kemp, 1987; Alexander *et al.*, 1989) (this paper) or with stearyl methyl glycerol (AMG.C_{16}) (Howell *et al.*, 1989), a glycerol diether previously shown to prevent the binding of phorbol esters and to inhibit protein kinase C (van Blitterswijk *et al.*, 1987; Kramer *et al.*, 1989). This indicates that the loss of affinity for Ca^{2+} is most likely due to spontaneous dephosphorylation after the almost infinite dilution of ATP during the permeabilization interval. Such dephosphorylation might also be encouraged by the leakage of endogenous phosphatase inhibitor proteins through the SL-O–induced membrane lesions.

To the extent that the inhibitory effects of AMG.C_{16} and PKC-I can be regarded as specific as reported (House and Kemp, 1987; van Blitterswijk *et al.,* 1987; Alexander *et al.,* 1989; Kramer *et al.,* 1989), then the recovery after addback to ATP is due to a phosphorylation reaction, mediated by protein kinase C. After such conditioning, Ca^{2+} alone (or on other occasions together with very low concentrations of GTP-γ-S), is then sufficient to induce exocytosis. When ATP is provided at the time of permeabilization, dependence on both Ca^{2+} and a guanine nucleotide is retained for at least 5 min (not shown). Thus the most clearly defined role for ATP can be seen as maintaining the sensitivity to Ca^{2+} and requirement for guanine nucleotide. Cells permeabilized in the absence of ATP lose their ability to recognize these effectors through spontaneous dephosphorylations. The finding of the present work is that late addition of ATP not only restores Ca^{2+} affinity but that the exocytosis at this stage occurs in the absence of guanine nucleotide.

Dephosphorylation occurring during the permeabilization interval is certainly not limited to a single protein. Using a rephosphorylation protocol and two-dimensional gel analysis (Kramer and Gomperts, unpublished results), we have found that the extent of labeling resulting from 1-min pulses of [^{32}P]-ATP increases with time after permeabilization. The possibility thus exists that, among the multiple dephosphorylation reactions that occur during the permeabilization interval, is one that allows progression along the exocytotic pathway. This might be the reason the guanine nucleotide becomes superfluous. We have to presume that rephosphorylation of this protein is slow compared with that which regulates the affinity for Ca^{2+}; otherwise, dependence on guanine nucleotide would be instantly regained.

This conclusion is well supported by the finding that dependence on guanine nucleotide is retained if the cells are pretreated with agents likely to inhibit protein dephosphorylation or to cause hyperphosphorylation. It may also provide a rational explanation for earlier observations showing inhibition by PMA of Ca^{2+}-induced, but not GTP-γ-S–induced, lysosomal enzyme secretion from permeabilized rabbit neutrophils (Barrowman *et al.,* 1986). In the experiments described here these conditions were generated by treating the cells before permeabilization with either okadaic acid or with PMA.

Although there have been some hints, the idea of protein dephosphorylation as an enabling reaction of the exocytotic pathway of mammalian cells lacks direct supporting evidence. Ca^{2+}-induced catecholamine secretion from permeabilized adrenal chromaffin cells can be inhibited by ATP-γ-S, suggesting a block resulting from stable thiophosphorylation (Brooks *et al.,* 1984; Brooks and Brooks, 1985), but we have not been able to produce a similar result in permeabilized mast cells (unpublished observation). In contrast, secretion of catecholamines occurs independently of Ca^{2+} from phaeochromocytoma (PC12) cells permeabilized in the presence of ATP-γ-S (Wagner and Vu, 1989). We have previously shown that in mast cells the onset of exocytosis induced by Ca^{2+}-plus-GTP-γ-S is delayed when ATP is provided, and we interpreted this in terms of an enabling reaction involving protein dephosphorylation (Gomperts and Tatham, 1988; Tatham and Gomperts, 1989). However, in connection with the present work, we have found no indication that pretreatment of mast cells with okadaic acid has any effect on the onset kinetics (experiments not shown). Furthermore, phorbol ester pretreatment has the effect of accelerating, not retarding, the onset of exocytosis (Tatham and Gomperts, 1989). This could be taken to mean that there are two steps in the exocytotic pathway involving protein dephosphorylation, only one of which is sensitive to okadaic acid. Alternatively, our interpretation of the kinetic results could be wrong.

In contrast with mammalian systems, a triggered dephosphorylation in the pathway regulating the trichocyst discharge reaction of *Paramecium tetraurelia* (a well-documented example of exocytosis [Plattner, 1987]) has been described (Zieseniss and Plattner, 1985). As with mast cells, the presence of ATP is not mandatory at the time of exocytosis, although a phosphorylation state is required to maintain secretory competence (Vilmart-Seuwen *et al.,* 1986). In this organism, direct microinjection of antibodies to calcineurin is inhibitory to discharge induced by aminoethyl dextran, whereas injection of alkaline phosphatase induces discharge directly. Immunologic (Stecher *et al.,* 1987) and genetic evidence (Zieseniss and Plattner, 1985) points to the importance of the soluble and integral membrane phosphoproteins pp63 ("parafusin") and pp65 as regulators of exocytosis, and there is evidence that homologues of both these proteins are present in the secretory tissues of mammals (Stecher *et al.,* 1987; Satir *et al.,* 1989).

We and others have proposed a role for GTP, and hence a role for a GTP-binding protein (G_E), in the terminal pathway of exocytosis (Gom-

perts, 1990a). Apart from the demonstration that the G-proteins mediating exocytosis and polyphosphoinositide hydrolysis (G_E and G_P) are distinct entities (Cockcroft *et al.*, 1987; Stutchfield and Cockcroft, 1988), the definition of G_E has relied entirely on measurements of secretion. The simplest conclusion to be drawn from the present results is that association of GTP with the hypothetical G_E results in protein dephosphorylation and that in permeabilized mast cells this late event is a necessary prelude to exocytosis. From this it follows that the effector unit of G_E is likely to be a phosphoprotein phosphatase, and that after activation by G_E the enzyme is no longer subject to inhibition by okadaic acid. Alternatively, if the inhibition of protein phosphatase by okadaic acid is irreversible, then the effect of G_E activation must be to open an alternative route, not involving protein dephosphorylation, to the terminal stage of exocytosis. These proposals are based on cell-physiological experiments, but either way they have profound biochemical implications that, if proven, are likely to extend to cellular processes far removed from exocytosis.

Methods

Mast cells were obtained by peritoneal lavage of large (>500 g) male Sprague Dawley rats. The cells were isolated from contaminating cell types by centrifugation through a cushion of Percoll (Pharmacia, Milton Keynes, Bucks, UK) as previously described (Tatham and Gomperts, 1990), washed twice by resuspension and centrifugation, and finally suspended in a buffered salt solution (pH 6.8) that comprised NaCl (137 mM), KCl (4 mM), PIPES (20 mM) and bovine serum albumin (1 mg · ml^{-1}). In most of the experiments described here, the strategy has been to permeabilize the cells under resting conditions and then apply the stimulus at set times afterwards (the permeabilization interval). Before permeabilizing the cells we treated them with metabolic inhibitors (2-deoxyglucose [6 mM] and antimycin-a [5 μM]) and a low concentration of calcium buffer (0.2 mM) to set [Ca^{2+}] at pCa7. They were then treated with streptolysin-O (0.4 IU · ml^{-1}) with other additions as described, and transferred after set times (the permeabilization interval) to tubes containing 3 mM calcium buffer (to regulate Ca^{2+} in the range pCa7–pCa5) and GTP-γ-S as indicated. At the end of 10 min the reactions were quenched by addition of 0.5 ml of ice-cold 0.15 M NaCl (containing 10 mM Kphosphate, pH7) and the cells sedimented by centrifugation. The supernatants were assayed for released *N*-acetyl-β-D-glucosaminidase (hexosaminidase) as previously described (Tatham and Gomperts, 1990).

Concentrated stock solutions in dimethyl sulfoxide (DMSO) of okadaic acid (2 mM) and PMA (100 μM) were prepared and were stored at −20°C. Appropriate additions of DMSO were made to control cells. SL-O (Wellcome Diagnostics, Dartford, Kent, UK) was purchased as a partially purified freeze-dried culture filtrate supplemented with buffer salts and reducing agent. It was dissolved as a clear solution in 2 ml water at a stock concentration of 20 IU · ml^{-1} and stored at 4°C. During several days the stock solution became cloudy, but this dispersed readily on dilution to 0.4 IU · ml^{-1}, and our experience (Howell and Gomperts, 1987) has been that there are no obvious effects of such aging on the ability of the cytolysin to cause membrane permeabilization. In the present work, however, in which the requirement for consistency may be more stringent, this could be a cause of some of the variability we have recorded.

Ca^{2+} was buffered at concentrations between 10^{-7} M and 10^{-5} M (pCa7 to pCa5) and Mg^{2+} was set at 2 mM by the use of EGTA buffers. These were prepared by mixing end-point-titrated equimolar solutions (100 mM nominal concentration) of EGTA and CaEGTA in proportions calculated with a computer program (Tatham and Gomperts, 1990) using the constants of Anderegg (Anderegg, 1964). Values of pK_a were converted (by addition of 0.11) to mixed constants for compatibility with the standard buffers used for pH electrode calibration (Martell and Smith, 1974). The calcium buffers were diluted in the buffered salt solution so that the final concentration of EGTA was 3 mM. Under these conditions the maximum error resulting from varying the concentration of ATP in the range 0–5 mM was <0.02 pCa.

Acknowledgments

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