Fusion between rat pancreatic zymogen granules and plasma membranes

Modulation by a GTP-binding protein

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At the moment, little is known about the molecular characteristics of the final step in the process of regulated exocytosis, i.e. the fusion of the membrane of a secretory vesicle with the plasma membrane. We have reconstituted this fusion event *in vitro*, using zymogen granules and plasma membranes from the exocrine pancreas of the rat. The membranes of zymogen granules were loaded with the lipid-soluble fluorescent probe octadecylrhodamine B, at a concentration that resulted in self-quenching of its fluorescence. The granules were then incubated with pancreatic plasma membranes at 37 °C, and fusion was measured through the dilution-dependent de-quenching of the fluorescence of the probe. Zymogen granules fused with pancreatic plasma membranes, but not with plasma membranes from liver or chromaffin cells; granules also fused with unlabelled granule membranes. The fusion of granules with plasma membranes was unaffected by variation of the Ca^{2+} concentration over a wide range, but fusion of granules with both plasma membranes and zymogen granule membranes was stimulated by GTP and, more potently, by guanosine 5'-[γ -thio]triphosphate (GTP[S]). The effect of GTP[S] was to increase the extent of fusion occurring at low concentrations of plasma membranes, without affecting the maximum signal obtained at high membrane concentrations. Pre-incubation of the plasma membranes with GTP[S] also enhanced their ability to fuse with zymogen granules. Our results indicate that membrane fusion during exocytosis may be under the direct control of a GTP-binding protein.

INTRODUCTION

Regulated exocytosis is a fundamental characteristic of many cell types (Burgess & Kelly, 1987). It is the process, for example, by which cells release enzymes, hormones and neurotransmitters in response to appropriate stimuli. The final step in exocytosis is the fusion of the membrane of a secretory vesicle with the plasma membrane, which leads to the release of the vesicle contents. Despite the ubiquity of this membrane fusion event, however, its molecular basis is obscure (Burgoyne, 1990).

The exocrine pancreas is a well-characterized system in which to study exocytotic membrane fusion (Palade, 1975). In the pancreatic acinar cell, digestive enzymes are stored in zymogen granules that are easily identifiable at the apical pole of the cell. Stimulation of the cells by secretagogues such as acetylcholine or cholecystokinin, which act at receptors on the basolateral domain of the plasma membrane, leads eventually to the fusion of the zymogen granules with the apical domain (Tartakoff & Vassalli, 1978). The response to secretagogues apparently occurs via a rise in the cytosolic Ca^{2+} concentration (Pandolfi *et al.*, 1985) that is oscillatory in nature (Tsunoda *et al.*, 1990), although it is at present unclear how the Ca^{2+} transients trigger exocytosis. Experiments with permeabilized pancreatic acini have provided further support for the central role of Ca^{2+} in the triggering of exocytosis, and have also pointed to the involvement of a GTP-binding protein, a suggestion based upon the enhancement of exocytosis produced by non-hydrolysable analogues of GTP, such as guanosine 5'-[γ -thio]triphosphate (GTP[S]) (Edwardson *et al.*, 1990; Padfield *et al.*, 1991). This concerted action of Ca^{2+} and GTP[S] in triggering exocytosis is in fact a feature of many cell types (Gomperts, 1990), which has led to the suggestion that membrane fusion in exocytosis may be under the direct control of a GTP-binding protein, that has been termed G_E .

A major problem in the elucidation of the molecular mechanisms of regulated exocytosis lies in distinguishing factors influencing 'upstream' events, such as the removal of cytoskeletal constraints on the vesicles, from those controlling membrane fusion itself (Lindstedt & Kelly, 1987). Using permeabilized cells as an experimental model is not particularly helpful in this respect. Consequently, we have developed an *in vitro* system based on isolated zymogen granules and plasma membranes in order to look specifically at membrane fusion (Nadin *et al.*, 1989). In the *in vitro* system, pancreatic plasma membranes trigger the release of granular enzymes, such as amylase. This interaction is specific to pancreatic membranes (liver plasma membranes, for example, are ineffective) and is sensitive to trypsinization of the plasma membranes, which indicates that proteins are involved. The interaction proceeds without any added agent and is insensitive to Ca^{2+} over a wide concentration range. It is, however, enhanced by the addition of GTP or GTP[S]. If this interaction is in fact modelling exocytotic membrane fusion, then the implications of its properties are first that fusion must normally be inhibited in the intact cell, second that the role of Ca^{2+} is upstream of fusion, and finally that a GTP-binding protein is involved at a late stage in exocytosis.

The evidence that the amylase release caused by the granule-plasma membrane interaction involves membrane fusion, and not simply granule lysis, consists of the demonstration that the lipid-soluble fluorescent probe octadecylrhodamine B chloride (R18) loaded into the plasma membranes undergoes a dilution-dependent de-quenching when the membranes are incubated with zymogen granules under conditions in which amylase release is known to occur. The problem with this method of detecting fusion is that only a small percentage of the plasma membrane fragments would be expected to participate in fusion. The majority of the membranes, for example, will be derived from the

Abbreviations used: GTP[S], guanosine 5'-[γ -thio]triphosphate; R18, octadecylrhodamine B chloride.

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basolateral domain, and a significant proportion will be present as vesicles sealed 'outside-out'. This paper reports the results of a series of experiments using a modified de-quenching assay, in which the probe has been loaded into the membranes of the zymogen granules, all of which should be capable of fusing with plasma membranes. We show that membrane fusion closely parallels the release of amylase under various conditions. Further, we demonstrate that zymogen granule membranes, in addition to pancreatic plasma membranes, are able to fuse with labelled zymogen granules, and that both types of fusion event are enhanced by GTP and GTP[S].

MATERIALS AND METHODS

Reagents

R18 was obtained from Molecular Probes, Inc. (Eugene, OR, U.S.A.), and GTP[S] was supplied as a 200 mM solution by Boehringer-Mannheim (Mannheim, Germany). All other reagents were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

Preparation of zymogen granules and zymogen granule membranes

Zymogen granules were prepared from rat pancreas by the method of Rogers *et al.* (1987). The method is simple and rapid (less than 1 h) and produces a granule suspension with only a low level of contamination by other organelles, as judged by electron microscopy. Granules from a single pancreas were suspended in approx. 300 μ l of 280 mM-sucrose/5 mM-Mes buffer, pH 6.0, to a protein concentration of 2–5 mg/ml. Protein was assayed by the method of Bradford (1976), using BSA as standard. Granule membranes were prepared by lysing granules in 170 mM-NaCl and 200 mM-NaHCO₃, pH 7.8 (1:3, v/v) at 4 °C, followed by centrifugation at 70000 *g* for 30 min at the same temperature. Membranes were resuspended in sucrose/Mes buffer, pH 6.5, to a protein concentration of approx. 1 mg/ml, and stored in aliquots at –20 °C.

Preparation of plasma membranes

Pancreatic plasma membranes and liver plasma membranes were prepared by the methods of Meldolesi *et al.* (1971a) and Emmelot *et al.* (1974) respectively, with the modifications described by Nadin *et al.* (1989). Chromaffin cell plasma membranes (kindly provided by Dr. T. R. Cheek, Department of Zoology, University of Cambridge, U.K.) were prepared by the method of Smith & Winkler (1967), with the modifications described by Geisow & Burgoyne (1982). All membranes were resuspended in sucrose/Mes buffer, pH 6.5, and stored in aliquots at –20 °C.

For one experiment, liver plasma membranes (2.6 mg/ml) were treated at 37 °C for 15 min with a high-speed supernatant (2.5 mg/ml) prepared by centrifugation of a pancreatic homogenate at 80000 *g* for 30 min at 4 °C. This high-speed supernatant contains, in addition to cytosolic proteins, substantial amounts of granular enzymes. A cocktail of proteinase inhibitors was also present during the incubation. The membranes were recovered by centrifugation at 70000 *g* for 30 min at 4 °C, washed in sucrose/Mes buffer, pH 6.5, and then resuspended in the same buffer.

Fluorescence de-quenching assay for membrane fusion

Membrane fusion was measured through the relief of self-quenching of the fluorescent probe R18, by the method of Hoekstra *et al.* (1984). R18 was stored at –20 °C as a 20 mM stock solution in ethanol. Probe (usually 1 μ l of the 20 mM stock solution) was added to 300 μ l of a zymogen granule suspension. Granules were incubated with probe at 37 °C for 5 min and then

collected by centrifugation at 900 *g* for 10 min. Labelled granules were resuspended in the original volume of sucrose/Mes buffer, pH 6.0. In a fusion assay, 10 μ l of labelled granule suspension was added to 700 μ l of sucrose/Mes buffer, pH 6.5, at 37 °C. Fluorescence was measured continuously at this temperature in a luminescence spectrometer (Models LS-50 and LS-3; Perkin-Elmer Corp.) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. After a short equilibration period (2.5 min), target membranes and other agents were added in a small volume and mixed with the labelled granule suspension, and the fluorescence was monitored for a further 7.5 min.

When no membranes were added to the labelled granule suspension, fluorescence rose slowly in a linear manner. Where appropriate, the size of the fluorescence de-quenching signal was taken as the additional increase in fluorescence above this baseline. De-quenching was expressed as a percentage of that occurring after solubilization of the labelled granules in 0.2% (v/v) Triton X-100.

RESULTS

Specificity of fusion

In the experiment illustrated in Fig. 1, labelled zymogen granules were incubated with pancreatic, liver or chromaffin cell plasma membranes. Whereas pancreatic membranes caused a clear de-quenching, which occurred with a half-time of about 1 min, neither liver membranes nor chromaffin cell membranes (the latter tested in both the absence and the presence of 10 μ M-Ca²⁺) caused any detectable de-quenching. Hence zymogen granules fuse only with plasma membranes derived from the pancreas, and not with membranes from other sources.

One potential problem with the use of the pancreas as a source of tissue is that, upon homogenization, lipases are released that act on membrane phospholipids, even on ice, to convert a substantial proportion of them to their lyso-derivatives (Meldolesi *et al.*, 1971b). This raises the possibility that it is this alteration in the phospholipids that is rendering the pancreatic plasma membranes fusogenic. To test this possibility, liver plasma membranes were treated with a high-speed supernatant of a pancreatic homogenate, which contained cytosolic proteins and granular enzymes. The membranes were recovered by centrifugation and then incubated with R18-labelled zymogen granules. No de-quenching was detected (results not shown). The fusogenic

Fig. 1. Specificity of fusion of zymogen granules with plasma membranes

R18-labelled granules were challenged at 2.5 min with plasma membranes (10 μ g of protein) obtained from pancreas, liver or chromaffin cells. Incubations with chromaffin cell membranes were carried out in both the absence and the presence of Ca²⁺ (10 μ M). Ca²⁺ was buffered with EGTA (1 mM), and the free Ca²⁺ concentration was calculated using a program devised by Fabiato & Fabiato (1979).

Fig. 2. Relationship between de-quenching and the amount of pancreatic plasma membrane protein added

(a) Fluorescence de-quenching traces. The 'spike' at 2.5 min is caused by the addition of membranes to the cuvette. The amount of membrane protein added (μg) is indicated alongside each trace. (b) Variation in signal size with membrane protein concentration. The signal was defined as the size of the deviation at 10 min from the slow linear increase in fluorescence that occurred in the absence of membranes.

Fig. 3. Relationship between de-quenching and the amount of zymogen granule membrane protein added

(a) Fluorescence de-quenching traces; (b) variation in signal size with membrane protein concentration. For further details, see the legend to Fig. 2.

activity of pancreatic plasma membranes cannot, therefore, be ascribed to the action of lipases.

The dependence of the extent of de-quenching on the amount of pancreatic plasma membrane protein added is illustrated in Fig. 2. De-quenching reached a peak of 0.8% at about 20 μg of protein/ml, with half-maximal de-quenching at 5 μg /ml.

Although the only plasma membranes so far known to be capable of fusing with zymogen granules are those from pancreas, we did find that zymogen granule membranes are also able to fuse with labelled zymogen granules (Fig. 3). De-quenching reached a peak of 2% at 15 μg of protein/ml, with half-maximal de-quenching at 4 μg /ml.

Extent of fusion

It has been shown previously (Hoekstra *et al.*, 1984) that the efficiency of self-quenching of R18 in dioleoyl-phosphatidylcholine bilayers rises linearly with increasing surface density, up to 80% at 9 mol%. Hence any decrease in surface density occurring as a result of fusion of labelled membranes with unlabelled target membranes will result in a proportional de-quenching. Unfortunately, in our system the signal received from the labelled granules at self-quenching values of less than 80% was so small that impractically large quantities of granules would be needed in order to obtain a quantifiable de-quenching. Consequently, it was necessary to label granules to a higher value of self-quenching (usually around 95%). At this level of self-quenching the de-quenching caused by a given dilution of R18 is less than at self-quenching levels below 80%, such that the signal

given by the plasma membranes (about 1%) and the zymogen granule membranes (about 2%) underestimates the extent of membrane fusion. In control experiments with zymogen granule membranes (Fig. 4), it was found that the size of the de-quenching signal rose to a maximum of about 9% as the initial self-quenching fell below 80%. For plasma membranes, the maximal de-quenching obtained at 80% self-quenching was about 5%. These values represent the maximal dilutions of R18 that can occur with the two types of membrane target. The above result also means that the relationship between self-quenching and R18 surface density becomes non-linear above 80% quenching. The de-quenching signals obtained are sufficiently small, though, that they will remain approximately proportional to the dilution of the probe.

The values for the maximal dilution of the R18 could be used to calculate the percentage of the granules undergoing fusion, if one also knew the mean size of the target membrane and the number of fusion events occurring per vesicle. At present, however, our information is not sufficiently complete to make such a calculation worthwhile. The saturation of de-quenching with increasing amounts of target membrane does in fact indicate that the number of fusion events occurring per granule is limited. The experiment illustrated in Fig. 5 provides further evidence for this. It can be seen that zymogen granule membranes caused greater de-quenching than an equivalent amount of plasma membranes. If granules fuse only once or a small number of times with target membranes, then the greater efficacy of the granule membranes may be a consequence of their larger average

Fig. 4. Relationship between the de-quenching obtained with zymogen granule membranes ($10 \mu\text{g}$ of protein) and the initial self-quenching of R18

Granules were incubated in various concentrations of R18 to produce different degrees of loading. The two sets of symbols represent the results obtained from two experiments with the same batch of granule membranes.

Fig. 5. Competition between plasma membranes and zymogen granule membranes for fusion with labelled granules

Labelled granules were challenged with plasma membranes (PM; $3.6 \mu\text{g}$ of protein), zymogen granule membranes (ZGM; $3.6 \mu\text{g}$ of protein) or both together.

Fig. 7. Enhancement of de-quenching by GTP[S]

(a) Labelled granules were challenged with GTP[S] ($120 \mu\text{M}$), plasma membranes (PM; $10 \mu\text{g}$ of protein) or both together. (b) Labelled granules were challenged with either plasma membranes (PM; $3.6 \mu\text{g}$ of protein) or zymogen granule membranes (ZGM; $3.6 \mu\text{g}$ of protein) in the absence or presence of GTP[S] ($120 \mu\text{M}$).

size, which will result in a greater dilution of the probe. If this is the case, then one should see competition between the two types of target; that is plasma membranes (which give the smaller de-quenching) should reduce the signal given by zymogen granule membranes (which give the larger de-quenching). This is precisely what happened. Why the number of fusion events per granule should be so restricted is at present unclear, although it is possible, for example, that granule integrity (that may be lost after a single fusion event) is an important requirement.

Ca^{2+} sensitivity

Although Ca^{2+} is a major factor involved in exocytosis in the acinar cell, we have shown previously that it is not required for the triggering of amylase release from the granules by plasma

Fig. 6. Concentration-effect relationship for Ca^{2+}

Labelled granules were challenged with plasma membranes ($10 \mu\text{g}$ of protein) at different free Ca^{2+} concentrations, as indicated. Ca^{2+} was buffered with EGTA (1 mM). (a) Fluorescence de-quenching traces from a single experiment, separated vertically for clarity. (b) Variation in signal size with Ca^{2+} concentration. Values are means $\pm$ S.E.M. from seven separate experiments.

Fig. 8. Concentration-effect relationships for GTP[S] (a) and GTP (b)

In all cases 1 μg of plasma membrane protein was added. The size of the enhancement of de-quenching produced is expressed as a percentage of the fluorescence obtained in the presence of 0.2% (v/v) Triton X-100. Values are means $\pm$ S.E.M. from six (a) or eleven (b) separate experiments.

membranes (Nadin *et al.*, 1989). This is also the case for granule-membrane fusion as measured by de-quenching (Fig. 6). Free Ca^{2+} concentrations were varied from nominally zero (i.e. no added Ca^{2+} and 1 mM-EGTA) up to 1 mM, and no significant change was seen in the de-quenching signal given by the plasma membranes.

Involvement of a GTP-binding protein

The effect of GTP[S] on fusion between labelled granules and target membranes is shown in Fig. 7. Fig. 7(a) shows that GTP[S] (120 μM) alone caused little de-quenching, but was able to increase significantly the signal given by pancreatic plasma membranes. Fig. 7(b) shows that this stimulatory effect of GTP[S] occurred with both granule-plasma-membrane and granule-granule membrane fusion. The concentration-dependence of the effects of both GTP[S] and GTP on the fusion of granules with plasma membranes, at a low plasma membrane concentration, is shown in Fig. 8. It can be seen that the enhancement of fusion by GTP[S] was half-maximal at about 20 μM and maximal at about

Fig. 9. Effect of GTP[S] on the relationship between de-quenching and the amount of plasma membrane protein added

●, Membranes added alone; ▲, membranes added in the presence of GTP[S] (100 μM). In each experiment de-quenching was expressed as a percentage of the value obtained with the optimal amount of membrane protein (20 μg) in the absence of GTP[S]. Values are means $\pm$ S.E.M. from seven separate experiments. Asterisks indicate points at which GTP[S] significantly enhanced de-quenching, as indicated by Student's unpaired *t* test ($P < 0.05$).

Fig. 10. Effect of pre-incubation of plasma membranes with GTP[S]

Labelled granules were challenged with plasma membranes (PM; 0.5 μg of protein), plasma membranes plus GTP[S] (120 μM) or membranes that had been pre-incubated with GTP[S] (120 μM) (PM_{pre-inc}) at 37 °C for 15 min.

60 μM; for GTP the corresponding values were 40 μM and 200 μM respectively. The values for GTP[S] are very close to those for the enhancement of plasma membrane-triggered amylase release (Nadin *et al.*, 1989); GTP, on the other hand, is apparently more potent in the present system. Guanosine 5'-[β-thio]diphosphate did not cause any enhancement of release at concentrations up to 100 μM (results not shown). Fig. 9 illustrates the effect of GTP[S] (100 μM) on the relationship between de-quenching and the amount of plasma membrane protein added. GTP[S] increased the extent of fusion occurring at low concentrations of plasma membrane, without enhancing the maximum signal obtained at high concentrations of membrane.

The fact that GTP[S] enhances fusion between labelled granules and unlabelled granule membranes indicates that a GTP-binding protein on the granule membrane must be involved in the control of fusion. In order to determine whether a similar protein operates on the plasma membrane, membranes were pre-incubated with GTP[S] at 100 μM and then incubated with labelled granules. The dilution of the membranes into the incubation mixture would have reduced the GTP[S] concentration to 1 μM, i.e. below that at which it becomes effective at

enhancing fusion (see Fig. 8). It was found (Fig. 10) that plasma membranes pre-incubated with GTP[S] were more effective at enhancing fusion than control membranes, which at the low concentration used in this experiment caused little de-quenching by themselves. Indeed, the pre-incubated membranes caused almost as much de-quenching as control membranes incubated with granules in the presence of GTP[S] (120 μ M). It would appear, therefore, that the GTP-binding proteins controlling fusion are present on both the granule membrane and the plasma membrane. The same conclusion was reached on the basis of previous results using the amylase release assay (Nadin *et al.*, 1989).

DISCUSSION

The results reported here lend strong support to our earlier contention that pancreatic zymogen granules and plasma membranes interact *in vitro* in a physiologically relevant manner. Fusion between the two membranes was measured directly and was found to have properties very similar to those of a system based on measurement of amylase release from zymogen granules (Nadin *et al.*, 1989).

The membrane fusion event exhibits specificity, as plasma membranes from both liver and chromaffin cells were unable to fuse with zymogen granules. The negative result with liver plasma membranes is perhaps not surprising, since this tissue does not undergo regulated exocytosis. The inability of chromaffin cell plasma membranes to fuse with zymogen granules is more interesting, since these cells have been used extensively as a model of regulated exocytosis (Burgoyne, 1991). Their inactivity suggests that membrane fusion in exocytosis may have at least some different requirements in different cell types. Indeed, the specificity seen in our experiments may reflect fundamental differences between exocrine and neurally derived cells. It may be of interest, therefore, to carry out this type of 'mix-and-match' experiment using plasma membranes from an exocrine tissue, such as the parotid gland.

Efficient fusion between zymogen granules and plasma membranes in the absence of any triggering agent does not, of course, reflect the situation in the intact acinar cell, where exocytosis occurs only after stimulation by secretagogues such as acetylcholine and cholecystokinin. The implication of our results is that fusion is inhibited in the cell and that cell stimulation is required to remove this inhibition. The finding that zymogen granule membranes also are able to fuse with intact granules requires a similar explanation. It has been demonstrated previously that the ability of zymogen granules to aggregate, and possibly fuse together, *in vitro* in response to high cation concentrations can be inhibited by a protein fraction prepared from pancreatic cytosol (Rogers *et al.*, 1987). These authors, too, suggested that membrane fusion may normally be suppressed within the cell.

It is widely believed that Ca^{2+} is a major trigger for exocytosis in the pancreatic acinar cell. This belief is based on findings such as the ability of agonists like acetylcholine and cholecystokinin to cause rapid rises in the cytosolic Ca^{2+} concentration (Pandolf *et al.*, 1985), and the stimulation of secretion in intact acini by Ca^{2+} ionophores (Merritt & Rubin, 1985) or in permeabilized cells by Ca^{2+} concentrations in the micromolar range (Edwardson *et al.*, 1990; Padfield *et al.*, 1991). The absence of any significant effect of changes in Ca^{2+} concentration on fusion between zymogen granules and pancreatic plasma membranes *in vitro* suggests that Ca^{2+} may not in fact be required for membrane fusion itself. What, then, might it be doing? The major difference between exocytosis *in vitro* and in the cell is that in the former case all physical constraints on the interacting membranes have

been removed. It is known that in several cell types secretory vesicles are anchored to the cytoskeleton, and that dramatic changes in cytoskeletal organization occur when secretion is triggered (reviewed by Lindstedt & Kelly, 1987, and by Burgoyne, 1991). Our results are consistent with the idea that at least part of the role of Ca^{2+} in the acinar cell is to bring about this reorganization, and to free the granules from the cytoskeleton, allowing them to approach and fuse with the plasma membrane; this role may be mediated through Ca^{2+} -dependent protein kinases/phosphatases (Cohen, 1988).

It has been shown recently, using a de-quenching method similar to the one described here, that chromaffin granules fuse with chromaffin cell plasma membranes *in vitro* (Karli *et al.*, 1990). Fusion required pre-treatment of the plasma membrane vesicles with phospholipase A_2 , a Ca^{2+} -dependent enzyme. Once the membranes had been primed in this way, however, fusion continued in the absence of both the enzyme and Ca^{2+} . It was concluded that membrane fusion proceeded in two stages: a Ca^{2+} -dependent activation of phospholipase A_2 , which caused the generation of fusogenic fatty acids from phospholipids, followed by a Ca^{2+} -independent fusion event between granules and plasma membranes. In this system also, therefore, Ca^{2+} plays a role upstream of fusion.

The enhancement of fusion between zymogen granules and plasma membranes by GTP[S] occurs over the same concentration range as the enhancement both of membrane-triggered amylase release (Nadin *et al.*, 1989) and of exocytosis in permeabilized acini (Edwardson *et al.*, 1990). This suggests that the same effect of these agents is being detected in the three systems. An effect of GTP and its analogues on exocytosis has been reported for many cell types (reviewed by Gomperts, 1990) and it has been proposed that exocytosis is under the direct control of a GTP-binding protein, termed G_ex . Our observation of an effect of GTP/GTP[S] on membrane fusion *in vitro* is certainly consistent with the operation of a GTP-binding protein very close to the final fusion event, and downstream of the effect of Ca^{2+} . The exact location and nature of the GTP-binding protein(s), though, remain to be determined. The demonstration that pre-incubation of plasma membranes with GTP[S] enhances their ability to fuse with zymogen granules points to the effect of a GTP-binding protein on the plasma membrane. On the other hand, the enhancement of fusion between granules and granule membranes by GTP[S] argues for the involvement of a GTP-binding protein on the granule membrane. Our investigation of the effects of GTP[S] on plasma membrane-triggered amylase release produced a similar picture (Nadin *et al.*, 1989).

It is becoming clear that intracellular membrane traffic is regulated through the operation of a family of small GTP-binding proteins (Balch, 1990), and it has been proposed that GTP hydrolysis by these proteins drives the fusion between transport vesicles and their target organelle (Bourne, 1988). Evidence for the control of vesicle budding through the operation of GTP-binding proteins has also been reported (Tooze *et al.*, 1990). GTP[S] has an inhibitory effect on both budding and fusion, presumably by preventing GTP hydrolysis. With regard to regulated exocytosis, considerable attention has been paid recently to the GTP-binding protein rab3A, which in neurones is localized to synaptic vesicles (Fischer von Mollard *et al.*, 1990). This protein has been shown to dissociate from the vesicles membrane when the cells are stimulated (Fischer von Mollard *et al.*, 1991), and it has been suggested that this protein may be involved in the control of exocytosis in neurones. Zymogen granule membranes, too, contain a group of GTP-binding proteins with molecular masses of 21–29 kDa (Lambert *et al.*, 1990; Padfield & Jamieson, 1991), that may mediate the effect of GTP[S] on exocytosis. None of them, however, has yet been

characterized. An important difference between regulated and constitutive exocytosis, of course, is that the former is almost always stimulated and not inhibited by GTP[S] (Gomperts, 1990). In fact, the GTP-binding proteins involved in the control of regulated exocytosis behave much more like the heterotrimeric G-proteins that couple receptors to second messenger systems; these proteins remain active while GTP is bound and are inactivated when GTP is hydrolysed as a consequence of the G-protein's intrinsic GTPase activity. It is possible, therefore, that the small GTP-binding proteins may not have exclusive control over intracellular membrane fusion events.

It was reported recently that GTP[S] stimulates phospholipase A₂ activity in zymogen granule membranes, an observation that led to the suggestion that this effect may underly the stimulatory effects of GTP[S] on granule-plasma-membrane fusion (Rubin *et al.*, 1991). Unfortunately, any attempt to link these events has to contend with the large differences in the potency of GTP[S] in producing the two effects (50 % maximal effect at about 100 nM for stimulation of phospholipase A₂, as opposed to 12 µM for enhancement of granule-plasma-membrane fusion).

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