A Specific Interaction In Vitro between Pancreatic Zymogen Granules and Plasma Membranes: Stimulation by G-Protein Activators But Not by Ca^{2+}

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Abstract. The molecular details of the final step in the process of regulated exocytosis, the fusion of the membrane of the secretory granule with the plasma membrane, are at present obscure. As a first step in an investigation of this membrane fusion event, we have developed a cell-free assay for the interaction between pancreatic zymogen granules and plasma membranes. We show here that plasma membranes are able to trigger the release of the granule contents, and that this effect is specific to pancreatic membranes, involves membrane fusion, requires membrane proteins, and is stimulated by activators of G-proteins but not by Ca^{2+}. The assay is simple, reliable, and rapid, and should permit the identification of proteins that are involved in the exocytotic fusion event.

During the process of regulated exocytosis, cell activation generates intracellular signals that trigger fusion between the membranes of secretory vesicles and the plasma membrane, leading to the release of the vesicle contents. Although the mechanisms involved in signal generation have been well characterized, virtually nothing is known about the membrane fusion event itself (Burgoyne, 1987, 1988; Lindstedt and Kelly, 1987). Permeabilized cell systems have been introduced as a way of dissecting the exocytotic process (Baker and Knight, 1978), and have been used to examine the role in exocytosis of Ca^{2+} (Baker and Knight, 1978; Howell and Gomperts, 1987), G-proteins (Barrowman et al., 1986; Howell et al., 1987; Cockcroft et al., 1987), and protein phosphorylation (Baker and Knight, 1978; Howell et al., 1987). Because of the inherent complexity of these systems, however, it has proven impossible either to elucidate the precise sequence of events occurring during exocytosis or to identify proteins that are involved in the final membrane fusion event. Further significant progress will depend on the reconstitution of the final stages in the exocytotic sequence in a cell-free system composed of well-defined subcellular fractions. Only with such a system will it be possible to look directly at the requirements of the membrane fusion event in the absence of complications introduced by "upstream" events, such as the release of the secretory vesicles from the cytoskeletal network (Linstedt and Kelly, 1987).

Several in vitro systems for regulated exocytosis have been described (Davis and Lazarus, 1976; Milutinovic et al., 1977; Konings and De Potter, 1981; Whitaker and Baker, 1983), of which the most impressive is the sea urchin egg system of Jackson and co-workers (Haggerty and Jackson, 1983; Crabb and Jackson; Jackson et al., 1985). Unfortunately, even this last system is probably too cumbersome to be applicable to a detailed study of the molecular mechanisms underlying the exocytotic fusion event. The work of Rothman and colleagues on protein transport within the Golgi complex (see, for example, Balch et al., 1984) has demonstrated that a simple, rapid, and reliable assay is an absolute necessity. We set out to develop such an assay. The tissue we chose to work with, the exocrine pancreas, is perhaps the most thoroughly characterized model system for regulated exocytosis (Palade, 1975). When pancreatic acinar cells are stimulated by secretagogues such as acetylcholine or cholecystokinin that interact with receptors on the basolateral domain of the plasma membrane, the contents of the zymogen granules, mainly hydrolytic enzymes, are released by compound exocytosis at the apical domain (Tartakoff and Vassalli, 1978). We show here that when zymogen granules and plasma membranes from rat pancreas are incubated together, an interaction occurs that results in the release of the granule contents. This effect is specific to pancreatic membranes, involves membrane fusion, requires membrane proteins, and is stimulated by activators of G-proteins but not by Ca^{2+}.

Materials and Methods

Reagents

All reagents were obtained from Sigma Chemical Co. (Poole, UK) unless otherwise stated.

Preparation of Zymogen Granules

Zymogen granules were prepared by the method of Rogers et al. (1987).

Rats (male Sprague-Dawley, 200–250 g, usually one per preparation) were killed by cervical dislocation. The pancreas was removed and placed in ice-cold Krebs-Henseleit solution. Adipose and lymphatic tissue was removed and the tissue was minced with scissors in 10 ml ice-cold 280 mM sucrose. All subsequent procedures were carried out at room temperature. The tissue was homogenized by three strokes in a glass-glass hand-driven homogenizer (Braun-Melsungen, Melsungen, FRG) and centrifuged at 400 *g* for 10 min. The supernatant was then centrifuged at 900 *g* for 10 min in an angle-head rotor. This procedure produced a tight white pellet of zymogen granules overlaid by a loose tan-colored pellet of mitochondria. The supernatant was aspirated and the mitochondrial layer removed by three washes with 1 ml 280 mM sucrose, 5 mM MES[1] buffer (2[*N*-morpholino]ethanesulfonic acid) (pH 6.0). Granules were then resuspended in the same buffer, to a protein concentration of 2–5 mg/ml, and used immediately.

Zymogen granule membranes were prepared by adding 170 mM NaCl and 200 mM $NaHCO_3$, adjusted to pH 7.8 with 1 M HCl (1 vol:3 vol), to lyse the granules and release the contents, and collected by centrifugation at 70,000 *g* for 30 min. The membranes were resuspended and washed with 500 mM KCl to remove peripheral proteins. They were then pelleted and resuspended in 280 mM sucrose, 5 mM MES buffer, pH 6.0 for determination of membrane protein content.

Preparation of Pancreatic Plasma Membranes

Rat pancreatic plasma membrane fractions were prepared by a modification of the method of Meldolesi et al. (1971). Pancreata (five per preparation) were collected and homogenized as described above. The homogenate was filtered through surgical gauze and centrifuged at 1,000 *g* for 12 min. Pellets were resuspended in a total volume of 15 ml 280 mM sucrose and homogenized by one stroke in a Teflon-glass homogenizer. The suspension was brought slowly to 1.6 M sucrose by dropwise addition of 48 ml 2 M sucrose and then overlaid with a total volume of 20 ml 300 mM sucrose in four centrifuge tubes. After centrifugation at 100,000 *g* for 1 h, the interface between the two layers was harvested and diluted by the addition of 150 ml of 300 mM sucrose. Membranes were pelleted by centrifugation at 70,000 *g* for 30 min. Each pellet was resuspended in 2 ml 170 mM NaCl and 6 ml 200 mM $NaHCO_3$, pH 7.8, to burst any contaminating zymogen granules and reduce the granule enzyme content of the membranes to negligible levels (Meldolesi et al., 1971). The membranes were repelleted by centrifugation at 70,000 *g* for 30 min and the final pellets were resuspended in 280 mM sucrose, 5 mM MES, pH 6.5 to a protein concentration of ~1 mg/ml, using a glass-glass Dounce homogenizer. Typically, 6 g of pancreas yielded 2 mg membrane protein. Aliquots were stored at −20°C until use. Each aliquot was used only once.

Preparation of Liver Plasma Membranes

Rat liver plasma membranes were prepared by the method of Emmelot et al. (1974) and were washed with 2 ml 170 mM NaCl and 6 ml 200 mM $NaHCO_3$, pH 7.8, as described for the pancreatic membranes. After pelleting, the membranes were resuspended in 280 mM sucrose, 5 mM MES, pH 6.5 to a protein concentration of ~5 mg/ml, divided into aliquots and stored at −20°C. Typically, 45 g of liver yielded 2.8 mg membrane protein.

Bile Canalicular Plasma Membranes

Rat liver bile canalicular plasma membranes were a gift from Dr. B. M. Mullock and Dr. J. P. Luzio (Department of Clinical Biochemistry, Cambridge University) and were prepared essentially as described previously (Hinton et al., 1970; Mullock et al., 1980).

Protein and Enzyme Assays

Protein was assayed by the method of Bradford (1976), using BSA as standard.

5′-nucleotidase was assayed by the method of Evans (1979), using adenosine monophosphate as substrate; phosphate was assayed by the method of Baginski and Zak (1960). NADPH cytochrome c reductase was assayed by the method of Omura and Takesue (1970). Cytochrome *c* oxidase was assayed by the method of Hodges and Leonard (1974), with the modification of Pacquet et al. (1982). Lactate dehydrogenase was assayed by the method of Bergmeyer et al. (1963). α-Amylase was assayed by the method of Rinderknecht et al. (1967), using amylose azure as substrate and porcine α-amylase as standard. Ribonuclease was assayed by the method of Takahashi (1961), using yeast RNA as substrate and bovine pancreatic ribonuclease as standard.

1. *Abbreviation used in this paper*: MES, 2(*N*-morpholino)ethanesulfonic acid.

Standard Incubation

Zymogen granules and plasma membrane fractions were incubated together at 37°C in 250 μl of 280 mM sucrose, 5 mM MES, pH 6.5, containing 1 mM DTT and the protease inhibitors benzamidine (17 μg/ml), pepstatin (1 μg/ml), PMSF (1 mM), antipain (1 μg/ml), soybean trypsin inhibitor (10 μg/ml), and bacitracin (50 μg/ml). Total granule protein concentration and granule membrane protein concentration in the incubation mix were typically 200 and 7 μg/ml, respectively. At the end of the incubation for time-course determinations, granules were pelleted by centrifugation for 1 min in an Eppendorf microfuge. In all other experiments, granules were incubated at 37°C for 15 min, and were pelleted by centrifugation at 900 *g* for 10 min. α-Amylase in the supernatant was assayed as described above. Total α-amylase in the incubation mix (typically 60 U) was assayed after lysing the granules by addition of 0.2% (wt/vol) Triton X-100. Results are expressed as a percentage of the total α-amylase. Each point was determined in triplicate. Values are means ± SD.

Assay for Membrane Fusion

Fusion of plasma membranes with zymogen granule membranes was monitored by following the relief of self-quenching of the fluorescent probe octadecyl rhodamine B-chloride (R18), by the method of Hoekstra et al. (1984). R18 was purchased from Molecular Probes, Inc. (Eugene, OR) and stored at −20°C as a 20 mM stock solution in DMSO. Probe was mixed with plasma membrane suspensions at room temperature to give a self-quenching concentration of between 5 and 8 mol%. Free probe was removed by passing the membranes over a Sepharose CL-4B column (Pharmacia Fine Chemicals, Piscataway, NJ) (0.7 × 25 cm) equilibrated with 280 mM sucrose, 5 mM MES buffer, pH 6.5. Labeled membranes, at a final concentration of 20 μg/ml protein, were then incubated both with and without zymogen granules at 37°C for various times in 500 μl of the standard incubation mix described above. Fluorescence was measured at 37°C in a luminescence spectrometer (model LS-5; Perkin-Elmer Corp., Norwalk, CT) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Any increase in fluorescence seen when labeled membranes were incubated alone was subtracted from that occurring in the presence of zymogen granules to obtain a value for the fusion-related change. A scale of fluorescence was produced by measuring the residual fluorescence of labeled membranes and fluorescence after addition of 0.5% (vol/vol) Triton X-100, the values being taken as 0 and 100% fluorescence, respectively.

Results

Characteristics of Zymogen Granule Preparations and Plasma Membrane Fractions

Zymogen granules were prepared by a method originally described by Rogers et al. (1987). This method is simple and rapid (<1 h) and produces a granule suspension that is essentially free of contamination by other subcellular organelles.

In a typical plasma membrane fraction, enrichment of the plasma membrane marker 5′-nucleotidase over the starting homogenate was eightfold (specific activity 8.2 μmol phosphate/mg protein per h). Enrichments of other enzymes were 1-fold for the microsomal marker NADPH-cytochrome *c* reductase (specific activity 1.4 nmol/mg protein per min); 0.5-fold for the mitochondrial marker cytochrome *c* oxidase (specific activity 2.0 nmol/mg protein per min) and 0.005-fold for the cytoplasmic marker lactate dehydrogenase (specific activity 1 mU/mg protein).

The $NaCl/NaHCO_3$ washing procedure reduced α-amylase contamination of plasma membrane preparations to 60 mU/μg protein. This represents a maximum of 1.7 U in the normal incubation, compared with 60 U contributed by the zymogen granules.

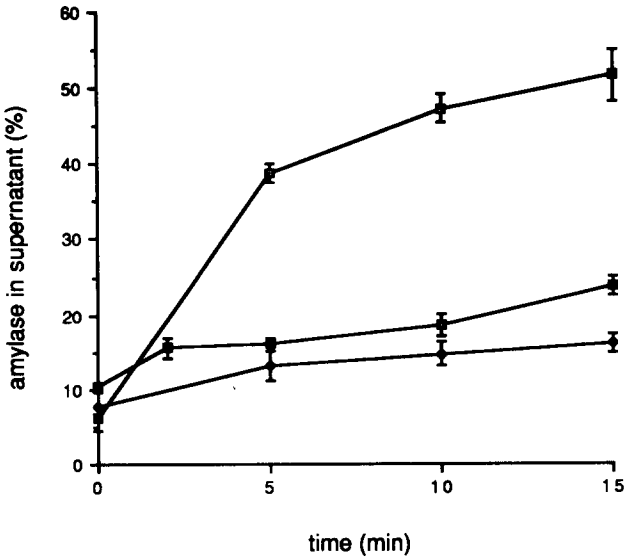

Figure 1. Time-course of release of α-amylase from zymogen granules. Zymogen granules were incubated at 37°C for various times either alone (□) or in the presence of 48 μg/ml of either pancreatic (⊡) or liver (♦) plasma membranes. At the end of the incubation, the granules were pelleted and the amount of α-amylase present in the supernatant was measured. Results are expressed as a percentage of the total α-amylase present in the incubation mix. 9.7 ± 0.3% of the α-amylase was present in the supernatant initially.

Principle of the Assay

If a zymogen granule interacts in vitro with the cytoplasmic face of a fragment of plasma membrane, then membrane fusion may occur, as it does in the intact cell. If the membrane fragment is in the form of a sheet, then the fusion event will cause the granule contents to be discharged into the incubation buffer. It should be possible, therefore, to measure the extent of fusion simply by following the transfer of a granular protein from a low-speed pellet to the supernatant. We chose α-amylase as our marker protein, both because it is abundant and because it can be assayed directly, because it is stored in its active form, unlike most of the hydrolytic enzymes of the pancreas, that are stored in the granules as zymogens.

Basic Properties of the System

The percentage of the total α-amylase that was present initially in the supernatant of the zymogen granule suspension varied from preparation to preparation. Usually the value was between 10 and 20%, although occasionally it was as high as 40%. In almost all cases, however, incubation with pancreatic plasma membranes at 37°C caused a release of a further 30–40% of the α-amylase (Fig. 1). The membrane-triggered release was time dependent, with a half-time of ~4 min. In the absence of plasma membranes α-amylase was released slowly from the granules: typically, 10% was released over a 15-min incubation period. In contrast to the effect of pancreatic membranes, liver plasma membranes did not trigger release of α-amylase. Rather they reduced enzyme release to a level below that for granules incubated alone. Bile canalicular plasma membranes also had an inhibitory effect (not shown).

The dependence of α-amylase release on the amount of membrane protein added is shown in Fig. 2 *a* for both pancreatic and liver membranes. There was some variation between preparations in the concentration dependence of the effects of the membranes. In the experiment illustrated, optimal stimulation of α-amylase release occurred at 20 μg/ml pancreatic membranes, and higher concentrations had no further effect. Liver membranes caused almost complete inhibition of enzyme release at a concentration of 80 μg/ml. In addition to inhibiting the loss of α-amylase from the zymogen granules, liver membranes also reduced the stimulatory effect of pancreatic membranes (Fig. 2 *b*).

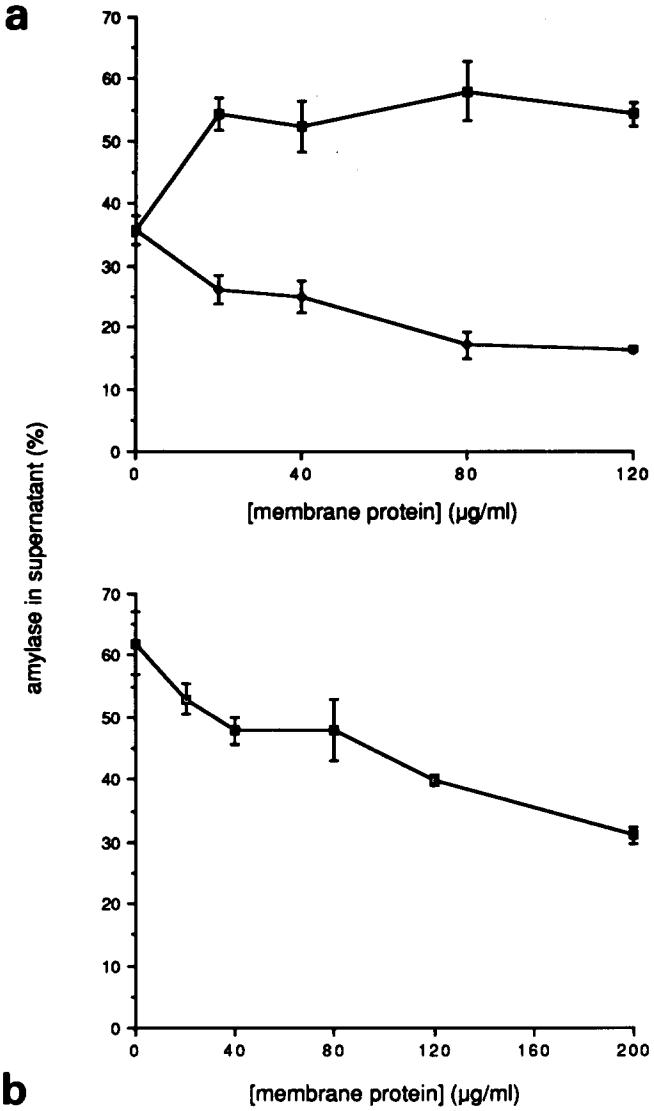

Figure 2. Dependence of the effects of pancreatic and liver membranes on membrane protein concentration. (*a*) Zymogen granules were incubated with various concentrations of pancreatic (⊡) and liver (♦) membranes, and the release of α-amylase was determined. 8.3 ± 1.2% of the α-amylase was present in the supernatant initially. (*b*) Inhibition of pancreatic membrane-induced stimulation by liver membranes. 27.9 ± 3.6% of the α-amylase was present in the supernatant initially. When granules were incubated with liver membranes only, 30.2 ± 1.3% of the enzyme was present in the supernatant at the end of the incubation. When granules were incubated alone, the figure was 35.4 ± 2.3%.

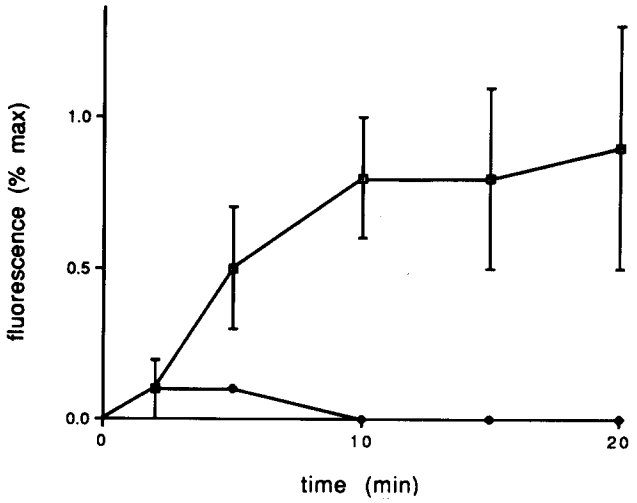

Figure 3. Fluorescence dequenching assay for membrane fusion. Pancreatic plasma membranes and liver bile canalicular plasma membranes were labeled with the fluorescent probe R18 and then incubated, at a protein concentration of 20 μg/ml, with zymogen granules at 37°C for various times. The increase in fluorescence at 590 nm was measured and expressed as a percentage of that obtained when the membranes were solubilized by addition of 0.5% Triton X-100. All values were corrected for any change occurring in the absence of zymogen granules. Values for the pancreatic plasma membranes (⊡) are the means (± SD) from three experiments. Values for the bile canalicular plasma membranes (♦) are the means from two experiments.

These results indicate that zymogen granules incubated at 37°C slowly lose their contents. This may be due to protein diffusion across the granule membrane (Rothman et al., 1974) or to granule lysis, that could occur either spontaneously or through granule-granule interaction. When pancreatic plasma membranes are present, release of granule contents is triggered. This effect is specific to pancreatic membranes, as liver membranes do not trigger release; indeed, they have a protective effect, probably through membrane dilution, which reduces the likelihood of a "productive" interaction.

Involvement of a Membrane Fusion Event in the Granule-Membrane Interaction

The physiological significance of the granule-membrane interaction hinges on the question of whether the release of granule contents is a consequence of a membrane fusion event. To answer this question, we used a separate assay that directly measures membrane fusion. The principle of the assay is that fusion of plasma membranes containing self-quenching concentrations of the fluorescent probe octadecyl rhodamine B-chloride (R18) with unlabeled zymogen granule membranes will result in dilution of the probe in the target membrane and cause a relief of self-quenching (Hoekstra et al., 1984). Pancreatic plasma membranes and liver bile canalicular plasma membranes were labeled with the probe and incubated with unlabeled zymogen granules under the same conditions as in the content-release assay. Fluorescence was measured at various times; an increase in fluorescence was taken to indicate that membrane fusion had occurred. The results obtained are shown in Fig. 3. The fluorescence of the pancreatic membranes increased on incubation with zymogen granules, up to a peak of ~1% of the maximum possible fluorescence after 15 min. No increase in fluorescence was seen with bile canalicular membranes. Hence, pancreatic plasma membranes, which trigger α-amylase release from zymogen granules, at the same time fuse with granule membranes. Furthermore, the time-course of fusion is identical to that of α-amylase release. In contrast, bile canalicular membranes neither trigger α-amylase release nor fuse with zymogen granule membranes.

These results provide a qualitative demonstration that incubation of pancreatic plasma membranes with zymogen granules results in fusion between the granule and plasma membranes and release of the granule contents. The extent of fusion occurring between pancreatic plasma membranes and granule membranes is difficult to assess, even in this apparently simple system. As discussed by Hoekstra et al. (1984), the extent of fluorescence dequenching observed depends on the relative amounts of labeled and target membranes, provided that the surface density of R18 in the bilayer is within certain limits. The ratio of labeled to target membranes used here is ~3:1, by protein. Consequently, if all of the plasma membranes fuse with zymogen granule membranes, an increase in fluorescence of 25% of the total possible increase would be expected. Another factor to consider is the proportion of the labeled membranes that are present as either inside-out vesicles or sheets, and that are therefore able to fuse with granule membranes. Usually <50% of plasma membrane vesicles are inside-out. For example, figures of <30% and ~15% have been reported previously for rat liver (Sips et al., 1982) and baby hamster kidney cells (Woodman and Edwardson, 1986), respectively. Furthermore, electron micrographs of samples of the pancreatic plasma membranes used in our experiments indicate that relatively little membrane (<5%) is in the form of sheets. Finally, only the apical plasma membrane of the pancreatic acinar cell would be expected to fuse with zymogen granule membranes, and this accounts for only ~4% of the total surface area of the cell (Rosenzweig et al., 1983). Taking all these factors into account, the maximum possible dequenching that would be expected in our system is probably of the order of 1% of the total.

Temperature Dependence of the Interaction

A specific binding reaction between plasma membranes and zymogen granules isolated from cat pancreas has been reported previously (Milutinovic et al., 1977). The initial rate of binding was shown to increase with temperature, being twice as high at 22 than at 4°C. In the light of these results, we decided to test the temperature dependence of our granule-membrane interaction. Pancreatic plasma membranes were incubated with zymogen granules at 4, 20, and 37°C, and the release of α-amylase was measured. In contrast to the results of Milutinovic et al., we found that α-amylase was released only at 37°C (data not shown). This observation provides further support for our contention that the membrane-triggered release of α-amylase involves more than a simple granule-membrane–binding reaction.

Requirement for Membrane Proteins

The observation that pancreatic, but not liver, plasma membranes stimulate the release of α-amylase from zymogen

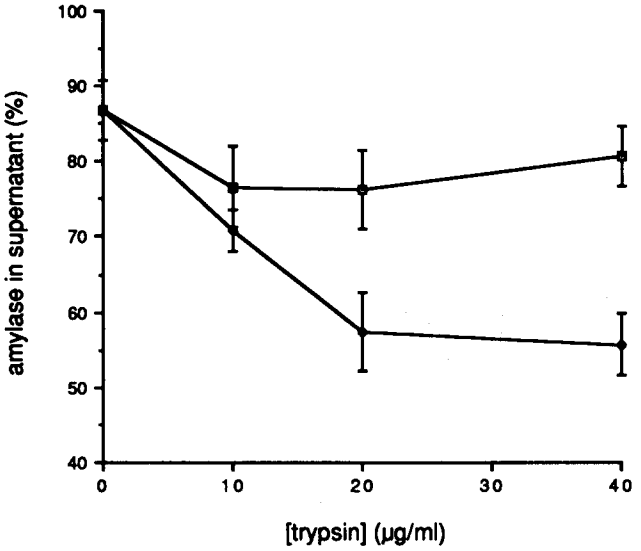

Figure 4. Effect of pretreatment of pancreatic plasma membranes with trypsin on granule-membrane interaction. A suspension of pancreatic plasma membranes (protein concentration 1 mg/ml) was treated with various concentrations of trypsin-TPCK (Cooper Biomedical, Malvern, PA) at 4°C for 1 h. Enzyme was present either alone (♦) or in the presence of soybean trypsin inhibitor, also at 1 mg/ml (⊡). At the end of the preincubation period, trypsin inhibitor was added to membranes that had been exposed to active enzyme. The membranes (32 μg/ml) were then incubated with granules and the release of α-amylase was determined. 43.7 ± 2.9% of the α-amylase was present in the supernatant initially. When granules were incubated alone, 56.0 ± 3.2% of the enzyme was present in the supernatant at the end of the incubation.

granules indicates that the in vitro interaction shows specificity, as would be expected of a physiologically significant event. Such specificity normally points to the involvement of proteins. To test this, we preincubated the pancreatic membranes with trypsin, at various concentrations, in the presence and absence of 100 μg/ml soybean trypsin inhibitor, before using them in the assay. We found that pretreatment of the membranes with trypsin reduced their efficacy in a dose-dependent manner, to a point where release was no greater than that for granules incubated alone (Fig. 4). Trypsin inhibited with soybean trypsin inhibitor had no significant effect on membrane-triggered α-amylase release. Thus the stimulatory effect of pancreatic plasma membranes requires the presence of membrane proteins.

Effect of Ca^{2+}

In many examples of regulated exocytosis, a rise in cytosolic free Ca^{2+} concentration is a major intracellular signal (Burgoyne, 1987). We therefore examined the effect of Ca^{2+} on the in vitro granule-membrane interaction. It was not possible to do this by assaying α-amylase release, since this enzyme contains a bound Ca^{2+} ion, removal of which causes inactivation, both directly (Vallee et al., 1959) and through increased sensitivity to proteases (Stein and Fischer, 1958). Instead, we used ribonuclease release as our marker for loss of granule integrity. Ribonuclease is released in parallel with α-amylase (not shown), but its activity is not sensitive to changes in Ca^{2+} concentration. In the range of concentrations normally found within cells capable of regulated exocytosis (100 nM–1 μM), Ca^{2+} did not stimulate the interaction between granules and pancreatic membranes (Fig. 5). At higher concentrations (>5 μM) Ca^{2+} inhibited the stimulatory effect of the membranes. Liver membranes did not trigger ribonuclease release at any of the Ca^{2+} concentrations used.

Involvement of a G Protein

In some cell types regulated exocytosis can be triggered by GTP analogues when the cytosolic free Ca^{2+} concentration is buffered at or below the physiological resting level of 10–100 nM (Barrowman et al., 1986). We investigated the possibility that such a control process operates in our in vitro system. Typically, G proteins are activated by GTP, and its nonhydrolysable analogues GTPγS and GMP-PNP, and also by the $[AlF_4]^-$ ion; they are inactivated by GDP and its nonhydrolysable analogue GDPβS (Gilman, 1987). We looked for an effect of these agents on α-amylase release produced by a sub-optimal concentration of membranes (16 μg/ml). We found that GTP, GTPγS, GMP-PNP, and $[AlF_4]^-$ all enhanced the effect of the membranes, increasing the percentage of the α-amylase released into the supernatant to between 70 and 80% (Fig. 6). The concentrations of the nucleotides giving 50% maximal enhancement of the membrane effect were 15 μM for GTPγS, 70 μM for GMP-PNP, and 200 μM for GTP (Fig. 6 *a*). GTPγS did not stimulate α-amylase release from granules alone or from granules in the presence of liver membranes. Neither GDPβS nor GDP (not shown) significantly stimulated α-amylase release at concentrations up to 100 μM, but at this concentration GDPβS antagonized the action of GTPγS, causing a fivefold parallel shift of the dose-response curve (Fig. 6 *b*). Aluminum (50 μM), in com-

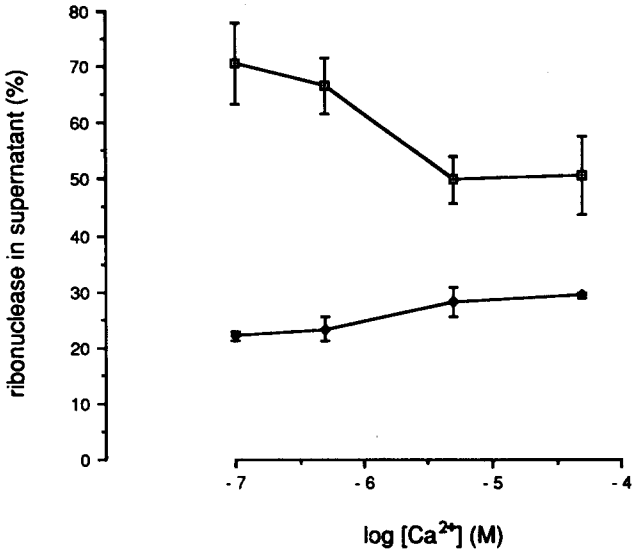

Figure 5. Effect of Ca^{2+} on the granule-membrane interaction. Zymogen granules were incubated with 64 μg/ml of pancreatic (⊡) or liver (♦) membranes, in the presence of various concentrations of Ca^{2+}, and the release of ribonuclease determined. Ca^{2+} buffering was achieved using 1 mM EGTA. Free Ca^{2+} concentrations were calculated using an iterative computer program based on that of Fabiato and Fabiato (1979). 16.9 ± 1.1% of the α-amylase was present in the supernatant initially.

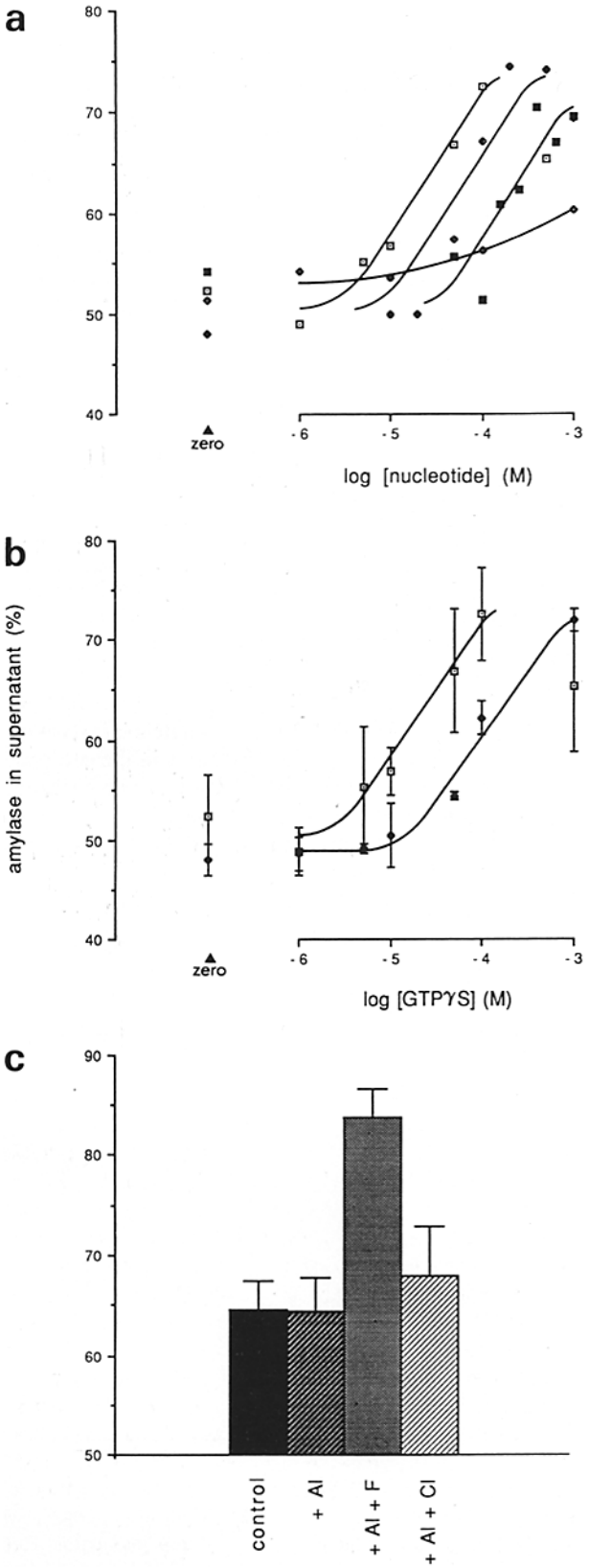

Figure 6. Evidence for the involvement of a G-protein in granule-membrane interaction. Zymogen granules were incubated with a suboptimal concentration of pancreatic plasma membranes (16 μg/ml) and α-amylase release was determined. (*a*) Dose-response curves to GTPγS, added as a tetralithium salt (⊡); GMP-PNP, added as a sodium salt (♦); GTP, added as a trisodium salt (■); and GDPβS, added as a trilithium salt (◇). Error bars are not shown for clarity. (*b*) Dose-response curves to GTPγS in the absence (⊡) and presence (♦) of GDPβS (100 μM). (*c*) Effect of aluminum (50 μM as aluminum ammonium sulfate), fluoride (10 mM as potassium fluoride); and chloride (10 mM as potassium chloride).

bination with fluoride (10 mM), caused a significant increase in membrane-triggered α-amylase release (Fig. 6 *c*). Neither agent was effective alone, and aluminum in combination with chloride (10 mM) was also ineffective.

These data are consistent with the notion that the in vitro interaction between zymogen granules and pancreatic plasma membranes can be stimulated through the operation of a G protein. In an attempt to pinpoint the location of this G protein, we looked in more detail at the effect of GTPγS on the dependence of α-amylase release on plasma membrane protein concentration. The results, shown in Fig. 7, were obtained with two different plasma membrane preparations, which had different concentration-effect characteristics. We found that GTPγS (100 μM) both shifted the concentration-release curve to lower membrane protein concentrations and also increased the maximum percentage release (Fig. 7 *a*). In addition, we showed that membranes preincubated with GTPγS triggered α-amylase release more effectively than control membranes (Fig. 7 *b*). Release was triggered still more effectively, however, when the GTPγS was present during the granule-membrane interaction. Our interpretation of these results is that part of the effect of GTPγS is exerted on the plasma membranes (hence the leftward shift of the concentration-release curve and the effect of preincubation of the membranes with GTPγS), but that this agent also has an effect on the zymogen granule (hence the increase in the maximum amount of α-amylase release and the requirement for GTPγS during the incubation to produce a maximal effect).

Finally, to see whether the effects of the nucleotide triphosphates described above was specific to guanine nucleotides, we examined the effect in our system of ATP, ATPγS, and CTP. We found that none of these other nucleotide triphosphates, at a concentration of 100 μM, caused any stimulation of the granule-membrane interaction (not shown). Hence, the effect is specific to guanine nucleotide triphosphates.

Discussion

Our ultimate goal is to understand the molecular details of the final step in the process of regulated exocytosis, that is granule-plasma membrane fusion. Our strategy is to develop a simple assay based on well-defined components that reproduces this step in vitro and to use this assay to identify the proteins responsible for exocytotic fusion and its control. In this paper we describe an interaction between pancreatic zymogen granules and plasma membranes that involves membrane fusion, and that is time-dependent, specific to pancreatic membranes and dependent on both the concentration of plasma membranes and the presence of intact proteins in the membranes. It appears, therefore, to fulfill the above criteria.

The in vitro interaction between zymogen granules and plasma membranes is stimulated by activators of G proteins,

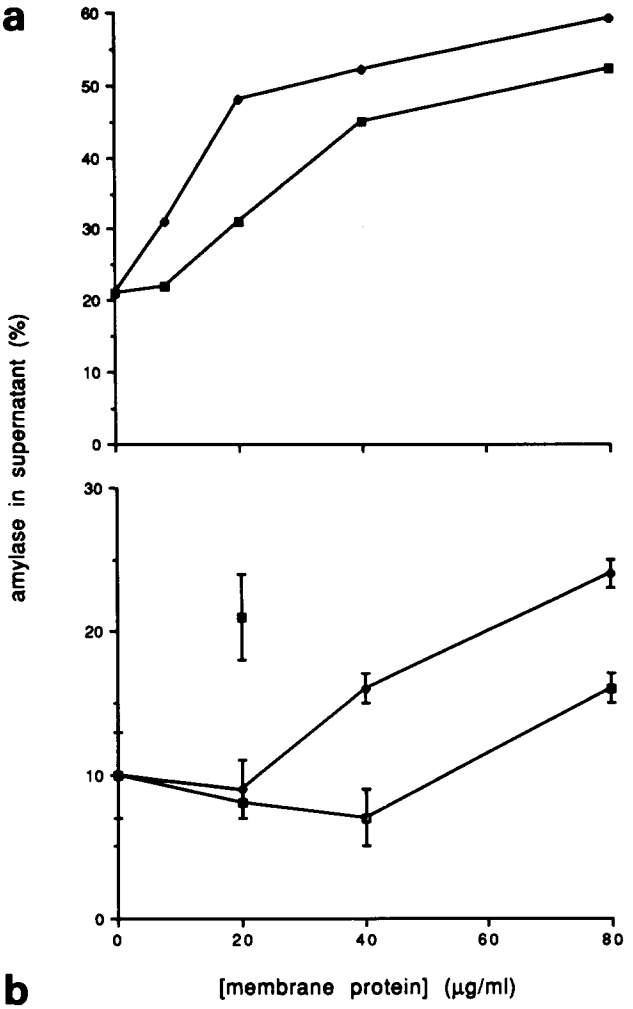

Figure 7. Effect of GTPγS on the dose dependence of the effect of pancreatic plasma membranes. (*a*) The concentration dependence of the effect of pancreatic plasma membranes on α-amylase release was examined in the absence (⊡) and presence (♦) of GTPγS (100 μM). Values are the means of two experiments. (*b*) Plasma membranes were pre-incubated either alone (⊡) or with GTPγS (100 μM; ♦) for 15 min at 37°C and then tested for their ability to release α-amylase from zymogen granules. In addition, GTPγS (100 μM) was included in the membrane-granule incubation at one membrane concentration (20 μg/ml; ■). 9.0 ± 1.0% of the α-amylase was present in the supernatant initially.

which are known to trigger exocytosis in several cell types. In permeabilized mast cells (Cockcroft et al., 1987), neutrophils (Barrowman et al., 1986), and adrenal chromaffin cells (Knight and Baker, 1985; Bittner et al., 1986), GTP analogues can regulate secretion by acting not only on the G protein that couples receptors to polyphosphoinositide hydrolysis at the plasma membrane, but also through a separate G protein, designated G_E (Gomperts, 1986) that operates late in the exocytotic sequence. G_E can be differentiated from the earlier G protein through its resistance to the effects of neomycin, which inhibits polyphosphoinositide hydrolysis. Using permeabilized cells, it has not been possible to determine the location of G_E, but it has been inferred that it is either on the secretory granule membrane or the plasma membrane (Gomperts, 1986). The stimulation of the interaction of isolated zymogen granules and pancreatic plasma membranes by GTP analogues and by $[AlF_4]^-$ ions reported here suggests that G proteins operate both on the granule membrane and also on the plasma membrane to control granule-membrane fusion. The presence of GTP-binding proteins on the membranes of secretory granules has been reported previously (Toutant et al., 1987), although their function was not investigated.

Ca^{2+}, at concentrations up to 5 mM, did not stimulate the in vitro interaction between zymogen granules and pancreatic plasma membranes. At first sight these data appear inconsistent with the role of Ca^{2+} as a direct mediator of stimulus-secretion coupling, a role inferred in pancreatic acinar cells from observations that agonists such as acetylcholine, cholecystokinin, and bombesin cause a rapid rise in cytosolic free Ca^{2+} concentration and Ca^{2+} efflux (Pandol et al., 1985), and also that secretion can be stimulated in intact cells by Ca^{2+}-ionophores (Merritt and Rubin, 1985) or in permeabilized cells by elevation of $[Ca^{2+}]$ to >1 μM (Knight and Koh, 1984). It is clear, however, that other exocytotic signals operate in the exocrine pancreas. For example, activators of protein kinase C, such as PMA and oleoylacylglycerol (Gunther, 1981; Rogers et al., 1988), or agonists such as vasoactive intestinal peptide and secretin, that elevate cAMP levels, stimulate exocytosis without elevating cytosolic $[Ca^{2+}]$. The effects of these agonists are probably mediated by protein phosphorylation, which may either trigger exocytosis directly or sensitize the exocytotic machinery to resting cellular levels of Ca^{2+}. In other cell types, permeabilized neutrophils or mast cells, for example, (Fernandez et al., 1984), GTP stimulates exocytosis at or below resting or subcellular Ca^{2+} levels. If these signals act at a site downstream from Ca^{2+} in the signalling pathway for exocytosis, for example at the level of granule-plasma membrane interaction, they might still operate in an in vitro system under circumstances in which the target for Ca^{2+} is lost.

The in vitro interaction described here occurs in the absence of cytosol and an energy source, unlike membrane fusion events occurring at other points in the intracellular transport pathway (Balch et al., 1984, 1987; Braell, 1987) and in constitutive exocytosis (Woodman and Edwardson, 1986). Furthermore, the interaction is merely stimulated by, and not absolutely dependent on, GTP and its analogues. These properties may reflect the rather special behavior of the secretory vesicle within the cell. Secretory vesicles form by budding from a compartment on the *trans* side of the Golgi complex, that has been termed the *trans* Golgi network (Griffiths and Simons, 1986), and then undergo a series of maturation events. The mature granule is stored, probably in association with the cytoskeleton, until an appropriate signal triggers exocytosis. It is quite likely, then, that at any one time, a significant population of granules in the cell exists in a "primed" state, and is able to interact immediately with the plasma membrane once freed from restraint. This situation is quite different from that in other cell-free assays, where only a very small pool of primed vesicles exists within the cell and where the in vitro system is asked to effect the transfer of vesicles between two unprimed compartments. The effects of GTP in this system may indicate that further priming above the basal level can be made to occur in vitro.

The ability to produce rapidly an almost pure preparation of zymogen granules that retain functional activity in a cell-

free system makes it possible to undertake an investigation of the role of proteins in the granule membrane in exocytosis. We hope that this approach will lead eventually to the elucidation of the molecular mechanisms underlying the process of regulated exocytosis.

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