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Pancreatic plasma membranes: promiscuous partners in membrane fusion

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We have developed a system in which the fusion of pancreatic plasma membranes with zymogen granules can be studied *in vitro*. We show here that pancreatic plasma membranes fuse not only with pancreatic zymogen granules but also with parotid secretory granules. In contrast, parotid membranes fuse only with parotid granules and not with pancreatic granules. The extent of fusion is insensitive to Ca^{2+} for all combinations of plasma membranes and granules. Guanosine 5′-[γ-thio]triphosphate (GTP[S]), on the other hand, stimulates fusion of pancreatic membranes with both pancreatic granules and parotid granules, but inhibits fusion between parotid membranes and parotid granules.

INTRODUCTION

We have shown that the membrane fusion event involved in regulated exocytosis in the exocrine pancreas can be reconstituted *in vitro*. Fusion between pancreatic plasma membranes and zymogen granules can be followed either through the release of granule content enzymes, such as amylase (Nadin et al., 1989), or by a fluorescence de-quenching technique that measures lipid mixing (MacLean and Edwardson, 1992). Fusion shows some degree of specificity, in that plasma membranes prepared from either liver or chromaffin cells do not fuse with zymogen granules. The negative result with liver membranes is perhaps not surprising, since this tissue is not known to undergo regulated exocytosis. Chromaffin cells, on the other hand, are used widely in the study of regulated exocytosis (Burgoyne, 1991), and the inability of chromaffin cell plasma membranes to fuse with pancreatic zymogen granules suggests that the molecular mechanisms of exocytosis in exocrine and neurally derived cells are different. In an attempt to pursue this question of specificity, we have extended the fluorescence de-quenching technique to a study of fusion between granules and plasma membranes from another exocrine gland, the parotid. This has enabled us to test whether fusion occurs between granules and plasma membranes from the two different tissues. We show here that pancreatic plasma membranes fuse with both types of granules, but that parotid membranes fuse only with parotid granules. In all cases, fusion is insensitive to Ca^{2+}. In contrast, guanosine 5′-[γ-thio]triphosphate (GTP[S]) stimulates pancreatic membrane/parotid granule fusion, as we have previously reported for pancreatic membrane/pancreatic granule fusion, but inhibits parotid membrane/parotid granule fusion.

MATERIALS AND METHODS

Octadecylrhodamine B-chloride (R18) was supplied by Molecular Probes Inc. (Eugene, OR, U.S.A.); GTP[S] was from Boehringer Mannheim G.m.b.H. (Germany). All other reagents were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

Rat pancreatic plasma membranes were prepared and characterized as we have described previously (Nadin et al., 1989). Rat parotid plasma membranes were prepared essentially as described previously (Arvan and Castle, 1982), except for the initial disruption of the tissue, which was carried out in a glass–glass homogenizer instead of a Polytron homogenizer. The degree of purification of plasma membranes was assessed by measuring the specific activities of the following enzymes relative to the starting homogenate: γ-glutamyl transpeptidase (plasma membrane marker; Tate and Meister, 1974), amylase (secretory granule content marker; Rinderknecht et al., 1967), cytochrome *c* oxidase (mitochondrial marker; Hodges and Leonard, 1974), and NADPH–cytochrome *c* reductase (endoplasmic reticulum marker; Omura and Takesue, 1970). Protein was assayed by the method of Bradford (1976), using BSA as standard. Plasma membranes were stored in aliquots at −20 °C and thawed immediately before use. Pancreatic zymogen granules and parotid secretory granules were both prepared by a procedure that we have described previously for pancreatic granules (Nadin et al., 1989). Granules were prepared fresh each day.

The membranes of secretory granules from a single rat (suspended in 280 mM sucrose, 5 mM Mes buffer, pH 6.0; total protein concentration 5 mg/ml) were loaded with the lipid-soluble fluorescent probe R18 (100 μM) by incubation at 37 °C for 5 min. Labelled granules were recovered by centrifugation at 900 *g* for 10 min and resuspended in the original volume of buffer. Fluorescence de-quenching assays were carried out using either a Perkin–Elmer (Beaconsfield, U.K.) LS-3 fluorescence spectrometer (Figures 2, 4 and 6) or a Hitachi F-2000 (Wokingham, U.K.) spectrometer (Figures 3, 5 and 7). An advantage of the latter machine is that membranes may be added through an injection port without interrupting the fluorescence trace. Wavelengths used were 560 nm (excitation) and 590 nm (emission). Samples of labelled granules (10 μl) were added to 700 μl of sucrose/Mes buffer, pH 6.5. Ca^{2+} was unbuffered except in experiments to determine the effect of Ca^{2+} on fusion. All de-quenching assays were carried out at 37 °C in an unstirred, thermostatted cuvette; de-quenching signals at lower temperatures (for example 20 °C) are smaller and slower (M. MacLean and J. M. Edwardson, unpublished work). Granules were incubated for 1 min to obtain a steady baseline. Plasma membranes were then added, with thorough mixing, and the fluorescence signal was followed for a further 4 or 5 min. De-quenching at the end of this period is expressed as a percentage of that achieved after solubilization of the membranes by addition of Triton X-100. All errors are S.E.M.s. As explained previously (MacLean and Edwardson, 1992), the relationship between de-quenching and the percentage of the granules undergoing membrane fusion is complex, so that de-quenching values can be regarded essentially as arbitrary units.

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

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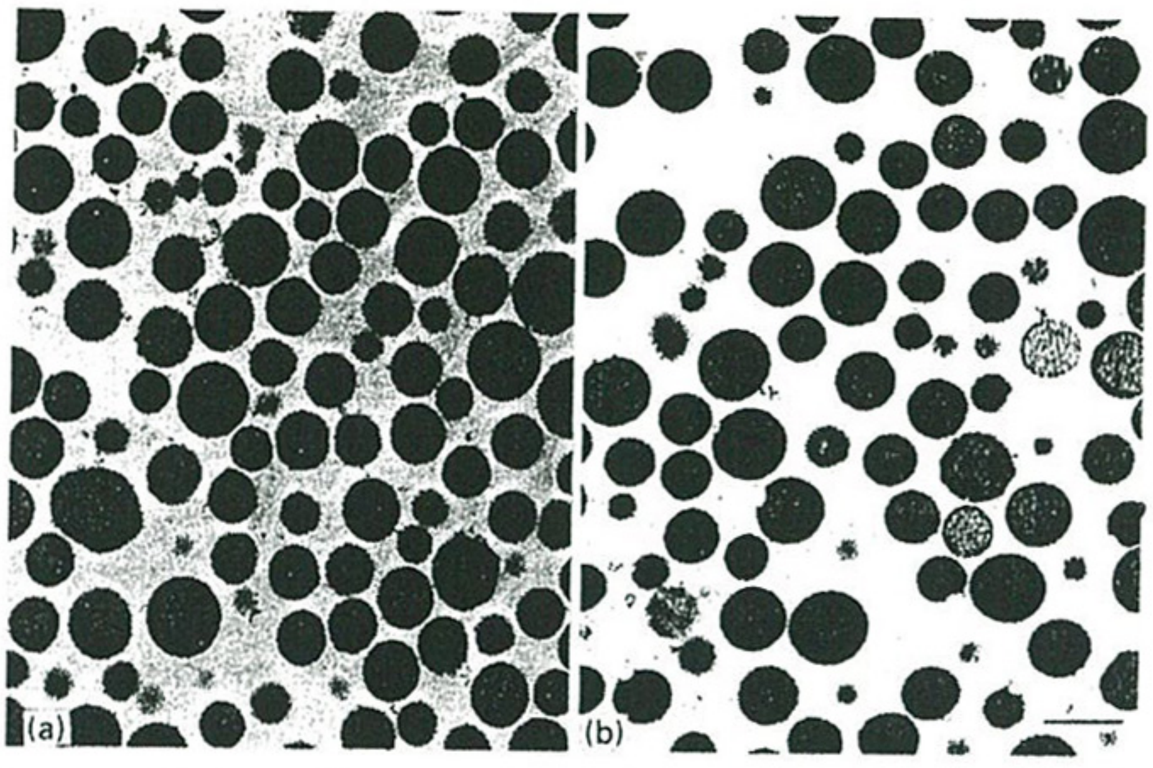

Figure 1 Electron micrographs of typical preparations of pancreatic zymogen granules (a) and parotid secretory granules (b)

The bar represents 1 μm.

RESULTS

Characterization of plasma membranes and secretory granules

Plasma membrane fractions were prepared from rat pancreas (Nadin et al., 1989) and rat parotid gland (Arvan and Castle, 1982) by previously reported procedures. We have shown that the method for preparation of pancreatic plasma membranes results in a mean enrichment of the plasma membrane marker 5′-nucleotidase of 8-fold over the starting homogenate, with no enrichment of markers for other organelles (Nadin et al., 1989). Many batches of pancreatic plasma membranes have been prepared that are active in membrane fusion; three batches were used in this study. The parotid plasma membrane fraction was enriched in the plasma membrane marker γ-glutamyl transpeptidase by 9.4-fold (mean value for four batches of membranes); mean enrichments of markers for other organelles were: amylase (secretory granule content), 0.2-fold; cytochrome *c* oxidase (mitochondria), 0.2-fold; NADPH–cytochrome *c* reductase (endoplasmic reticulum), 2.5-fold. Electron micrographs of typical preparations of the two types of secretory granule (Figure 1) showed that both are substantially free of contamination by other organelles, and also that the pancreatic zymogen granules (Figure 1a) and parotid secretory granules (Figure 1b) are almost identical in appearance and size (diameter approx. 1 μm).

Fusion of pancreatic plasma membranes with secretory granules

Fusion between plasma membranes and secretory granules was measured at 37 °C through the de-quenching of the fluorescence of the probe R18, which was loaded into the granule membrane. It was found that pancreatic plasma membranes gave a de-quenching signal when incubated with either parotid secretory granules (Figure 2a) or pancreatic zymogen granules (Figure 2b). The half-time of de-quenching for pancreatic membranes over the series of experiments carried out was in the range 20–40 s, and there was no obvious difference between the values given by parotid or pancreatic granules. The relationship between de-quenching at the end of the incubation and the concentration of pancreatic plasma membrane protein is shown in Figure 2(c). The concentration of membrane protein giving 50% of the maximum signal (EC_{50}), with the concentration of granules used, was 2 μg/ml for parotid granules and 3 μg/ml for zymogen granules. Since de-quenching with the higher concentrations of

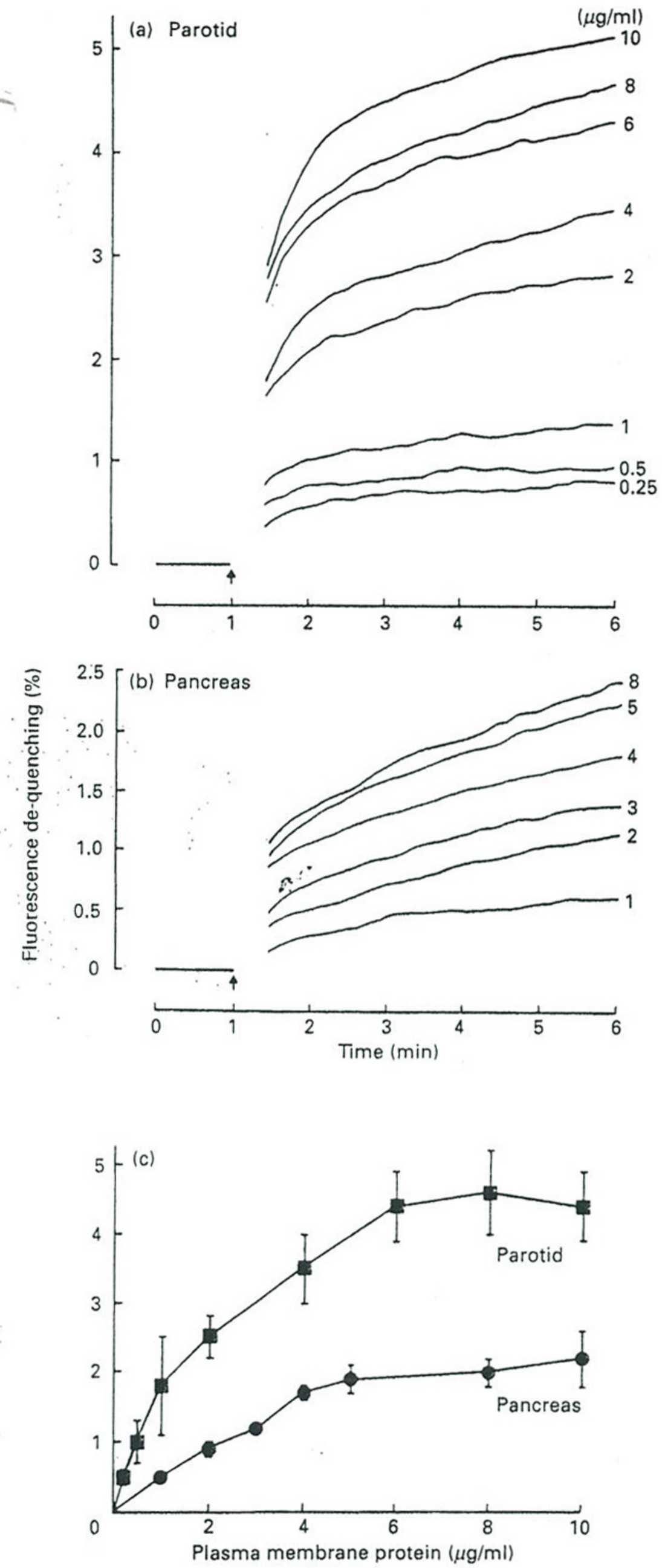

Figure 2 Promiscuous fusion of pancreatic plasma membranes

Membrane fusion was measured through the de-quenching of the fluorescence of the lipic-soluble probe R18 that had been loaded into the membranes of pancreatic zymogen granules or parotid secretory granules. For both types of granule, the final granule protein concentration was approx. 70 μg/ml. (**a**) Typical de-quenching traces obtained when pancreatic plasma membranes were added at various concentrations to labelled parotid secretory granules at 37 °C. Plasma membranes were added at the time indicated by the arrow. The basal fluorescence at the time of addition of the membranes is indicated by the bar. (**b**) Typical traces for pancreatic plasma membranes added to labelled pancreatic zymogen granules. (**c**) Relationship between de-quenching (at 6 min) and pancreatic plasma membrane protein concentration for parotid secretory granules (■; $n = 4$) or pancreatic zymogen granules (●; $n = 7$). De-quenching is expressed as a percentage of that achieved after solubilization of the membranes by addition of 0.2% Triton X-100.

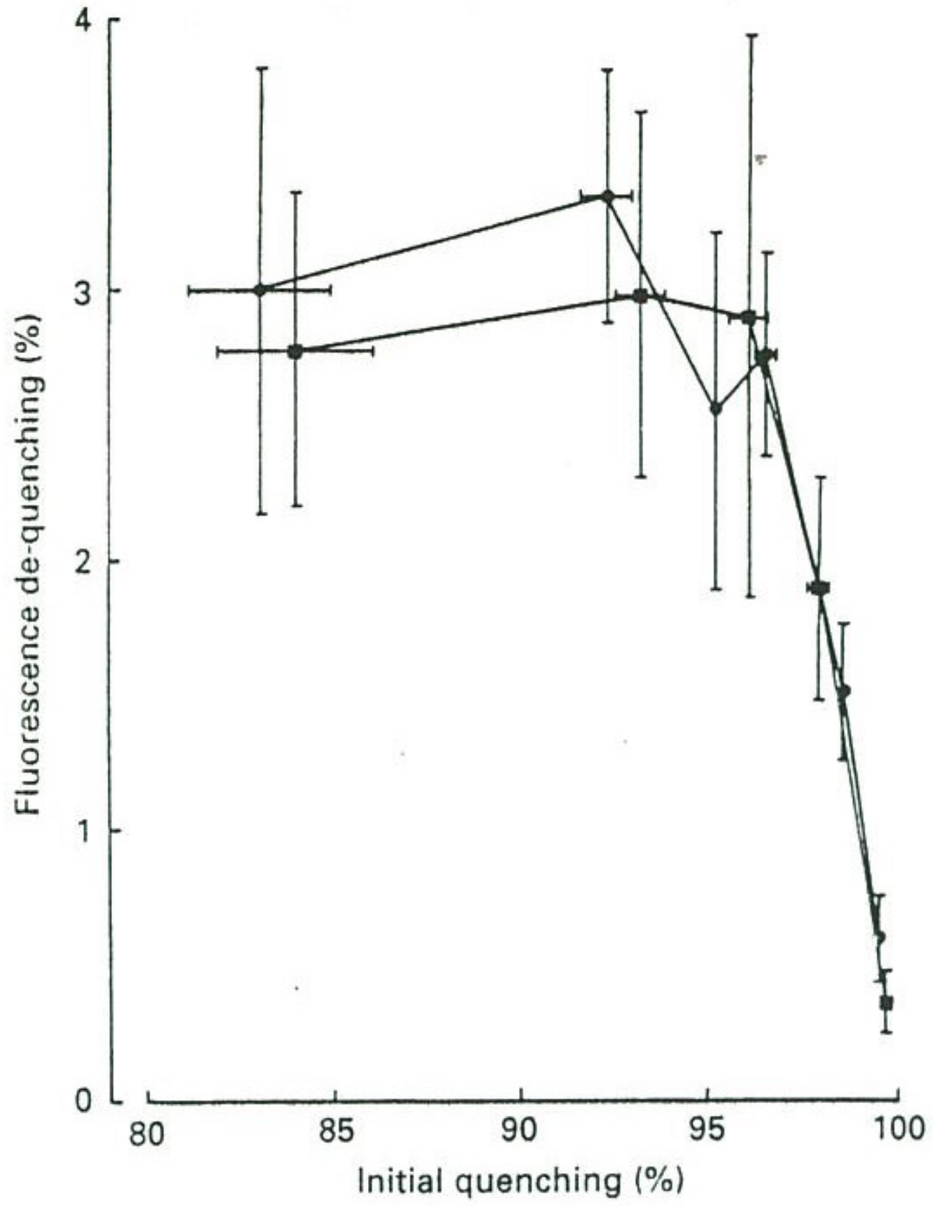

Figure 3 Relationship between de-quenching and initial quenching for pancreatic plasma membranes

Pancreatic zymogen granules and parotid secretory granules were incubated with various concentrations of R18, in order to produce various values for initial quenching. The granules were incubated with a maximal concentration of pancreatic plasma membranes (15 μg/ml) and de-quenching was determined. ●, Pancreatic zymogen granules; ■, parotid secretory granules. Data points were grouped together in the following ranges of initial quenching: pancreas, < 90%, 90–94%, 94–96%, 96–98%, 98–99%, 99–100%; parotid, < 90%, 90–94%, 94–97%, 97–99%, 99–100%. Numbers of points per group ranged from three to nine.

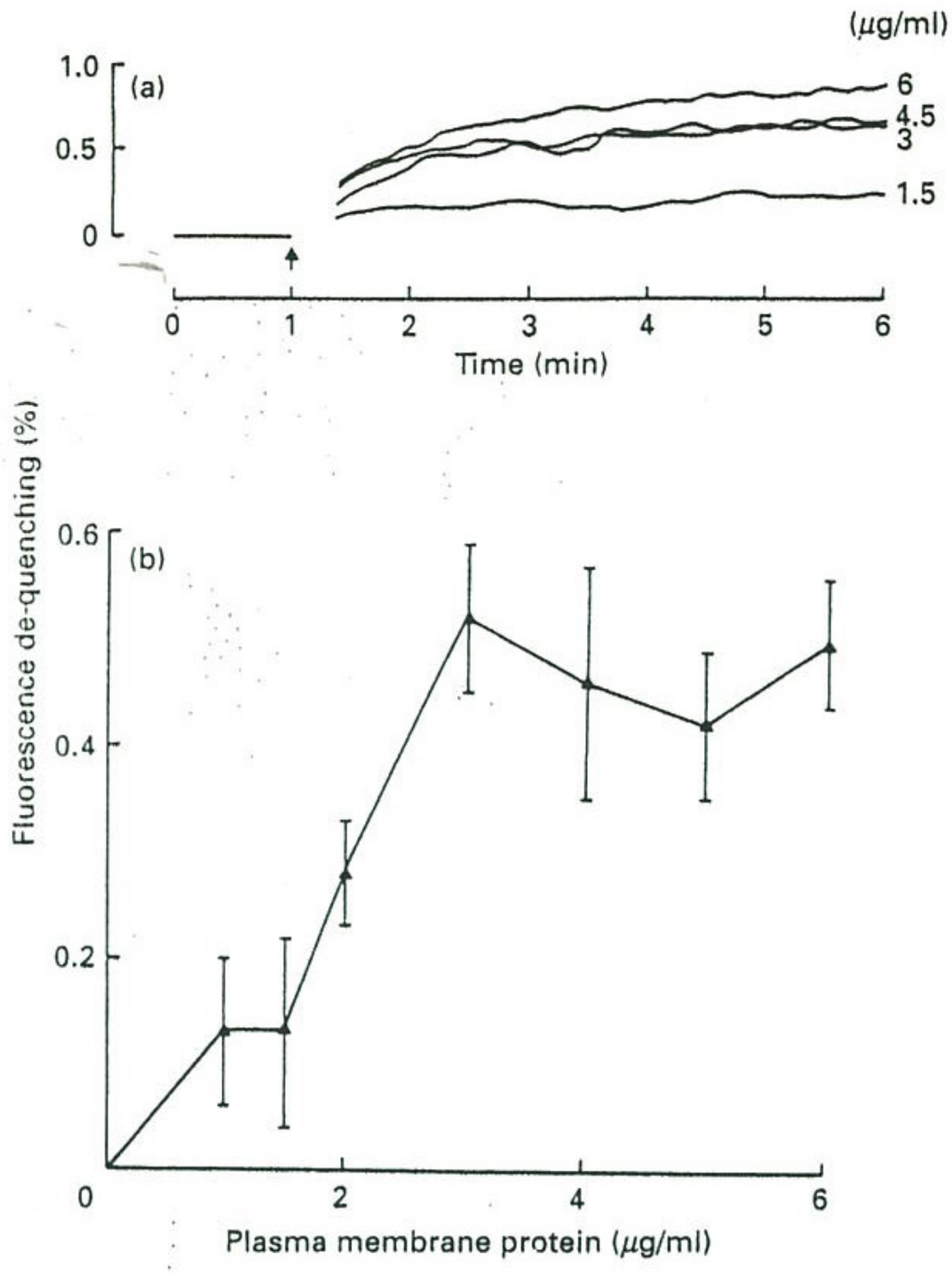

Figure 4 Fusion of parotid plasma membranes with parotid secretory vesicles

(**a**) Typical de-quenching traces obtained when parotid membranes were added at various concentrations to labelled parotid secretory granules. (**b**) Relationship between de-quenching (at 6 min) and parotid plasma membrane protein concentration ($n = 8$).

plasma membranes did not reach completion within the 6 min incubation period, a degree of caution is required in the interpretation of these EC_{50} values. Nevertheless, they are close to the EC_{50} of 4 μg/ml reported previously for fusion between pancreatic plasma membranes and pancreatic zymogen granules (MacLean and Edwardson, 1992).

One feature of the results shown in Figure 2 that requires explanation is the difference in the maximum de-quenching signals given by the two types of granule. Since the plasma membrane target was the same in both cases, the cause of the difference in de-quenching must lie with the granules. We have shown previously (MacLean and Edwardson, 1992) that the size of the maximum de-quenching signal, for pancreatic zymogen granules fusing with zymogen granule membranes, falls as the initial quenching rises above 90%. Since the mean values for the initial fluorescence quenching were 94 ± 1% ($n = 7$) for the pancreatic granules and 91 ± 1% ($n = 4$) for the parotid granules, it seemed possible that the difference in the maximum de-quenching signals was a result of the difference in the initial quenching. To test this, samples of pancreatic and parotid granules were incubated with various concentrations of R18, in order to achieve different values of initial quenching. The relationship between de-quenching (at a maximal concentration of pancreatic plasma membranes) and initial quenching was then determined. As found previously for zymogen granule/granule membrane fusion, de-quenching increased as initial quenching fell, reaching a plateau value below 90% quenching (Figure 3). The lines for the two types of granule are virtually superimposable, which indicates that the granules behave identically with respect to the pancreatic plasma membrane target, and supports our proposal that the difference in maximal de-quenching for the two types of granule (Figure 2) is a consequence of the different initial quenching.

Fusion of parotid plasma membranes with secretory granules

Parotid plasma membranes fused *in vitro* with parotid secretory granules (Figure 4a), but not with pancreatic zymogen granules. The half-time of de-quenching for parotid membranes in this study was 40–80 s, that is, somewhat longer than the value for pancreatic membranes. The relationship between de-quenching at the end of the incubation and the concentration of parotid plasma membrane protein is shown in Figure 4(b). The EC_{50} for parotid membrane protein was 2 μg/ml, i.e. identical to that for fusion between pancreatic membranes and parotid granules (Figure 2). The maximum de-quenching signal given by the parotid membrane/parotid granule combination was smaller than that for the pancreatic membrane/parotid granule pairing. There are two possible explanations for this. First, the mean value for the initial quenching of the probe in the series of experiments illustrated in Figure 4 was 96 ± 1% ($n = 8$), i.e., higher than that for the experiments with parotid granules shown in Figure 2. This would result in a smaller de-quenching signal (see above). Secondly, if the number of fusion events per granule is limited, as appears to be the case (MacLean and Edwardson, 1992), then the size of the de-quenching signal will depend on the mean area of the target plasma membrane. If the mean area of the parotid plasma membranes is less than that of the pancreatic

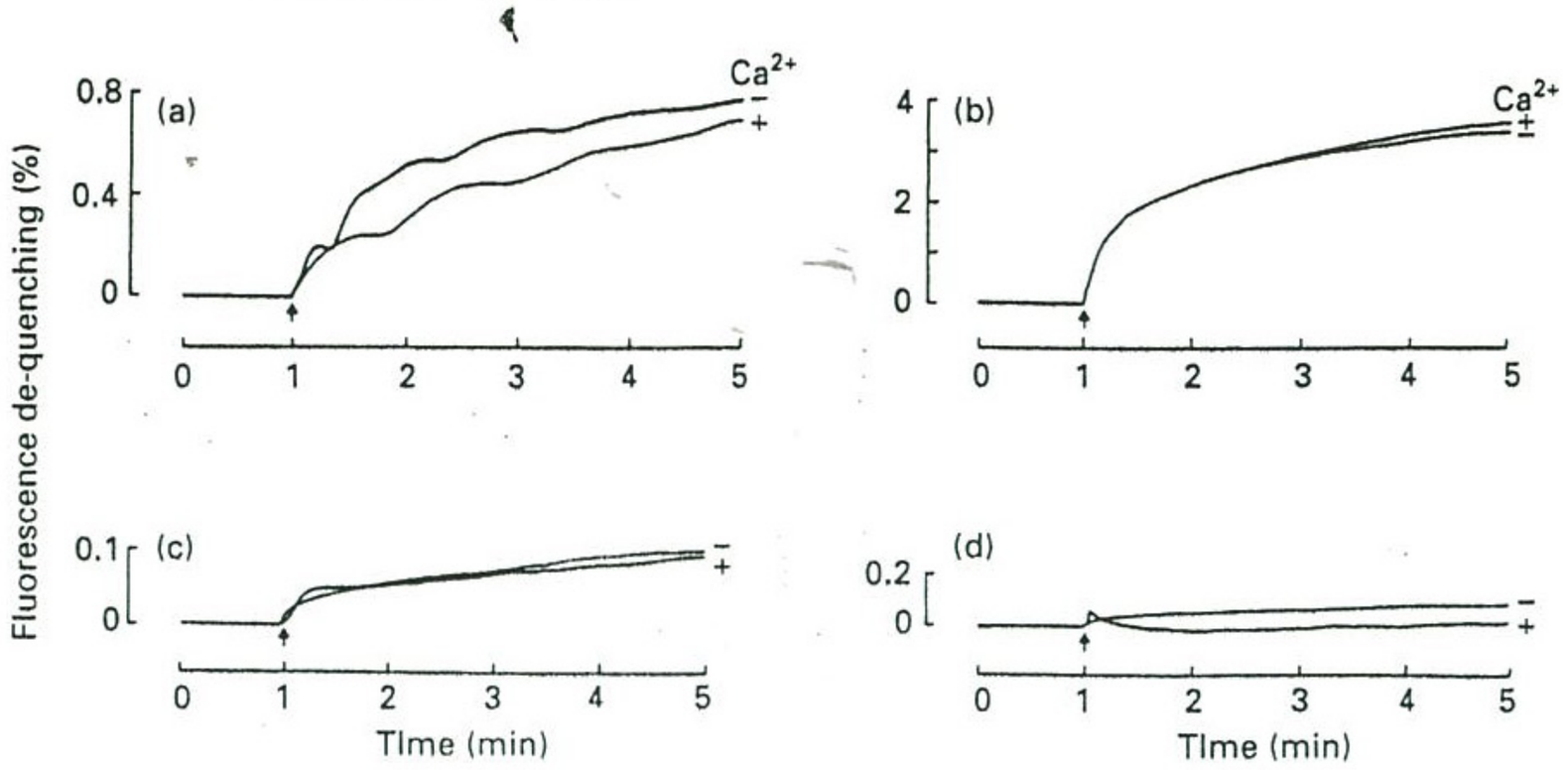

Figure 5 Effect of Ca^{2+} on membrane fusion

The four combinations of plasma membranes and secretory granules were incubated together in the absence and presence of Ca^{2+} (10 μM). Ca^{2+} was buffered with EGTA (1 mM), and free Ca^{2+} concentration was calculated according to Fabiato and Fabiato (1979). (**a**) Pancreatic plasma membranes (15 μg/ml)/pancreatic zymogen granules; (**b**) pancreatic plasma membranes/parotid secretory granules; (**c**) parotid plasma membranes (10 μg/ml)/parotid secretory granules; (**d**) parotid plasma membranes/pancreatic zymogen granules.

membranes, a smaller de-quenching signal would result. Unfortunately, we have no information about the dimensions of the membrane fragments used in this study. Consequently, we are unable to assess the relative importance of these two sources of variation in the size of the de-quenching signal.

Effect of Ca^{2+} on membrane fusion

Despite the fact that exocytosis in permeabilized pancreatic acini requires the presence of Ca^{2+} at concentrations in the micromolar range (Edwardson et al., 1990; Padfield et al., 1991), fusion *in vitro* between pancreatic plasma membranes and zymogen granules is insensitive to Ca^{2+} over a wide range of concentrations (MacLean and Edwardson, 1992). This result was taken to indicate that the requirement for Ca^{2+} in exocytosis was upstream of the final membrane fusion event. Figure 5 shows the de-quenching signals produced by the four combinations of plasma membranes and granules in the absence and the presence of Ca^{2+} (10 μM). In all cases, both the time course and the extent of de-quenching were insensitive to Ca^{2+}, at least at this physiological concentration. Parotid membranes and pancreatic granules gave no significant signal in either the absence or the presence of Ca^{2+} (Figure 5d).

Effect of GTP[S] on membrane fusion

Fusion between pancreatic plasma membranes (at sub-maximal concentrations) and zymogen granules is enhanced by GTP[S], the non-hydrolysable analogue of GTP, with an EC_{50} of 20 μM (Nadin et al., 1989; MacLean and Edwardson, 1992). This result indicates that exocytotic membrane fusion is under the control of a GTP-binding protein. Figure 6 shows that GTP[S] also increased the extent of the signal given by pancreatic membranes and parotid granules, to a maximum of 180 % of the control, with an EC_{50} of approx. 40 μM. In contrast, GTP[S] reduced the de-quenching signal given by parotid membranes and parotid granules (Figure 7) to a minimum of 50 % of the control, with an IC_{50} of approx. 10 μM. In neither case was there any detectable effect of GTP[S] on the kinetics of de-quenching.

DISCUSSION

The reconstitution of exocytotic membrane fusion *in vitro* permits a direct study of the characteristics of this process in the absence of complications introduced by other cellular events, such as second messenger generation and cytoskeletal reorganization. An additional advantage of an *in vitro* system is that it makes possible the 'mix-and-match' experiments reported here. This approach should reveal the degree of overlap between the

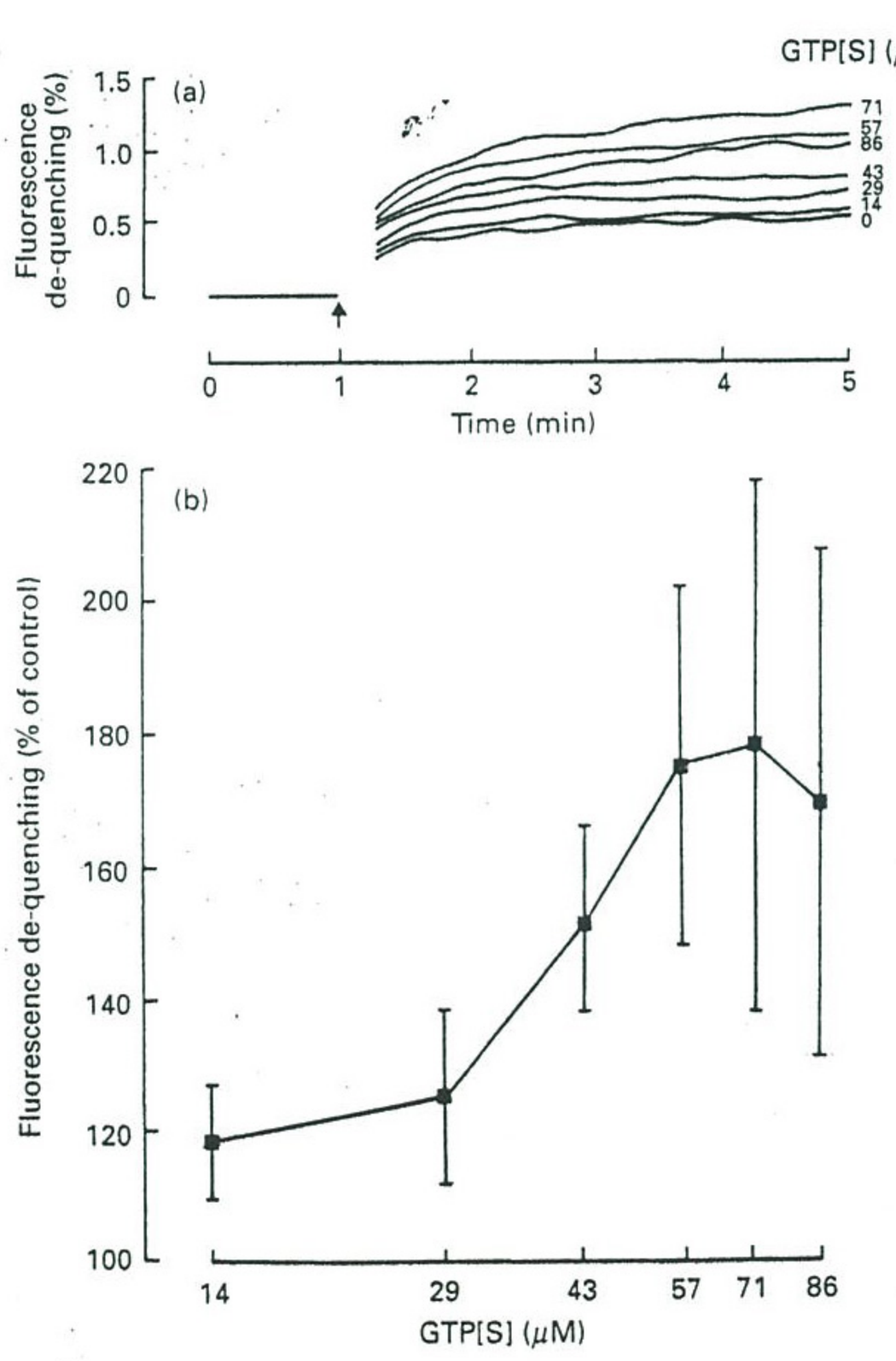

Figure 6 Effect of GTP[S] on fusion between pancreatic plasma membranes and parotid secretory granules

GTP[S] was added to the incubation mixture immediately before addition of plasma membranes (4 μg/ml). (**a**) Typical traces obtained with various concentrations of GTP[S]. (**b**) Relationship between de-quenching (at 5 min) and GTP[S] concentration ($n = 5$).

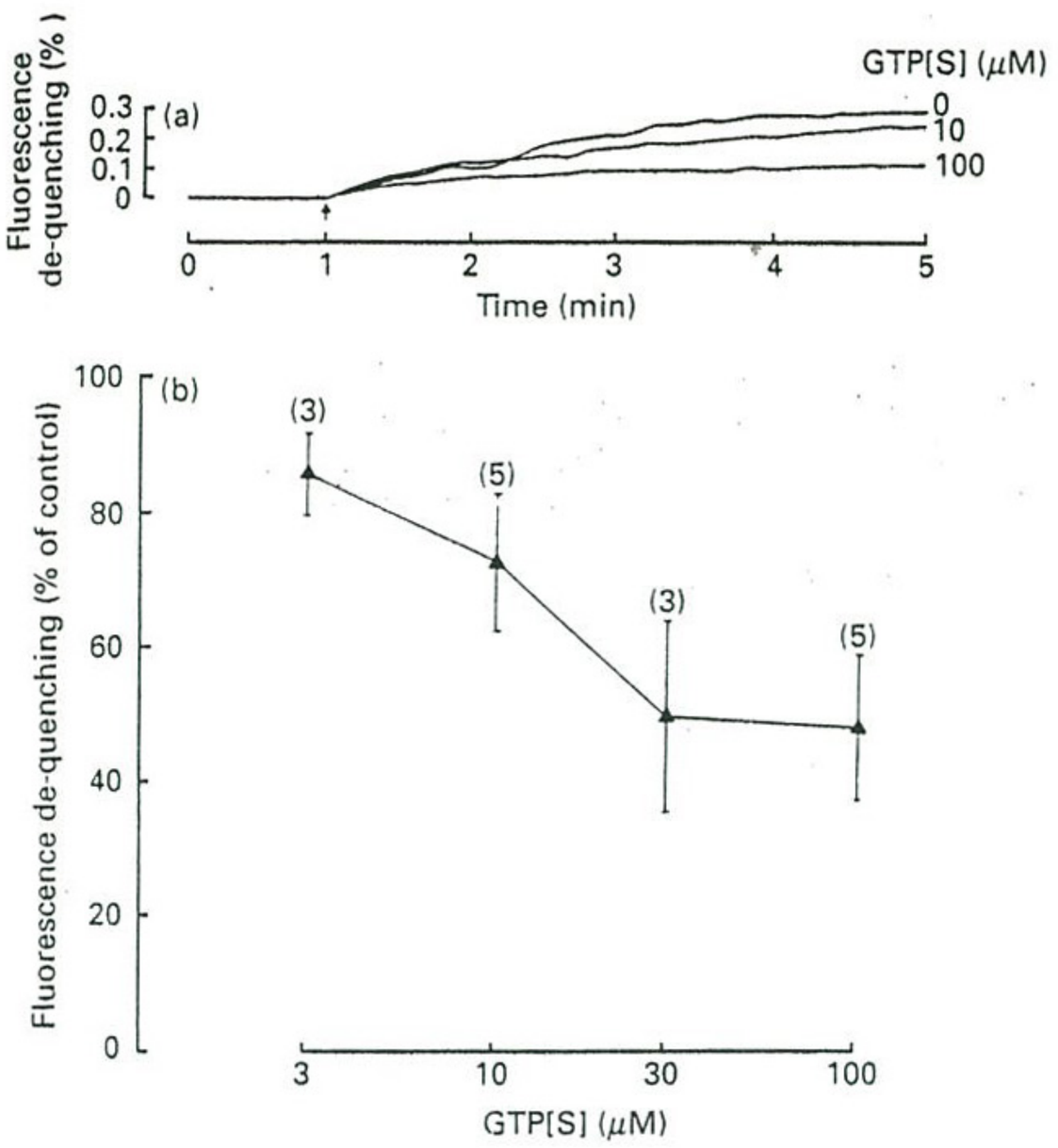

Figure 7 Effect of GTP[S] on fusion between parotid plasma membranes and parotid secretory granules

GTP[S] was added to the incubation mixture immediately before addition of plasma membranes (4 μg/ml). (**a**) Typical traces obtained with various concentrations of GTP[S]. (**b**) Relationship between de-quenching (at 5 min) and GTP[S] concentration. The numbers of determinations made are shown in parentheses above each point.

mechanisms of exocytotic membrane fusion in different cell types.

We show in this paper that the fluorescence de-quenching protocol for measuring membrane fusion, which was originally developed using pancreatic zymogen granules and plasma membranes (MacLean and Edwardson, 1992), can be extended to the plasma membranes and secretory granules of the parotid gland. In addition, we show that pancreatic plasma membranes are promiscuous, in that they will fuse not only with pancreatic zymogen granules but also with parotid granules; parotid plasma membranes, on the other hand, will fuse only with parotid granules. It seems reasonable to expect that, in both pancreatic and parotid acinar cells, exocytosis involves the specific docking of the secretory granule with the apical domain of the plasma membrane, followed by the activation of a mechanism that causes the membranes to fuse. It is likely that the initial docking event requires mutual recognition between proteins on the cytoplasmic surfaces of the interacting membranes. Components of the fusion machinery are believed to be recruited from the cytosol (Rothman and Orci, 1992) and may be present on either, or both, of the two membranes. The promiscuity of pancreatic plasma membranes would then indicate that the docking markers on the two types of granule are either identical or similar and also that the plasma membranes are able to construct a complete fusion apparatus when supplemented with either type of granule. The inability of the parotid plasma membranes to 'cross over' may be a result of a failure either to dock with pancreatic zymogen granules (perhaps because they cannot recognize the zymogen granule docking marker) or to fuse with them (possibly because they cannot form a complete fusion apparatus). As a next step it would be interesting to test whether docking does in fact occur, a question that we plan to address.

As we had found previously with pancreatic membrane/zymogen granule fusion (Nadin et al., 1989; MacLean and Edwardson, 1992), there was no effect of Ca^{2+} (10 μM) on fusion between any of the combinations of membranes and granules tested in this study. This provides further evidence that Ca^{2+} has an upstream role in the control of exocytosis. Recently we have shown that in the exocrine pancreas, Ca^{2+} triggers the dephosphorylation of a phosphoprotein (p45) on the zymogen granule membrane (MacLean et al., 1993). It is possible, therefore, that this dephosphorylation event might be the link between the intracellular Ca^{2+} signal and exocytosis in this tissue. Interestingly, p45 is also present on the membrane of the parotid secretory granule (S. J. Marciniak, C. M. MacLean and J. M. Edwardson, unpublished work).

The similarity between the characteristics of pancreatic membrane/pancreatic granule and pancreatic membrane/parotid granule fusion extends to the stimulatory effect of GTP[S], which occurs with approximately the same potency in the two cases. In contrast, parotid membrane/parotid granule fusion is inhibited by GTP[S], again over a similar concentration range. Both fusion events involving pancreatic membranes, therefore, are stimulated by GTP[S], whereas GTP[S] has opposing effects on the fusion of parotid granules with pancreatic membranes and with parotid membranes. Taken together, these results indicate that the major determinant of the effect of GTP[S] on fusion is the nature of the plasma membrane. We have shown previously (Nadin et al., 1989; MacLean and Edwardson, 1992) that pre-incubation of pancreatic plasma membranes with GTP[S] enhances their ability to fuse with pancreatic zymogen granules, which suggests that a significant part of the effect of GTP[S] on this fusion event is a result of its interaction with a GTP-binding protein on the pancreatic plasma membrane. On the other hand, pre-incubation of the plasma membranes with GTP[S] is not as effective as including it in the membrane/granule incubation, and furthermore GTP[S] stimulates fusion between zymogen granules and zymogen granule membranes, results which point to an additional action of GTP[S] on the zymogen granule membrane. The presence of both small, monomeric and large, heterotrimeric GTP-binding proteins on the membrane of the zymogen granule (Padfield and Jamieson, 1991; Schnefel et al., 1992), and of heterotrimeric GTP-binding proteins (mainly G_s) on the membrane of the parotid secretory granule (Watson et al., 1992) is consistent with an additional effect of GTP[S] on the membranes of both types of granules. In the case of the parotid membrane/parotid granule combination, however, it is an inhibitory GTP-binding protein, most likely on the plasma membrane, which is the predominant mediator of the effect of GTP[S] on membrane fusion.

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