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Studies on Isolated Subcellular Components of Cat Pancreas

II. A Ca^{++}-Dependent Interaction Between Membranes and Zymogen Granules of Cat Pancreas

S. Milutinović, B.E. Argent*, I. Schulz, and G. Sachs**

Max-Planck-Institut für Biophysik, 6000 Frankfurt (Main), Germany

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Summary. A membrane and zymogen granule fraction of cat pancreas has been purified on an exponential ficoll-sucrose gradient in a zonal rotor. A Ca^{++}-dependent interaction between the membranes labelled with ^{125}I or ^{14}C-*p*-chloromercuribenzoate or N-ethyl(2,3-^{14}C)maleimide and zymogen granules has been observed by measuring the amount of membrane protein, enzymes, and peptides which stay associated with the granules after centrifugation through a 31% sucrose cushion. The interaction was a function of the Ca^{++} concentration, starting at 1×10^{-6} M and being saturated at 2×10^{-5} M of free Ca^{++} (apparent $K_m = 6.5 \times 10^{-6}$ M), and showed preference for Ca^{++} over other divalent cations with a selectivity sequence (at 0.5 mM of total cation concentration): Ca^{++} 100, Mg^{++} 35, Ba^{++} 25, Sr^{++} 20. The interaction between membranes and granules was specific for cat pancreatic membranes as opposed to cat liver membranes, and for pancreatic zymogen granules as opposed to pancreatic mitochondria. Only 30% of the membrane fraction was bound at saturating levels of zymogen granules and the bound fraction contained alkaline phosphatase, but not other pancreatic plasma membrane markers such as adenylate cyclase or 5′-nucleotidase. After the interaction, removal of Ca^{++} by the calcium chelator EGTA only partially (about 30%) reversed binding of labelled membranes to the zymogen granules. The process appears to be dependent on the membrane proteins, since brief trypsinization of membranes prior to the assay completely abolished the Ca^{++}-induced interaction. It is concluded that 1) the observed binding may reflect an initial Ca^{++}-dependent event in the process of fusion of zymogen granules with the apical plasma membranes of acinar cells, and 2) protein recognition sites on the interacting membranes are essential for this process.

Ca^{++} has been shown to be essential for secretion of zymogens by intact pancreas [3, 8], pancreatic slices [21, 22, 32], and isolated acinar

* *Present address:* Department of Physiology, University Medical School, Newcastle upon Tyne, NE1 7RU, England.

** *Present address:* Laboratory of Membrane Biology, University of Alabama in Birmingham, Birmingham, Alabama 35294.

cells [40], and to be a second messenger of pancreatic secretion [16]. It can also induce enzyme secretion in the absence of added hormone in the isolated perfused cat pancreas [36]. Furthermore, it has been shown that cellular Ca^{++} influx is augmented by the secretagogs acetylcholine and pancreozymin as well as by other substances which evoke enzyme secretion [23]. Because the fusion of β secretory granules with plasma membranes [12], β-granules with β-granules [10], secretory vesicles with secretory vesicles from rat liver [17], liposomes with liposomes [30] and muscle cells with muscle cells [38] can be induced by Ca^{++} it has been assumed that Ca^{++} directly triggers fusion processes.

Fusion of the zymogen granule with the apical membrane of the acinar cell is the ultimate step of enzyme secretion. This paper presents data demonstrating a Ca^{++}-dependent interaction between a specific pancreatic membrane fraction and isolated zymogen granules *in vitro*. The results therefore could be interpreted as evidence that Ca^{++} may be involved in the final stages of stimulus-secretion coupling in the pancreas. Furthermore they indicate the presence of special recognition sites on the interacting membranes.

Materials and Methods

Materials

All reagents used were of analytical purity. Ficoll (approximate mol wt 400,000), ethyleneglycol-bis(β-aminoethyl ether) N,N′-tetraacetic acid (EGTA), phenylmethyl sulfonyl-fluoride (PMSF) and poly-L-lysine hydrobromide were obtained from Sigma, St. Louis, Mo. Pyruvate kinase (crystalline, suspension), adenosine 5′-triphosphate (ATP), adenosine 5′-monophosphate (AMP), adenosine 3′,5′-cyclic monophosphoric acid (cAMP), guanosine 5′-triphosphate (GTP), guanosine 3′,5′-cyclic monophosphate (cGMP), phospho(enol)pyruvate, Na^+ succinate, trypsin, soybean trypsin inhibitor, glucose oxidase, and the protein calibration kit for gel electrophoresis were obtained from Boehringer, Mannheim, Germany. G-strophantin (crystalline) was obtained from Merck, Darmstadt, Germany, and lactoperoxidase from Calbiochem, Luzern, Switzerland. Dowex 50 WX (200–400 mesh, hydrogen form) and *p*-chloromercuribenzoic acid were purchased from Serva, Heidelberg, Germany. ^{125}Iodide (carrier-free) was obtained from Hoechst, Frankfurt, Germany, and ^{14}C-*p*-chloromercuribenzoate, N-ethyl(2,3-^{14}C)maleimide, adenosine 5′-[α-^{32}P]triphosphate (2–5 C/mmole sodium salt) and [8-^{3}H]adenosine 3′,5′-cyclic phosphate (27 C/mmole ammonium salt) were obtained from Radiochemical Centre, Amersham, U.K.

Methods

I. Separation of different fractions of pancreatic homogenate. The procedure used for the fractionation of tissue is described in the provious paper [28], and it will be only outlined here. It permits plasma membranes and zymogen granules to be separated from other cell organelles by a single centrifugation step in a zonal rotor.

Cat pancreas was homogenized by hand using a loose teflon-glass homogenizer in 5 volumes of 10% (w/w) sucrose containing 10 mM Tris-Cl, pH 7.4, 0.1 mM ethyleneglycol-bis(β-aminoethyl ether) N,N′-tetraacetic acid (EGTA) and 0.1 mM phenylmethyl sulfonyl-fluoride (PMSF). To remove nuclei and cell debris the homogenate was centrifuged by accelerating the SS34 rotor on a Sorvall RC 2B centrifuge to 2,000 rpm and then shutting off the motor. After the pellet was removed, supernatant was centrifuged through an exponential gradient between 5% ficoll in 0.25 M sucrose and 45% sucrose with a 60% sucrose cushion in a Beckman Ti 14 rotor, run for 3.5 hr at 43,000 rpm.

The supernatant distributed among four protein bands, which were collected, diluted and centrifuged as follows: peak I at $20{,}000 \times g$ for 20 min, peak II at $20{,}000 \times g$ for 20 min followed by centrifugation of the supernatant from the first run at $100{,}000 \times g$ for 1 hr, peak III at $20{,}000 \times g$ for 20 min and peak IV at $1{,}000 \times g$ for 15 min. Material derived from peak I was enriched in plasma membrane markers (hormone-stimulated adenylate cyclase, $(Na^+ + K^+)$-ATPase, and alkaline phosphatase among others; *see also* reference [28]), peak II (lighter part) was enriched in markers for endoplasmic reticulum (RNA, rotenone-insensitive NADH-cytochrome-*c*-reductase), peak III was enriched in the mitochondrial marker succinic dehydrogenase and peak IV in the zymogen granule markers trypsin and amylase. Liver plasma membranes were isolated using the method of Aronson and Touster [4].

II. Isolation of zymogen granule membranes. Following the zonal ficoll-sucrose-gradient centrifugation and pelleting the material recovered in peak IV for 15 min at $1{,}000 \times g$, zymogen granule membranes were prepared according to the method of Meldolesi *et al.* [26] with some modifications which are described in the accompanying paper [28].

III. SDS gel electrophoresis. Plasma membranes (Fraction I) and zymogen granule membranes (*see above*) were dissolved in SDS mercaptoethanol by incubating for 10 min at 37 °C. The solution was electrophoresed in 7.5% acrylamide gels by previously described procedures [35]. Other fractions obtained as detailed below were also studied by gel electrophoresis.

IV. Labelling of membranes. Three procedures for labelling the membranes were compared. Membranes were incubated for 10 min at 22 °C in a buffer containing 0.3 M sucrose, 10 mM Tris-Cl, pH 7.4, and 10^{-6} M of either ^{14}C N-ethylmaleimide or ^{14}C-*p*-hydroxy mercuribenzoate. Alternatively membranes were iodinated with ^{125}I using glucose-glucose oxidase and lactoperoxidase [37]. No difference in binding to zymogen granules was found between the membranes labelled by the different methods. Consequently, the ^{125}I method was used because of its convenience and high specific activity. This method entailed the following steps:

Iodination was performed using about 10 mg of protein in 10 ml of a medium containing 0.3 M sucrose, 10 mM Tris chloride, pH 7.4, 42 µg glucose oxidase, 6 mg glucose, 180 µg lactoperoxidase, and 100 µC of carrier-free ^{125}I (8–15 C/mg NaI) with 0.1 mM KI. The reaction was initiated by the simultaneous addition of ^{125}I, KI (final specific activity 100 µC/10^{-7} M KI) and glucose and carried out for 5 min at 4 °C. Then the sample was diluted to 140 ml with 0.3 M sucrose, 10 mM Tris-Cl, pH 7.4, spun down and washed 3 times by alternate resuspension and centrifugation. The centrifugal force used in the washing procedure depended on the type of membranes labelled, i.e. $20{,}000 \times g$ for Fractions I and II, and $100{,}000 \times g$ for the light part of Fraction II and zymogen granule membranes. The final pellet was taken up in 0.3 M sucrose and the final protein concentration was 5 mg/ml. The amount of iodine incorporated into membranes at saturating iodine concentrations was 1.5×10^{-8} M/mg protein. This labelling procedure was used for Fractions I and II, zymogen granule membranes and liver plasma membranes. The membranes had been stored overnight at −40 °C before labelling and were used immediately after iodination. Zymogen granules had been kept overnight at +2 °C in 45% sucrose.

V. Zymogen granules-plasma membranes interaction assay. Incubations were carried out for 10 min at 22 °C in plastic test tubes. The incubation medium contained 0.3 M sucrose, 50 mM acetate buffer, pH 6.6, zymogen granules (250–300 μg of protein) with Ca^{++} or other divalent cations or nucleotides as indicated in the legends to the Figures and Tables. To ensure a stable free Ca^{++} concentration in the incubation medium containing high tissue protein concentrations, a Ca^{++}-EGTA buffer was used. The ratio $Ca^{++}_{total}/EGTA_{total}$ was calculated at pH 6.6 for each free Ca^{++} concentration according to Portzehl *et al.* [31]. Pancreatic plasma membranes (50–70 μg of protein) suspended in 0.3 M sucrose were added to make the reaction volume 500 μl. In some experiments an excess of EGTA (EGTA to calcium 6:1) was added at the end of incubation and the samples were incubated for an additional 10 min. After incubation, the reaction mixture was layered on 2.5 ml of ice-cold 31% (w/w) sucrose, contained in glass test tubes. The tubes were centrifuged immediately in a Sorvall centrifuge (SS34 rotor) at $8{,}000 \times g$ for 15 min (4 °C). Following centrifugation two fractions were recovered: the pellet of zymogen granules, containing bound membranes, and a band at the 10 and 31% interface containing membranes which had not reacted with zymogen granules. The supernatant was aspirated using a Pasteur pipette, and 0.3 ml of 0.1 M NaOH was added to the pellet. The material was dissolved and transferred to a plastic vial for counting in a Packard gamma counter. The extent of zymogen granule lysis was monitored by comparing zymogen granule protein recovered in the pellet with the amount of protein originally present in the zymogen granules in the incubation mixture. About 80–90% of zymogen granules were recovered in the pellet, the rest were lysed. The quantity of membranes bound to the zymogen granules as assessed by cpm appearing in the pellet was corrected for this loss of zymogen granules.

In some experiments, following incubation with zymogen granules and centrifugation through the sucrose cushion, the material remaining at the 10 and 31% sucrose interface was assayed for adenylate cyclase, 5′-nucleotidase and alkaline phosphatase. Also the pellet was treated with EGTA (10^{-3} M), layered over 31% sucrose and recentrifuged as above. The band reappearing at 10/31% sucrose interface was also assayed for the above-mentioned enzymes. Only 7.4% of the amount of membrane protein originally bound to the zymogen granules was released by the EGTA treatment.

The pellet obtained after interaction by centrifugation over the 31% sucrose cushion was lysed by 150 mM Tris, pH 7.8, as above to release zymogen granule contents. The residual material containing zymogen granule membranes and interacted membranes were pelleted by centrifugation at $100{,}000 \times g$ for 1 hr, dissolved in SDS-mercaptoethanol and electrophoresed as above. When the effect of trypsinization on zymogen granule-plasma membrane interaction was checked plasma membranes were preincubated with trypsin (1 mg/ml) for 5 min at 37 °C.

VI. Zymogen granule-zymogen granule membrane interaction. The same procedure as described above (sections IV. and V.) was applied using zymogen granule membranes to study interaction of these membranes with zymogen granules.

VII. Enzyme assays. 5′-Nucleotidase was measured as described previously [41], adenylate cyclase by a previously described method [24], alkaline phosphatase by the Mercko-Test method, and protein by the method of Lowry *et al.* [25].

Results

I. Fractionation

As described in the previous paper [28] zonal centrifugation produced well defined and purified fractions corresponding to plasma membranes (Fraction I), a mixture of plasma membranes and endoplasmic reticulum

(Fraction II), mitochondria (Fraction III), and zymogen granules (Fraction IV). As no specific marker enzyme is known for zymogen granule membranes a possible contamination of zymogen granule membranes by plasma membranes and the membranes derived from endoplasmic reticulum was estimated by comparing the gel patterns of these three fractions.

As shown in the foregoing paper the gel pattern of zymogen granule membranes is quite distinctive and different from the gel pattern of plasma membranes (Fraction I). The gel pattern of Fraction II also showed distinctive bands [28]. Electron microscopy confirmed the possible origin of fractions analyzed by enzyme markers [28].

II. Binding of Membranes to Granules

A. Effect of Ca^{++} ion. In the absence of added Ca^{++}, recovery of ^{125}I labelled plasma membranes (Fraction I) in the zymogen granule pellet was less than 5% of the added counts (Fig. 1 and Table 1). In

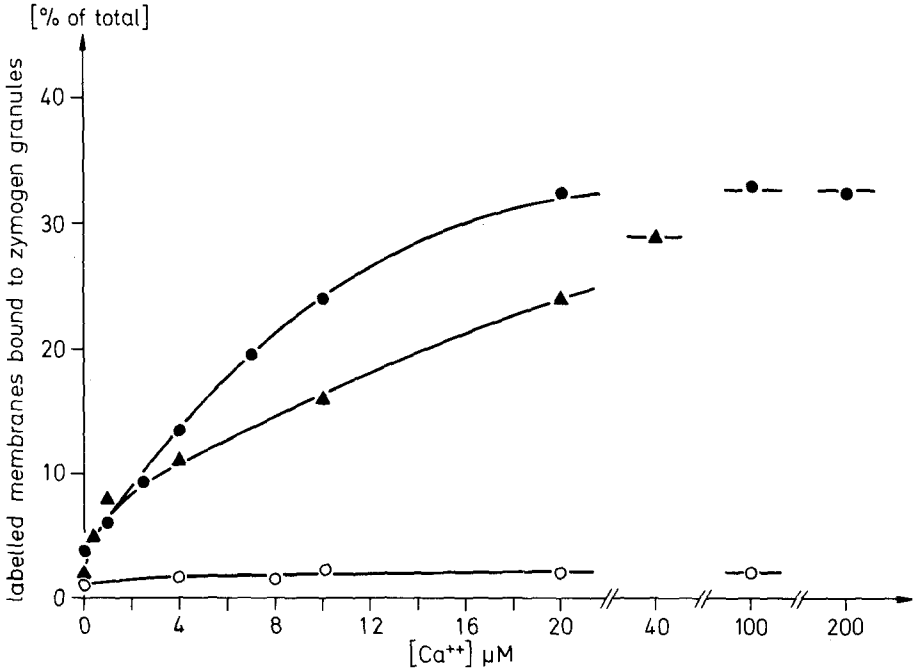

Fig. 1. The effect of varying Ca^{++} concentration on binding (expressed as % of total counts appearing in pellet of sucrose cushion, ●—● of plasma membrane Fraction I to zymogen granules, ▲—▲ of zymogen granule membranes to zymogen granules, ○—○ of plasma membranes having been preincubated with trypsin (1 mg/ml) for 5 min at 37 °C to zymogen granules. The apparent K_m for plasma membrane binding was 6.5×10^{-6} M

Table 1. Effect of Ca^{++} (5×10^{-4} M) on binding of different membrane fractions (~50 μg protein/tube) to zymogen granules or mitochondria (~1,000 μg/tube)

Carrier particles	Carried labelled particles	Per cent bound		Stimulation by Ca^{++} (ratio)
		without Ca^{++}	with Ca^{++}	
Zymogen granules	plasma membranes (Fraction I)	4.2	33.0	8.25
Zymogen granules	plasma membranes (boiled for 2 min)	2.0	2.8	1.40
Zymogen granules	plasma membranes (trypsin treated, 1 mg/ml for 5 min at 37 °C)	1.5	1.6	1.10
Zymogen granules	endoplasmic reticulum (Fraction II)	6.3	15.8	2.50
Zymogen granules	zymogen granule membranes	4.4	30.8	7.00
Zymogen granules	liver microsomes	1.4	1.6	1.10
Mitochondria	plasma membranes (Fraction I)	3.5	2.0	0.57

the presence of 1×10^{-6} M free Ca^{++}, binding of labelled membranes to zymogen granules was increased. Further increase in free Ca^{++} concentration resulted in a concentration-dependent increase of binding, saturation occurring at 2×10^{-5} M. At this concentration binding was increased by eightfold over that in the absence of Ca^{++}. Half-maximal stimulatory concentration of Ca^{++} was 6.5×10^{-6} M. Addition of EGTA in a sixfold excess over total calcium concentration to the incubation medium resulted in only 30% reversal of membrane binding showing the apparent irreversibility of the major part of binding (not shown).

B. Membrane selectivity. In the absence of Ca^{++} comparable amounts of Fraction I and Fraction II bound to the zymogen granules. However, the addition of Ca^{++} resulted in only 2.5-fold increase in binding of Fraction II (endoplasmic reticulum) and was significantly less than that obtained with Fraction I (plasma membranes) which increased 8.2-fold over the control (Table 1).

No binding of pancreatic mitochondria with pancreatic membranes or binding of liver membranes to zymogen granules was detected with this technique (Table 1). Hence the Ca^{++}-dependent interaction of the fraction tested was confined to the fraction containing the highest enrich-

ment of plasma membranes and to the zymogen granule fraction of the pancreas. Ca^{++} also enhanced the binding of labelled zymogen granule membranes to the intact granules (Table 1 and Fig. 1).

C. Dependence of binding on the amount of zymogen granules and membranes. Increasing the amount of zymogen granules with a fixed concentration of plasma membrane protein (50 μg/500 μl) in the incubation medium showed that the binding was saturable. No further binding occurred after 30% of the plasma membranes present had been bound (Fig. 2*A*).

In the opposite procedure, adding increasing amounts of plasma membranes to an excess amount of zymogen granules (1050 μg protein), the amount of membranes bound was directly proportional to the concentration of membranes in the medium (Fig. 2*B*). Under these conditions at all concentrations of plasma membranes tested the amount of plasma membranes bound approached ~30% of the membranes added to the incubation mixture.

D. Membrane specificity. Fraction I almost certainly contains a mixture of apical and basal membranes of the pancreatic cell and about 10% of endoplasmic reticulum contamination [28], as well as possibly zymogen granule membranes, originating from the process of lysis of zymogen granules during homogenization. It would seem likely that the fusion process could distinguish between membranes from different cell surfaces, so it was necessary to show that the interaction was not due solely to Ca^{++}-dependent interaction of zymogen granule membranes with intact granules.

The distribution of membrane marker enzymes in the nonreacting membrane fraction which remained at the 10–31% sucrose interface and in the membranes associated with zymogen granules (pellet) was checked (Table 2). Only a small quantity of adenylate cyclase and 5′-nucleotidase was transferred to the pellet. This was confirmed by the considerable enrichment of these two enzyme activities in the membrane fraction which had not reacted with zymogen granules and by the absence of these enzymes in the membrane fraction recovered after EGTA treatment of the membrane-zymogen granule pellet. In contrast, a considerable fraction of the alkaline phosphatase was found in the zymogen granule-membrane pellet. Consequently, this enzyme disappeared from the 10–31% interface.

Hence it would appear that the membrane fragments containing adenylate cyclase and 5′-nucleotidase do not interact with zymogen granules and are presumably basal or baso-lateral. On the other hand, the fraction

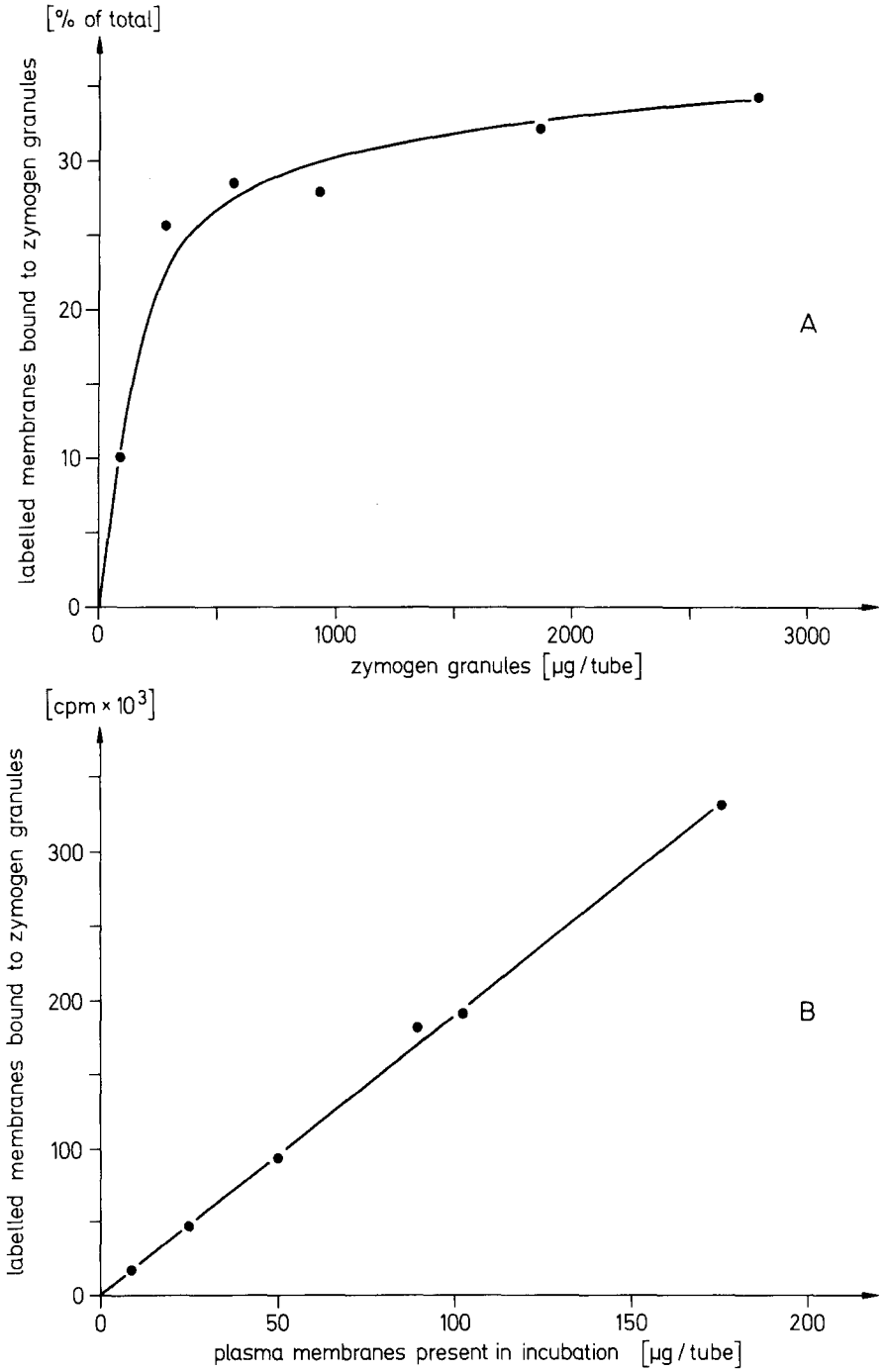

Fig. 2. (*A*) The effect of increasing zymogen granule fraction at a fixed concentration of plasma membrane protein (50 µg/tube) on binding of the membrane fraction. The binding is expressed as % of total counts. (*B*) The effect of increasing concentrations of labelled plasma membranes at a fixed concentration of zymogen granule protein (1,050 µg/tube) on binding to zymogen granules

Table 2. Difference in enzyme marker distribution in plasma membranes bound to zymogen granules (interacting fraction) and those remaining at the interface (noninteracting fraction) in the presence of 5×10^{-4} M $[Ca^{++}]$ (one out of three similar experiments)

	Protein (%)	Adenylate cyclase		5′-nucleotidase		Alkaline phosphatase	
		enrichment[a] (*a*)	(%) recovery (*b*)	*a*	*b*	*a*	*b*
Plasma membranes (Fraction I)	100	1	100	1	100	1	100
Interacting fraction	33	0	0	0.15	11	1.56	58
Noninteracting fraction	67	1.91	100	2.10	89	0.90	42

[a] Specific activity of the fraction divided by specific activity of Fraction I (plasma membranes).

that did interact contained alkaline phosphatase which is located in the apical cell membrane similar to other tissues [19]. A difference as found for enzyme activities did not show up in the electropherograms of noninteracting and interacting membranes. The gel patterns, however, were different from those of all other membranes shown in Fig. 5 of the accompanying paper [28] (results not shown).

Labelled zymogen granule membranes also interact with zymogen granules in a Ca^{++}-dependent fashion (Table 1). Re-examination of the gel pattern of Fraction I and the gel pattern of the granule membranes, however, showed that granule membrane contamination of Fraction I would be too small to account for the degree of binding observed (compare with the previous paper [28]). Hence the Ca^{++}-dependent reaction is specific for only part of the membranes.

E. Other requirements (cations, nucleotides, pH, temperature). Various other cations were tested instead of Ca^{++}. The order of effectiveness in promoting binding of plasma membranes of Fraction I to zymogen granules (at 0.5 mM) was Ca^{++} 100, Mg^{++} 30, Ba^{++} 30, Sr^{++} 25. At 10^{-3} M La^{++} was 20% more effective than Ca^{++}.

ATP, GTP, cAMP, cGMP, or polyvalent cations (e.g. poly-L-lysine, mol wt 24,000) were inactive and did not affect the Ca^{++}-dependent binding.

There was a broad pH optimum of Ca^{++}-dependent interaction between 5.5 and 7.0, but at pH 7.0 and above, decrease in size of the pellet was observed due to lysis of the granules.

The binding reaction was increased by temperature, the initial rate of reaction being twice as high at 22 °C compared to 4 °C. At 37 °C significant granule lysis occurred. Boiling the membranes abolished the zymogen granule-membrane interaction.

Trypsinization of membranes completely abolished Ca^{++}-induced increase in binding to zymogen granules, showing the importance of membrane peptides for this process (Fig. 1 and Table 1).

Discussion

Ca^{++} probably plays a crucial role in pancreatic enzyme secretion. Experiments performed on the intact organ and pancreatic slices showed that omission of Ca^{++} from the extracellular medium leads to a reversible abolition of hormone-induced enzyme secretion [3, 21, 22, 32]. On the other hand, a sudden increase in extracellular Ca^{++} concentration which is likely to increase cytoplasmic Ca^{++} concentration leads to enzyme secretion even in the absence of secretagogues [36]. Furthermore, it was also shown that hormones which stimulate enzyme secretion increase the membrane permeability for Ca^{++} [23]. All these observations support the view that Ca^{++} is a common intracellular mediator in the process of hormone-induced enzyme secretion. Although it is probable that Ca^{++} is a regulatory ligand in the process of hormone-induced enzyme secretion, the underlying mechanism of the action of Ca^{++} leading to the final effect, that is discharge of zymogens from the acinar cells into the lumen of the exocrine duct system, is not clear. Although an alternative hypothesis for the release of secretory products has been proposed [33], the generally accepted model supports the view that release occurs by fusion of the zymogen granule membrane with the luminal acinar cell membrane with subsequent extrusion of the granule content [29]. In a variety of systems in which fusion has been studied the requirement for Ca^{++} has been demonstrated [10, 17, 30].

The present study was undertaken in an attempt to assess a direct role of Ca^{++} in the last step of the stimulus-secretion coupling involving zymogen granule and plasma membrane interaction. For this purpose purified plasma membranes and zymogen granules as identified by specific marker enzymes were used. To detect specific regions of the cell membrane able to interact with zymogen granules in a Ca^{++}-dependent fashion we took advantage of the large differences in density between membranes and zymogen granules. This made the separation of mem-

branes bound to zymogen granules from unreacted membranes possible. It is shown in Fig. 1 that Ca^{++} in low concentrations (1×10^{-6} to 2×10^{-5} M, $K_m = 6.5 \times 10^{-6}$ M) mediates binding between membranes and zymogen granules, indicating high sensitivity of this interaction for Ca^{++}. Reanalysis of this curve in a form of a double reciprocal plot shows the presence of a single type of high affinity Ca^{++} binding site. High affinity Ca^{++} sites in this range are characteristic for Ca^{++} binding to proteins [39], whereas Ca^{++} binding to phospholipids would be expected in a higher concentration range [1, 5]. Further support for the involvement of proteins in this interaction was obtained by demonstrating the sensitivity to trypsinization. Fig. 1 shows that after treatment of membranes with trypsin the interaction was completely abolished. The finding that at saturating conditions the process was saturable with respect to Ca^{++}, zymogen granules and membranes (Fig. 1), of total membranes only 30% present in the incubation medium was bound to zymogen granules, indicates that this Ca^{++}-mediated process can distinguish between different membranes. This was further confirmed by determination of membrane marker distribution. Since it is generally accepted that peptide hormone receptors are located on the cell membrane facing the blood side, allowing hormones to bind without crossing the cell membrane, we used pancreozymin-stimulated adenylate cyclase [34] as the most reliable marker of baso-lateral plasma membranes of acinar cells. Membranes containing this enzyme as well as 5′-nucleotidase did not react with zymogen granules. However, alkaline phosphatase-containing membranes were bound to zymogen granules. Therefore it appears that only one type of membrane, most likely the luminal plasma membrane, has interacted with zymogen granules. It should be mentioned, however, that besides different distribution of membrane marker enzymes also the inside-out and right-side out membranes may contribute to the partial interaction found. Zymogen granule membranes were also able to interact with zymogen granules in a Ca^{++}-dependent fashion. So the question arises whether the interaction observed with Fraction I (enriched in plasma membranes) could be accounted for exclusively by a contamination with membranes of zymogen granules lysed during tissue homogenization. Three observations make this assumption unlikely: (1) as shown in Fig. 1 the half-maximal stimulating Ca^{++} concentration is higher for zymogen granule membranes than for plasma membranes. (2) At saturating Ca^{++} and zymogen granule concentration a higher percentage of labelled plasma membranes as compared to labelled zymogen granule membranes was taken up by zymogen granules. But

it is difficult to completely exclude a contamination of zymogen granule membranes by plasma membranes, which may account for part of the interaction observed with the granule membrane fraction. (3) Analysis of gel patterns shows that two characteristic major bands of zymogen granule membranes make up at least 80% of the total protein. The same two bands appear only as a minor component in the gel pattern of Fraction I making up at the most only 10% of total protein. So cross-contamination would not contribute significantly to the results obtained.

Fusion between secretory granules has been demonstrated by electron microscopy in parotid [2], pancreatic β-cells [10], and secretory vesicles from liver [17]. In this respect it is not surprising that membranes originating from zymogen granules are able to interact also *in vitro* with zymogen granules. As a further support for a specificity of interacting membrane surfaces or regions, other cell components were shown not to interact. Pancreatic mitochondria and endoplasmic reticulum membranes did not bind to plasma membranes (Fraction I) and zymogen granules, respectively. This inability to interact was also shown for plasma membranes prepared from cat liver. The high sensitivity of this process for Ca^{++} compares with other systems in which Ca^{++} in μmolar concentration range regulates cellular activity as for example control of muscle contraction [7, 18], transmitter release from nerve terminals [27], or aggregation of blood platelets [9].

The properties of Ca^{++}-dependent membrane zymogen granule binding observed in this study are quite similar in stimulus-secretion and in excitation-contraction coupling. From all cations tested only Sr^{++} and Ba^{++} are able to stimulate directly both processes [11, 15, 20], whereas Mg^{++} inhibits the action of Ca^{++} [6, 11, 14]. The study of the effect of other cations in our system revealed a sequence of potency comparable to the selectivity sequence predicted by Sherry (discussed in [13]) showing the highest specificity for Ca^{++}.

The nature of this Ca^{++}-induced interaction, however, is not clear. Experiments using the Ca^{++} chelator EGTA in high concentrations in order to remove Ca^{++} after the interaction had been completed, showed that this process was not completely reversible.

On the basis of the presented data it is not possible to decide whether the underlying mechanism of the Ca^{++}-induced interaction is only binding of two membranes or fusion. Attempts to demonstrate Ca^{++}-induced fusion between isolated zymogen granules and plasma membrane fractions *in vitro* by electron microscopy have not yet been successful.

The data presented show that Ca^{++} mediates a membrane specific interaction between zymogen granules and a membrane fraction of cat pancreas which may be a model for at least the last step in protein secretion by this organ.

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