Biochimica et Biophysica Acta 856 (1986) 348–356
Elsevier

BBA 73037

Calcium binding properties of purified zymogen granule membrane of pig pancreas. Evidence for calcium binding proteins

Sylvain Phaneuf and Adrien R. Beaudoin *

Centre de Recherche sur les mécanismes de sécrétion, Faculté des sciences, Université de Sherbrooke, Sherbrooke, Québec J1K 2R1 (Canada)

(Received October 7th, 1985)

Key words: Ca^{2+}; Zymogen granule; Calcium binding protein; (Pig pancreas)

Ca^{2+} binding properties of purified zymogen granule membranes of pig pancreas have been measured: Binding increased linearly with Ca^{2+} concentration in the medium up to the micromolar range; in the millimolar range a sharp rise in binding capacity was observed. Binding increased with pH both at low and high concentrations of Ca^{2+}. It was insensitive to Na^+ and K^+ ions at concentrations up to 100 mM. Mg^{2+} was inhibitory in the millimolar range whereas La^{2+} and Tb^{3+} were inhibitory in the micromolar range. The Ca^{2+} binding components of zymogen granule membranes were identified by two methods: (1) by measuring $^{45}Ca^{2+}$ binding after counter-ion electrophoresis and (2) by Stain's-all (forms a complex with Ca^{2+} binding proteins absorbing maximally at 600 nm), after SDS-polyacrylamide gel electrophoresis. The first method, counter-ion electrophoresis, indicated that most of the $^{45}Ca^{2+}$ was associated with an acidic band which could be subsequently subfractionated by SDS-polyacrylamide gel electrophoresis in five bands: 66, 57, 30, 27 and 22.5 kDa. The second method, Stain's-all, revealed six positive polypeptides after SDS-polyacrylamide gel electrophoresis of native zymogen granule membranes' two were unreactive after neuraminidase treatment (130 and 92 kDa, respectively), whereas four other bands were still reactive (66, 57, 43, 30 kDa, respectively.) Ca^{2+} binding was also measured on intact zymogen granules: the binding capacity was higher than for zymogen granule membranes. Among the Ca^{2+} binding proteins of the zymogen granule membrane only one is apparently located on the granule external surface: the 30 kDa polypeptide. If Ca^{2+} directly facilitates fusion of zymogen granules with plasma membrane by a Ca^{2+}-protein interaction, then this protein is a presumptive candidate to play such a key role.

Introduction

The action of cholinergics or pancreozymin-like peptides on the pancreatic acinar cell is believed to be in great part mediated by a rise in cytosol Ca^{2+} [1]. Binding of the secretagogue to the cell surface causes depolarization and mobilization of Ca^{2+} from one or several intracellular pools [2]. During this initial phase of secretion, concentration of Ca^{2+} in the cytosol rises from 0.1 μM up to about 1.0 μM [3–6].

In the past few years, considerable work has been devoted to the comprehension of the molecular mechanisms which elicit the exocytosis phenomenon. Despite these research efforts, this cellular phenomenon is still poorly understood. Various modes of action have been proposed for Ca^{2+} in different secretory cells: (a) binding of calcium to fusion sites would neutralize surface charges, thereby reducing the energy barrier limiting mem-

* To whom correspondence should be addressed.

Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

brane-membrane interaction [7,8]; (b) calcium could be implicated in the fusion process by interacting with the microfilament network; microfilaments would prevent zymogen granule to reach the luminal plasmalemma [9]; (c) calcium could favor formation of bridges between secretory granules and plasma membrane as observed in bovine adrenal medullary gland [10]; such connections between zymogen granule and luminal plasma membrane have been observed in pancreatic exocrine cells (unpublished observations); (d) calcium could also stimulate phosphorylation reactions of zymogen granule membrane proteins [11].

The main purposes of the present work were (a) to examine the Ca^{2+} binding properties of the zymogen granule membrane and to evaluate some of the parameters which could influence this binding and (b) to identify the Ca^{2+} binding components of zymogen granule membrane.

Materials and Methods

Materials

Hepes, unlabelled calmodulin and ionophore A23187 were purchased from Calbiochem-Behring Corp., La Jolla, CA, U.S.A. Hepes buffer was passed through a Chelex-100 column to suppress traces of Ca^{2+} according to Crouch and Klee [12]. Chelex-100 column, and all the material used for electrophoresis were bought from Bio-Rad Lab, Richmond, CA, U.S.A. Tergitol (Nonidet P40) and neuraminidase isolated from *Clostridium perfringens* were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Ca^{2+} solutions were prepared from dehydrated $CaCO_3$. Calmodulin [^{125}I]RIA kit and $^{45}Ca^{2+}$ (4–30 Ci/g) were supplied by New England Nuclear, Lachine, Québec, Canada. Scintillation solutions, Bio-Solv-3, Ready-Solv-NA and toluene were provided by Beckman Instruments Inc., Montréal, Québec, Canada. Stain's-all was bought from Eastman Kodak, Rochester, NY, U.S.A. All other reagents were commercial preparations of the highest obtainable purity. Solutions were freshly prepared with bidistilled deionized water the conductivity of which was superior to 15 MΩ.

Methods

Zymogen granule and membrane preparations. Pig pancreases were obtained from a local slaughterhouse; pancreases were removed within 5 min after death, and kept at 0°C in a Krebs-Ringer-phosphate medium supplemented with 0.2 mM $CaCl_2$ and 0.2% glucose. Upon arrival at the laboratory (60 min later), excess fat was removed and the tissue was minced in a meat grinder. Homogenization, preparation of zymogen granules, and zymogen granule membranes were carried out according toPâquet et al. [13], except that the final zymogen granule membrane 240 000 × *g* pellet was suspended in 20 mM Hepes (pH 7.4), and zymogen granules were suspended in 0.65 M sucrose in the same buffer. Prior to assays, Ca^{2+} was removed by passing the zymogen granule membranes onto a Chelex-100 column [12]. Column efficiency was tested by adding $^{45}Ca^{2+}$ to the homogenate and measuring the residual Ca^{2+} in purified zymogen granule membrane. Less than 0.0045% of the $^{45}Ca^{2+}$ added to the homogenate was found in the zymogen granule membrane preparation after treatment. Zymogen granule membranes were used immediately for binding studies or stored at −20°C for electrophoresis experiments. To prevent proteolytic digestion, 0.1 mM phenylmethylsulfonyl fluoride (PMSF) was routinely added to all the solutions.

$^{45}Ca^{2+}$ binding assay. For the assays on zymogen granule membranes, unless otherwise stated, about 15 μg of protein were suspended in 20 mM Hepes buffer (pH 7.4), containing $^{45}Ca^{2+}$ in a final volume of 1 ml. Non-specific binding was measured by subtracting the $^{45}Ca^{2+}$ binding observed in the presence of 300 mM Ca^{2+} from total $^{45}Ca^{2+}$ binding. Incubation was carried out in 1.5 ml Eppendorf centrifuge tubes at 37°C for 30 min with gentle shaking. For the assays on intact zymogen granules, 1–2 mg of protein were suspended in 20 mM Hepes (pH 6.0), containing 0.65 M sucrose and 10 μM Ca^{2+} in a final volume of 1 ml. Incubation was carried out at room temperature (21°C) for 30 min. Non-specific binding was measured by adding 300 mM Ca^{2+} as for the zymogen granule membranes. After centrifugation at 14 000 × *g* for 20 min with a Sorvall RC-5B equiped with GSA rotor, the supernatants were discarded and the pellets were either suspended in a mixture of Bio-Solv-3/toluene/Ready Solv-NA (1 : 2 : 3, v/v) for radioactivity counting, or in an appropriate buffer for protein, or for ATP diphos-

phohydrolase assays. The assays were carried out in triplicate.

Identification of Ca^{2+} binding protein bands after counter-ion electrophoresis. To identify the Ca^{2+} binding components of the zymogen granule membrane, counter-ion electrophoresis was carried out according to Ueng and Bronner [14] and Schibeci and Martonosi [15] under non-denaturing conditions with Nonidet P-40. Stacking gel acrylamide concentration was 5% at pH 6.8; whereas in separating gel, it was 8% or 12% acrylamide at pH 8.3; $CaCl_2$ (1 μM, spec. act. 10 to 30 μCi/mmol) was added to the lower buffer; zymogen granule membrane proteins (70–150 μg) were solubilized in 2% Nonidet P-40/mg of protein per ml and were introduced on top of each cylindrical gel. Methyl green was used as a tracking dye, but since it bound Ca^{2+} it was not included with the samples on the gels where $^{45}Ca^{2+}$ binding was measured. Denatured zymogen granule membranes (90°C for 2 min) were also run in each series of experiments. For each series, four gels were cut in 3-mm thick slices with a Bio-Rad slicer (model 190) and slices were dissolved in 1 ml of 2% periodic acid, for 30 min, at room temperature. Radioactivity was measured with the scintillation solution described in the preceding section. Duplicate gels were treated for 1 h with 10% trichloroacetic acid and stained with Coomassie blue for 3 to 4 h. Gels were destained and scanned on a Gilford spectrophotometer 240 equipped with a gel scanner module.

Subfractionation of the Ca^{2+} binding bands by SDS-polyacrylamide gel electrophoresis. For the molecular weight determinations of the Ca^{2+} binding bands separated by counter-ion electrophoresis, protein from the band located at the extremity of the gel were eluted from the gel according to Hunkapiller et al. [18]: after dialysis against 1 mM Tris (pH 8.5), samples were lyophilyzed and separated by SDS-polyacrylamide gel electrophoresis according to Laemmli [17]. For this purpose, an acrylamide gradient from 8 to 15% was used. Proteins were either stained by Coomassie blue or by the cationic dye Stain's-all [18]. The dye stock solution 0.1% (w/v) was prepared in formamide. Staining was carried out as follows: gels were washed in 25% isopropanol (v/v) for 48 h then Stain's-all was added at 0.0025% in 25% isopropanol, 7.5% formamide and 30 mM Tris at pH 8.8 for 48 h. Staining and destaining steps were carried out in the dark. Gels were rinsed in distilled water for 48 h and finally scanned with a Beckman DU 8B spectrophotometer.

Influence of neuraminidase treatment on staining by Stain's-all. For these experiments, zymogen granule membranes were incubated in the presence of Stain's-all, 0.001% in 1% formamide and 10 mM Tris (pH 8.0) for 60 min at room temperature. Alternatively zymogen granule membranes were stained after neuraminidase treatment according to King and Morrison [19]: zymogen granule membranes (70 to 100 μg of protein/ml) were incubated for 3 h at 30°C with 1 mM $CaCl_2$, 150 mM NaCl, 0.1 mM PMSF, 10 mM Tris (pH 7.0) and 0.1 unit (NAN-lactose) of neuraminidase/ml. Solutions were then centrifuged at 200000 $\times g$ for 1 h. Pellets were suspended in water and kept at −20°C until used. Controls were run without neuraminidase. This treatment removes sialic acid residues which give a positive reaction with Stain's-all.

Binding of Ca^{2+} to phospholipids. Zymogen granule membranes (75 μg of protein/ml) were incubated with 100 μM $CaCl_2$ (1.0–1.2 μCi) in a volume of 8 ml for 30 min at 37°C. Incubation media were then centrifuged at 14000 $\times g$ for 20 min. Supernatants were discarded, and pellets were extracted three times with a mixture of methanol/chloroform (1 : 1, v/v) according to Burger et al. [20]. Insoluble proteins were recovered as a pellet after a 1000 $\times g$ centrifugation for 10 min. Protein extract was heated at 90°C for 30 min in 1 M NaOH to solubilize protein for radioactivity counting. Lipid extract was mixed with chloroform and water according to Folch et al. [21]. Organic phosphorus and Ca^{2+} radioactivity were measured in the lipid extract, protein and $^{45}Ca^{2+}$ radioactivity were determined in the protein, and in the aqueous phases after evaporation of the organic solvents. Radioactivity was measured in a mixture of Bio-Solv-3/toluene/Ready-Solv-NA (1 : 2 : 3, v/v) as a scintillation cocktail.

Biochemical assays. Proteins were determined according to Lowry et al. [22] after 10% trichloroacetic acid precipitation at 4°C. Bovine serum albumin was used as protein standard. ATP-diphosphohydrolase was assayed according to

Laliberté et al. using ADP as the substrate [23]. Inorganic phosphorus was measured by the method of Bartlett [24]. Calmodulin was detected with [^{125}I]RIA kit according to Chafouleas et al. [25] and Dedman et al. [26]. Assays were carried out in triplicate.

Results

Kinetic of Ca^{2+} binding to zymogen granule membrane

The influence of Ca^{2+} concentration on its binding by purified zymogen granule membrane was first studied. Total binding varied from one preparation to the other. Table I describes the variations observed in 12 different preparations. The binding curve followed essentially the pattern illustrated in Fig. 1. A linear relationship between binding and concentration was observed at concentration up to the micromolar range (inset), then it increased sharply in the millimolar range. It was independent of protein concentration between 5 to 75 μg protein/ml (data not shown), it was fast and reached a plateau within 20 min of incubation. To make sure that the apparent binding did not correspond to an uptake of $^{45}Ca^{2+}$ by closed vesicles, the ionophore A23187 was added to the incubation medium. Binding was not reduced by the ionophore at Ca^{2+} concentrations of 10 and 100 μM, whereas it was slightly increased at 1 μM. Binding varied linearly with pH at the three concentrations tested: between pH 6.2 and 8.2 it increased by 3-fold at 100 μM (47 vs. 145 nmol/mg of protein) by 8-fold at 10 μM (5 vs. 41 nmol/mg of protein) and by 20-fold at 1 μM Ca^{2+} (0.2 vs. 4.4 nmol/mg of protein). At concentrations ranging from 1 μM to 100 mM, Na^+ and K^+ ions did not affect $^{45}Ca^{2+}$ binding observed at 1, 10 or 100 μM. Higher concentrations of the monovalent cations decreased Ca^{2+} binding at the three concentrations tested (data not shown). In contrast an inhibition of 95% was observed with 10 mM Mg^{2+} (Fig. 2). As expected 1 μM La^{3+} reduced Ca^{2+} binding at 1 and 10 μM Ca^{2+} whereas a higher concentration of La^{3+} (10 μM) was required to produce a significant inhibitory effect at 100 μM Ca^{2+} (Fig. 3). Terbium produced essentially the same type of inhibition as La (data not shown).

TABLE I

Ca^{2+} BOUND BY ZYMOGEN GRANULE MEMBRANE AT DIFFERENT CONCENTRATIONS

The figures are presented as means ± S.E. of 12 preparations.

Ca^{2+} (μM)	Ca^{2+} bound (nmol/mg protein)
1	2.1 ± 0.1
10	14.9 ± 1.0
100	76.6 ± 3.8

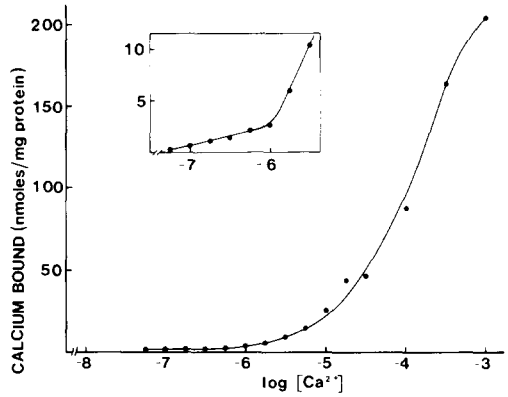

Fig. 1. Ca^{2+} binding to zymogen granule membrane as a function of Ca^{2+} concentration. Protein concentration was 9 μg/ml. Inset: linear relationship between binding and low Ca^{2+} concentration. Conditions of the assays are described in Materials and Methods. Each point is the mean of a triplicate determination.

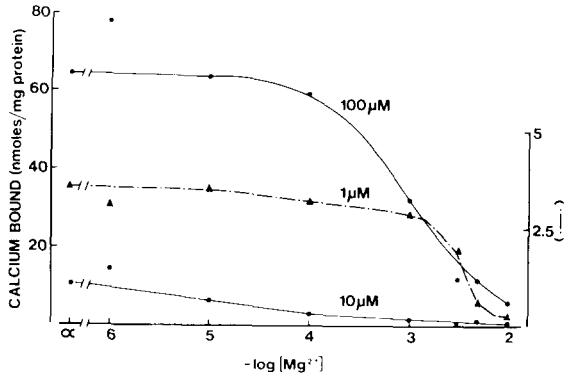

Fig. 2. Effect of Mg^{2+} on Ca^{2+} binding to zymogen granule membrane at 1, 10 and 100 μM Ca^{2+}. Protein concentration was 15 μg/ml. Each point is the mean of a triplicate determination. The replicates were within 5% of the highest value.

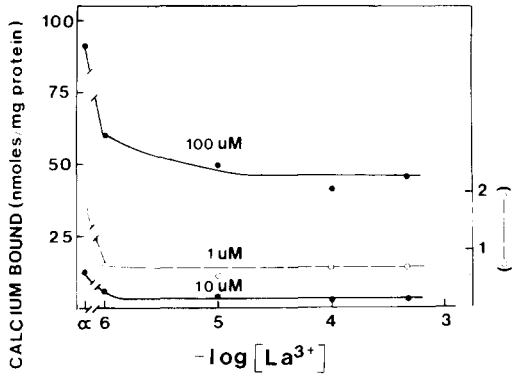

Fig. 3. Effect of La^{2+} on Ca^{2+} binding to zymogen granule membrane at 1, 10 and 100 μM Ca^{2+}. Protein concentration was 15 μg/ml. Each point is the mean of a triplicate determination. The replicates were within 5% of the highest value.

Identification of the Ca^{2+} binding sites of zymogen granule membrane after counter-ion electrophoresis

Ca^{2+} binding on zymogen granule membrane can be attributed to polypeptides or lipids. In the micromolar range very little Ca^{2+} is expected to be bound by phospholipids, even at 100 μM as shown in Table II, 65% of total Ca^{2+} was associated to protein. To identify the protein components responsible for Ca^{2+} binding, electrophoresis in non-denaturing conditions was carried out. Fig. 4 shows a typical electrophoretogram of Nonidet P-40 solubilized proteins separated on an 8% DATD-acrylamide gel. Six major bands and several minor bands were revealed by Coomassie blue staining, $^{45}Ca^{2+}$ binding is also illustrated. Net

TABLE II

DISTRIBUTION OF $^{45}Ca^{2+}$ BOUND TO ZYMOGEN GRANULE MEMBRANE FOLLOWING PHOSPHOLIPID EXTRACTION

Zymogen granule membranes were incubated with 100 μM Ca^{2+} as stated in Materials and Methods. Protein concentration was 75 μg/ml. After centrifugation following the incubation in the presence of ^{45}Ca, the pellet was extracted. These experiments were done in triplicate on two different zymogen granule membrane preparations. n.d., not determined.

Fraction	Amount recovered (% of total)		
	Ca^{2+}	Proteins	P_i
Aqueous phase	2, 8	n.d.	n.d.
Protein extract	65, 6	95	n.d.
Organic phase	31, 5	n.d.	96, 6

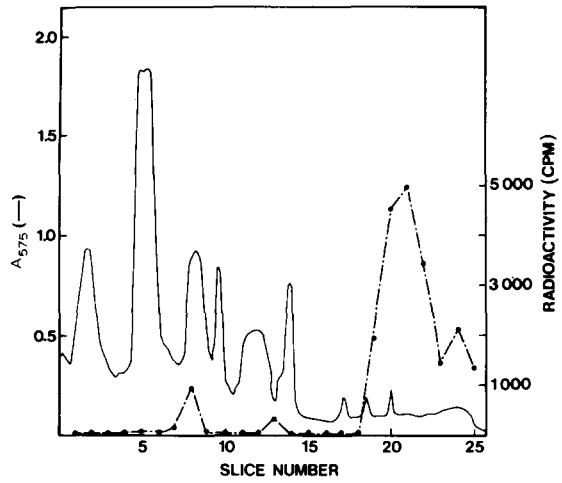

Fig. 4. Polyacrylamide gel electrophoresis (8%) of zymogen granule membrane proteins solubilized in Nonidet P-40. Coomassie blue stained gels were scanned with a Gilford spectrophotometer. Dashed line: radioactivity of corresponding gel slices. Solid line: a sample of 100 μg of protein was loaded onto the gel. Other details of the procedure are described in Materials and Methods. *A*, absorbance.

counts were obtained after subtracting the counts measured for denatured zymogen granule membrane which gave an identical electrophoretogram. Most of the Ca^{2+} bound was associated to a highly mobile protein band, the peak of radioactivity coinciding with a minor band revealed by Coomassie. The latter was not detected on electrophoretograms of the zymogen granules content. Binding components were not lipids since after electrophoresis, in a more reticulated gel (12%

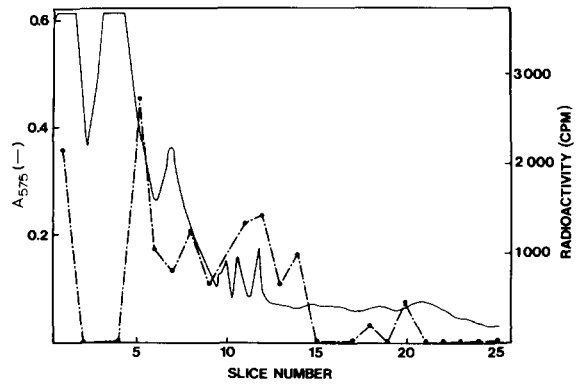

Fig. 5. Polyacrylamide gel electrophoresis (12%) of zymogen granule membranes. $^{45}Ca^{2+}$ binding is illustrated (dashed line). See details of the procedure in Fig. 4. *A*, absorbance.

acrylamide), radioactivity was found in several bands located in the middle instead of the extremity of the gel (Fig. 5). In addition, after extraction of phospholipids from the zymogen granule membrane, most of the Ca^{2+} remained associated with protein (Table II). The possibility that one of the polypeptides was calmodulin was verified: on a 8% acrylamide gel, calmodulin migrated at level of the tracking dye. In parallel with these experiments calmodulin radioimmunoassays indicated that zymogen granule membrane contained 20 to 35 ng of calmodulin per mg of protein. That amount would not be detectable after Coomassie staining. Considering the amount of calmodulin and its binding capacity, its contribution in the zymogen granule membrane binding assay was negligible.

Stain's-all identification of Ca^{2+} binding protein

The Ca^{2+} binding proteins were identified with Stain's-all, a stain which absorbs between 600 and 650 nm. Calmodulin staining increased non linearly with concentration up to 5 μg per ml, whereas with zymogen granule membrane a linear relationship was observed up to 570 μg of protein per ml. Glycoprotein sialic acid residues react with the stain and absorb at 600 nM. As indicated in Table III treatment of zymogen granule membrane with neuraminidase caused a slight reduction of the absorbance at this wavelength but it was not related to the amount of zymogen granule membrane proteins.

Having confirmed that some of the proteins of zymogen granule membranes were forming complexes with Stain's-all we then separated the polypeptides by two different electrophoretic systems. Firstly the system used for counter-ion electrophoresis (see Fig. 4) was tested. As shown by the absorbance at 600 nm a major band was observed at the extremity of this gel confirming the results of ^{45}Ca binding. Secondly zymogen granule membranes were separated by SDS-polyacrylamide gel electrophoresis and stained with Stain's-all (Fig. 6). Six major bands were revealed after scanning at 600 nm: Bands (A) 130, (B) 92, (C) 66, (D) 57, (E) 43, and (F) 30 kDa, respectively. After neuraminidase treatment of the zymogen granule membrane, the peaks corresponding to polypeptides A and B disappeared indicating that they were sialoglycoproteins. When these gels were scanned at 450 nm, a large peak appeared at the extremity confirming the presence of phospholipids. Finally at 500 nm all the Ca^{2+} binding polypeptides and several other polypeptides as well as phospholipids were revealed. To determine if the Ca^{2+} binding band located at the extremity of the gel after counter-ion electrophoresis could be subfractionated by SDS-polyacrylamide gel electrophoresis (see Fig. 4) the band was eluted, submitted to electrophoresis, and stained with

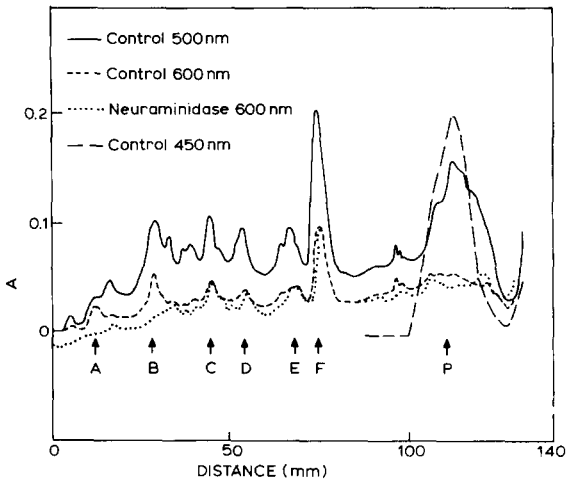

Fig. 6. SDS-polyacrylamide gel electrophoresis of zymogen granule membranes. Electrophoretogram after staining with Stain's-all at 500 nm (red) 600 nm (blue) or at 450 nm (yellow). By comparison with standards the molecular weights are: Band A, 130000; B, 92000; C, 66000; D, 57000; E, 43000 and F, 30000. Phospholipids are identified by the letter P. 100 μg of protein were applied on the gel. *A*, absorbance.

TABLE III

EFFECT OF NEURAMINIDASE ON THE STAINING (STAIN'S-ALL) OF ZYMOGEN GRANULE MEMBRANE (ZGM)

Values are the means of two separate experiments carried out on different zymogen granule membrane preparations. Assays were done in triplicate.

ZGM (μg of protein/ml)	Absorbance (600 nm)	
	control	+ neuraminidase
20	0,029	0,020
30	0,054	0,047
40	0,088	0,074
60	0,151	0,139

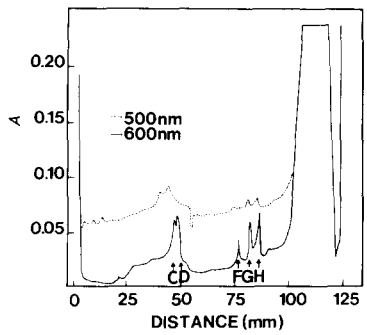

Fig. 7. SDS-polyacrylamide gel electrophoresis of the protein band responsible for Ca^{2+} binding. The band which binds Ca^{2+} on counterion electrophoresis gel was eluted from ten gels dialyzed, and submitted to SDS separation. SDS gel was treated with Stain's-all. As evaluated by densitometry, five protein bands are revealed at 600 nm: (66 kDa), D (57), F (30), G (27) and H (22.5). *A*, absorbance.

Stain's-all. As shown in Fig. 7 the band was a complex of five polypeptides corresponding to C, D, F of Fig. 6 and two other bands G (27 kDa) and H (22.5 kDa).

We also compared Ca^{2+} binding on intact zymogen granule with that of purified zymogen granule membrane. Because of their instability, granules could not be washed on the Chelex column, and binding could not be carried out at alkaline pH. Secondly granules contained large amounts of secretory proteins as compared to membrane proteins. To circumvent these problems binding was carried out on intact zymogen granule, at pH 6.0, and results were expressed on the basis of their content in ATP diphosphohydrolase activity. Determinations were carried out on different preparations of zymogen granule and zymogen granule membrane. At a concentration of 10 μM Ca^{2+} 82.9 $\pm$ 8.7 nmol of Ca^{2+} were bound per unit of ATP diphosphohydrolase whereas in isolated zymogen granule membrane there were 38.6 $\pm$ 5.0 nmol/unit of enzyme.

Discussion

In the first part of this study we have shown that zymogen granule membrane can bind Ca^{2+} in the submicromolar range and that this binding was not attributable to phospholipids. The possibility that this apparent binding would correspond to an uptake by closed vesicles was ruled out by adding the ionophore A23187 to the assay medium.

Ca^{2+} binding increased with pH, changes were relatively important especially at 1.0 μM Ca^{2+}, where a 20-fold increase in specific binding was observed with a 100-fold reduction in H^+ concentration. Since we noticed that pH also affected binding at higher concentration of Ca^{2+}, but to a lesser degree, it suggests that there has been some structural modifications of the membrane favoring the access of the cation to its binding sites. Binding was relatively insensitive to large fluctuations of Na^+ and K^+ whereas with Mg^{2+} binding was inhibited in the millimolar range. In that range it cannot be excluded that the divalent cation would have induced some structural alterations of the membrane such as membrane stacking thereby impairing Ca^{2+} binding. In contrast, the trivalent cations La^{3+} and Tb^{3+} were inhibitory in the micromolar range. Even though lanthanide ions are known to possess structural characteristics and chemical properties similar to those of Ca^{2+}, such as calmodulin activation [27], they do not appear to totally displace the Ca^{2+} ions. In the present study there was a lanthanide-resistant component, i.e. sensitive to the La^{3+} concentration. In relation to the above-mentioned properties, it is noteworthy that in isolated mouse pancreas acini, secretagogues do not modify the intracellular concentration of total Na^+ and exchangeable K^+ and Cl^- and do not influence cell pH [28].

To our knowledge, Ca^{2+} binding to zymogen granule membrane has not yet been reported in the literature, but it has been studied with microsomal membranes of rat pancreas [29]. These authors separated the microsomes in a light and a heavy fraction, the light fraction presumably contains some zymogen granule membrane. In the latter fraction, they found two classes of binding sites.

In this work we identified the Ca^{2+} binding polypeptides by two different methods. Stain's-all and counter-ion electrophoresis with ^{45}Ca. Counter-ion electrophoresis of the zymogen granule membrane using 8% and 12% polyacrylamide gel concentrations indicated that in the micromolar range most of the radioactive ^{45}Ca was bound by several protein bands. We ruled out the possibility that calmodulin was among these after measuring its concentration with a radioimmunoassay. In-

deed its level was extremely low and thereby would not account for the Ca^{2+} binding properties of zymogen granule membrane. Secondly we found that the electrophoretic properties of calmodulin were different from the protein bands detected on our gels. With these same electrophoretic systems and extraction studies we also ruled out the possibility that phospholipids would contribute significantly to the binding of Ca^{2+} by zymogen granule membrane. Stain's-all, the dye used to identify the Ca^{2+} binding protein [18], was applied after SDS-polyacrylamide gel electrophoresis of zymogen granule membrane. Six polypeptides reacted with the stain (A, B, C, D, E, F). Treatment with neuraminidase caused the disappearance of the 92 (A) and 130 kDa (B) polypeptide staining on gels, these must therefore be considered as sialoglycoproteins. The 92 kDa band is presumably the protein originally described as GP_2 by Ronzio et al. [30–32], these authors measured about 102 nmol of sialic acid/mg of zymogen granule membrane protein in the rat [30]. When the Ca^{2+} binding band, obtained by counter-ion electrophoresis in 8% non-denaturing gel, was submitted to SDS-polyacrylamide gel electrophoresis five polypeptides were separated (C, D, F, G, H). These are not necessarily Ca^{2+} binding proteins, but as mentioned above, three of them C, D and F behave as such with Stain's-all. The D polypeptide has been recently identified as the ATP diphosphohydrolase (Vachereau et al., manuscript in preparation).

One can wonder if and how these proteins, that bind Ca^{2+}, are related to the exocytosis phenomenon? Several years ago, Milutinovic et al. [33] reported a Ca-dependent interaction between plasma membrane and zymogen granules of rat pancreas. They found an apparent K_m for Ca^{2+} of 6.5 μM at pH 6.6 for the interaction between these membranes. From their study they proposed that this interaction could reflect the initial event in the fusion process between zymogen granules and plasma membrane. In the present study intact zymogen granules bind large amounts of Ca^{2+}, probably more than isolated zymogen granule membranes. Among the proteins that react positively to Stain's-all after SDS gel electrophoresis, and among the polypeptide that are associated with the Ca^{2+} binding band after counter-ion electrophoresis, only one would apparently have access to the external surface of the zymogen granule [34]. This is band F the 30 kDa polypeptide. Therefore, this protein would be responsible for the Ca^{2+} binding to intact zymogen granules and would be a presumptive candidate to play a role if a Ca^{2+}-binding protein is involved in the fusion of zymogen granules with luminal membrane.

What are the mechanisms that elicit fusion of these membranes? In 1970 Matthews proposed the hypothesis that in the islet cells the secretory granules and inner surface of the cell membrane are equally charged, then an electrostatic repulsive force field would be generated when a secretory particle approached the membrane. Unless the kinetic energy of translational motion exceeds the electrostatic energetic barrier, contact-collision of the granule would not occur [34,36]. It was postulated that one function of Ca^{2+} ions in exocytosis was to partially neutralize the surface negative charges and consequentially diminish the energetic barrier; only those granules with sufficiently large kinetic energies would make lasting contact with the membrane thus permitting adhesion. It was found later that 1 mM Ca^{2+} reduced by only 11% the surface charge of isolated guinea pig granules [37]. A fortiori, a concentration of 1 μM Ca^{2+} would not reduce significantly the surface charge of the zymogen granules. From these observations one can propose that if Ca^{2+} is directly involved in the interaction of zymogen granules with luminal membrane it is not through a neutralization of surface charges. Perhaps there is another way by which Ca^{2+} could be directly involved: as a chelating agent, it could favor the intra and the intermembrane aggregation of a specific protein (for example the 30 kDa protein) thereby facilitating the anchoring of the zymogen granules to plasma membrane.

While this work was in progress Haase et al. [38] detected by electron microscopy the occasional presence of Ca^{2+} deposits at the zymogen granule surface when the acinar cell was stimulated by carbachol. These deposits were not seen in unstimulated cells. These observations substantiate the possibility of a direct interaction of Ca^{2+} with a Ca^{2+} binding protein in the zymogen granule membrane during stimulation.

Acknowledgements

This work was supported by grants from 'Le Conseil de la recherche en sciences naturelles et en génie du Canada' and 'Le Fonds F.C.A.R. du Québec'. We thank Carolyn Rancourt for typing the manuscript and Marielle Martin for the drawings. We are grateful to Andrée Jean for carrying out the calmodulin RIA analysis. We express our gratitude to Dr. E.E. Daniels from McMaster University, Hamilton, Ontario, Canada for helpful discussion.

References

1 Chandler, D.E. (1978) Life Sci. 23, 323–334
2 Case, R.M. (1978) Biol. Rev. 53, 211–354
3 O'Doherty, J. and Stark, R.J. (1982) Am. J. Physiol. 242, G513–G521
4 Streb, H. and Schulz, I. (1983) Am. J. Physiol. 243, G347–G357
5 Ochs, D.L., Korenbrot, J.I. and Williams, J.A. (1983) Biochem. Biophys. Res. Commun. 117, 122–128
6 Dormer, R.L. (1983) Biosci. Rep. 3, 233–234
7 Dean, P.M. (1974) in Secretory Mechanism of Exocrine Glands (Thorn, N.A. and Petesen, O.H., eds.), pp. 152–165, Munkesgaard, Copenhagen
8 Matthews, E.K. and Nordmann, J.J. (1976) Mol. Pharmacol. 12, 778–788
9 Orci, L., Galbay, K.H. and Malaisse, W.J. (1972) Science 175, 1128–1130
10 Aunis, D., Hesketh, J.E. and Devilliers, G. (1979) Cell Tissue Res. 197, 433–441
11 Lambert, M., Camus, J. and Christophe, J. (1974) FEBS Lett. 49, 228–332
12 Crouch, T.H. and Klee, C.B. (1984) Biochem. 19, 3692–3698
13 Pâquet, M.R., St-Jean, P., Roberge, M. and Beaudoin, A.R. (1982) Eur. J. Cell Biol. 29, 20–26
14 Ueng, T.H. and Bronner, F. (1979) Arch. Biochem. Biophys. 197, 205–217
15 Schibeci, A. and Martonosi, A. (1980) Anal. Biochem. 104, 335–342
16 Hunkapiller, M.W., Lujan, E., Ostrander, F. and Hood, L.E. (1983) Methods Enzymol. 91, 227–236
17 Laemmli, U.K. (1970) Nature 227, 680–685
18 Campbell, K.P., MacLennan, D.H. and Jorgensen, A.O. (1983) J. Biol. Chem. 258, 11267–11273
19 King, L.E. and Morrison, M. (1976) Anal. Biochem. 71, 223–230
20 Burger, S.P., Fuji, T. and Hanaham, D.J. (1968) Biochem. 7, 3682–3700
21 Folch, J., Lees, M. and Sloane-Stanley, G.H. (1957) J. Biol. chem. 226, 497–509
22 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
23 Laliberté, J.F., St-Jean, P. and Beaudoin, A.R. (1982) J. Biol. Chem. 257, 3869–3874
24 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466–468
25 Chafouleas, J.G., Dedman, J.R., Munjaal, R.P. and Means, A.R. (1972) J. Biol. Chem. 254, 10262–10267
26 Dedman, J.R., Welsh, M.J. and Means, A.R. (1978) J. Biol. Chem. 253, 7515–7521
27 Wallace, R.W., Tallant, E.A., Dockter, M.E. and Cheung, W.Y. (1982) J. Biol. Chem. 257, 1845–1854
28 Preissler, M. and Williams, J.A. (1981) J. Physiol. 321, 437–448
29 Argent, B.E., Case, R.M., Hirst, F.C. and Scott-Wilson, C.J. (1977) Proc. Physiol. Soc., Sept. 69P–70P
30 MacDonald, R.J. and Ronzio, R.A. (1972) Biochem. Biophys. Res. Commun. 449, 377–382
31 Lewis, D.S., MacDonald, R.J., Kronquist, K.E. and Ronzio, R.A. (1977) FEBS Lett. 76, 115–120
32 Ronzio, R.A., Kronquist, K.E., Lewis, D.S. MacDonald, R.J., Mohrlok, S.H. and O'Donnel, J.J., Jr. (1978) Biochim. Biophys. Acta 508, 65–84
33 Milultinovic, S., Argent, B.E., Schulz, I. and Sachs, G. (1977) J. Membrane Biol. 36, 281–295
34 LeBel, D. and Beattie, M. (1984) Biochim. Biophys. Acta 769, 622–624
35 Matthews, E.K. (1970) in Calcium and Cell Function (Cuthbert, A.W., ed.), pp. 163, MacMillan, London
36 Matthews, E.K. (1970) Acta Diabetol. Lat. 7, 83–70
37 Dean, P.M. (1974) Diabetologia 10, 427–430
38 Haase, W., Friese, W. and Heitmann, K. (1984) Cell Tissue Res. 235, 683–690