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Electrogenic Calcium Transport in Plasma Membrane of Rat Pancreatic Acinar Cells

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Summary. ATP-dependent $^{45}Ca^{2+}$ uptake was investigated in purified plasma membranes from rat pancreatic acinar cells. Plasma membranes were purified by four subsequent precipitations with $MgCl_2$ and characterized by marker enzyme distribution. When compared to the total homogenate, typical marker enzymes for the plasma membrane, (Na^+,K^+)-ATPase, basal adenylate cyclase and CCK-OP-stimulated adenylate cyclase were enriched by 43-fold, 44-fold, and 45-fold, respectively. The marker for the rough endoplasmic reticulum was decreased by fourfold compared to the total homogenate. Comparing plasma membranes with rough endoplasmic reticulum, Ca^{2+} uptake was maximal with 10 and 2 μmol/liter free Ca^{2+}, and half-maximal with 0.9 and 0.5 μmol/liter free Ca^{2+}. It was maximal at 3 and 0.2 mmol/liter free Mg^{2+} concentration, at an ATP concentration of 5 and 1 mmol/liter, respectively, and at pH 7 for both preparations. When Mg^{2+} was replaced by Mn^{2+} or Zn^{2+} ATP-dependent Ca^{2+} uptake was 63 and 11%, respectively, in plasma membranes; in rough endoplasmic reticulum only Mn^{2+} could replace Mg^{2+} for Ca^{2+} uptake by 20%. Other divalent cations such as Ba^{2+} and Sr^{2+} could not replace Mg^{2+} in Ca^{2+} uptake. Ca^{2+} uptake into plasma membranes was not enhanced by oxalate in contrast to Ca^{2+} uptake in rough endoplasmic reticulum which was stimulated by 7.3-fold. Both plasma membranes and rough endoplasmic reticulum showed cation and anion dependencies of Ca^{2+} uptake. The sequence was $K^+ > Rb^+ > Na^+ > Li^+ >$ choline$^+$ in plasma membranes and $Rb^+ \geq K^+ \geq Na^+ > Li^+ >$ choline$^+$ for rough endoplasmic reticulum. The anion sequence was $Cl^- \geq Br^- \geq I^- > SCN^- > NO_3^-$ > isethionate$^-$ > cyclamate$^-$ > gluconate$^-$ > $SO_4^{2-} \geq$ glutarate$^-$ and $Cl^- > Br^-$ > gluconate$^-$ > SO_4^{2-} > $NO_3^- > I^-$ > cyclamate$^- \geq SCN^-$, respectively. Ca^{2+} uptake into plasma membranes appeared to be electrogenic since it was stimulated by an inside-negative K^+ and SCN^- diffusion potential and inhibited by an inside-positive diffusion potential. Ca^{2+} uptake into rough endoplasmic reticulum was not affected by diffusion potentials. We assume that the Ca^{2+} transport mechanism in plasma membranes as characterized in this study represents the extrusion system for Ca^{2+} from the cell that might be involved in the regulation of the cytosolic Ca^{2+} level.

Key Words electrogenic Ca^{2+} transport · plasma membrane · rough endoplasmic reticulum · pancreatic acinar cells

Introduction

An increase in the intracellular free Ca^{2+} concentration plays an important role in the sequence of events leading to stimulation of enzyme secretion from the exocrine pancreas [8, 12, 13, 14, 18, 22, 27, 41, 42, 45, 53]. From studies with a Ca^{2+}-selective electrode in permeabilized cells we know that the rough endoplasmic reticulum can regulate the free Ca^{2+} concentration to 4×10^{-7} mol/liter [3, 55]. Since in intact acinar cells active Ca^{2+} extrusion mechanisms in the plasma membrane should determine the cytosolic steady-state Ca^{2+} concentration, the properties of the plasma membrane Ca^{2+} transport is an important prerequisite for the understanding of cellular Ca^{2+} metabolism at rest and during stimulation. It is therefore essential to isolate the plasma membrane and investigate its contribution to the regulation of the cytosolic free Ca^{2+} concentration.

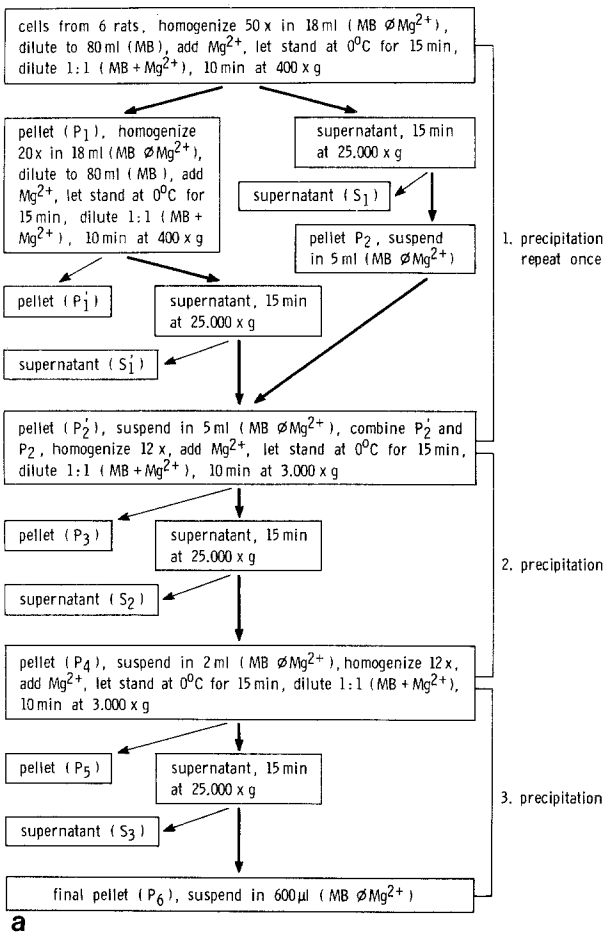
Thus far no methods for successful separation of plasma membranes from other cell organelles within reasonable time and with a protein yield sufficient to perform further experiments have been available for exocrine pancreas. Therefore, the role of the plasma membrane in calcium transport and in the regulation of the cytosolic free calcium concentration has yet remained unclear.

We now report on isolation of a pure plasma membrane fraction from pancreatic acinar cells. This enabled us to differentiate clearly between the Ca^{2+} transport characteristics of the plasma membrane and the rough endoplasmic reticulum.

Materials and Methods

Materials

All reagents were of analytical grade. Percoll was obtained from Pharmacia, Uppsala, Sweden. Collagenase (from clostridium histolyticum) type III was from Worthington, Freehold, N.J. Ethyleneglycol-*bis*-(β-amino-ethylether)-N,N-tetraacetic acid (EGTA), ethylene-diamine-tetra-acetic acid (EDTA), adenosine 5-triphosphate (ATP), guanosine 5-triphosphate (GTP), inosine 5-triphosphate (ITP), uridine 5-triphosphate (UTP), cytidine 5-



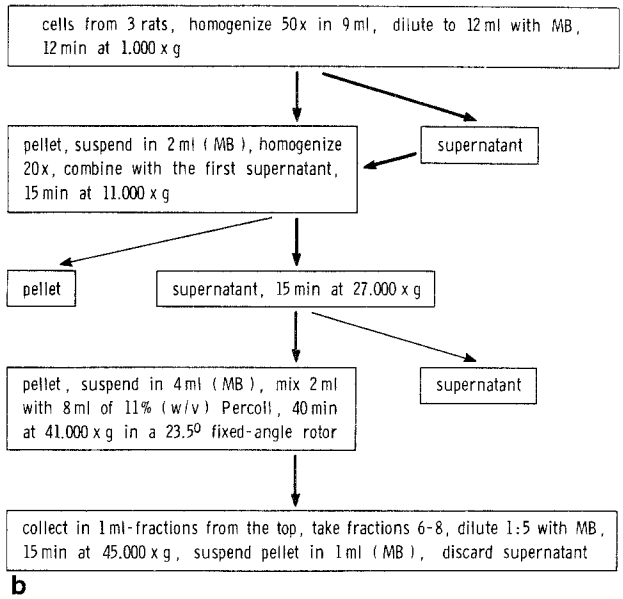


Fig. 1. *a.* Isolation scheme of plasma membrane vesicles derived from rat pancreatic acinar cells. MB = mannitol buffer. For further details *see* text. *b.* Isolation scheme of rough endoplasmic reticulum vesicles derived from rat pancreatic acinar cells. MB = mannitol buffer. Further details are described in the text and previously [3]

triphosphate (CTP) (as Tris or K_2 salts), phosphocreatine (sodium salt) the protonophore carbonyl cyanide *p*-trifluoromethoxyhydrazone (CFCCP) and benzamidine were from Sigma, München, W. Germany. Cytochrome *c*, lactate dehydrogenase (LDH), pyruvate kinase (PK), creatine kinase (CK), phosphoenolpyruvate (PEP), nicotine adenine dinucleotide (NADH), ribonucleic acid (RNA), trypsin inhibitor (from soybean) and valinomycin were from Boehringer, Mannheim, W. Germany. Antimycin A, oligomycin, Triton X, L-histidine, glutardialdehyde and bovine serum albumin were from Serva, Heidelberg, W. Germany. Sodium dithionite was from Merck, Darmstadt, W. Germany. The Ca^{2+} ionophore A23187, and the octapeptide of cholecystokinin CCK-OP were from Calbiochem, Giessen, W. Germany. $^{45}CaCl_2$ (4 to 50 Ci/g) was from New England Nuclear, Dreieich, W. Germany.

Preparation of Acinar Cells

Rat pancreatic acinar cells were prepared as described by Amsterdam and Jamieson [2] with modifications as described by Streb and Schulz [55]. Briefly, pancreatic tissue from 6 to 9 rats was digested in a collagenase (150 U/ml) containing Krebs-Ringer's solution for about 75 min at 37°C. Single cells were obtained by an interposed washing step with a 2 mmol/liter EDTA-containing solution subsequent to the initial 15 min of collagenase digestion.

Preparation of Plasma Membranes

All membrane preparation steps were performed at 5°C. Following isolation, cells were washed twice in a mannitol buffer (MB) containing in mmol/liter: 280 mannitol, 10 HEPES, 10 KCl, 1 $MgCl_2$, 1 benzamidine, pH 7.4 adjusted with Tris. Cells were then homogenized in a volume of 18 to 27 ml of MB in a tight-fitting motor-driven glass/Teflon Potter (Braun, Melsungen, W. Germany) by 50 strokes at 900 rpm. The homogenate was then divided to prepare either plasma membranes or rough endoplasmic reticulum (*see below*). To prepare plasma membranes the isolation scheme as shown in Fig. 1*a* was developed. The final Mg^{2+} concentration during the precipitation and separation steps was 11 mmol/liter. The second and third homogenizations were performed as described above, except the last step, that was performed in a glass/glass hand Potter (Braun, Melsungen, W. Germany). Three low-speed centrifugations to remove the aggregated material alternated with three high-speed centrifugations to collect the material from the plasma membrane-enriched supernatants. The first precipitation was repeated once to increase the final protein yield. The low-speed centrifugations of the first precipitation were performed with a JS 7.5 rotor in a Beckman J-21C centrifuge and the low-speed centrifugation of the second precipitation was performed in an SW 27 rotor in a Beckman L2 65B ultracentrifuge. The last low-speed centrifugation of the third precipitation was performed in an SW 50 rotor in the same ultracentrifuge. The first three high-speed centrifugations were car-

ried out in the SW 27 rotor and the last high-speed centrifugation in the SW 50 rotor in the above ultracentrifuge. To remove the Mg^{2+}, the final membrane pellet was washed in the mannitol buffer as described which was supplemented with 0.3 mmol/liter EGTA and 3 mmol/liter EDTA and centrifuged for 15 min at 50,000 × *g*.

Preparation of Rough Endoplasmic Reticulum

Rough endoplasmic reticulum was prepared as described in detail previously [3]. For short information see the isolation scheme (Fig. 1*b*). The mannitol buffer was composed as described above. The Percoll medium, 11% (wt/vol), contained additionally, in mmol/liter: 280 mannitol, 5 HEPES, 5 KCl, 0.5 $MgCl_2$, 0.5 benzamidine, pH 7.4 adjusted with Tris, and had a density of about 1.035 g/cm³. After isopycnic centrifugation, the self-generated Percoll gradient was fractionated in eight 1-ml fractions from the top. The purified rough endoplasmic reticulum was located in fractions 6 to 8 with an average density of about 1.055 g/cm³.

Measurement of Calcium Uptake

Calcium uptake was measured using $^{45}Ca^{2+}$. 20 to 40 μg of plasma membrane protein or 50 to 100 μg of rough endoplasmic reticulum protein were preincubated for 20 min in 500 μl at 25°C of an incubation medium containing basically in mmol/liter: 130 KCl, 30 HEPES, 0.01 antimycin A and 0.005 oligomycin, pH 7 adjusted with Tris. The amount of radioactivity varied from 4 to 30 μCi/ml according to the desired total Ca^{2+} concentrations. Uptake was initiated by adding Tris-ATP to a final concentration of 1 mmol/liter. The free Ca^{2+} and Mg^{2+} concentrations are given in the legends to the figures. At given time points, triplicate samples were filtered rapidly through cellulose nitrate filters with a pore size of 0.65 μm (Sartorius, Göttingen, W. Germany), which had been presoaked in isotonic KCl solution. Filters were washed with 4 ml of an ice-cold solution containing, in mmol/liter: 140 KCl, 10 HEPES, 1 $MgCl_2$, pH 7 adjusted with KOH. The radioactivity was quantitated using "Rotiscint 22" scintillator (Roth, Karlsruhe, W. Germany) in a Mark III Liquid Scintillation System, Model 6880 (Searle Analytic Inc., Des Plaines, Illinois). The values for ATP-driven Ca^{2+} transport into vesicles were calculated as the difference between Ca^{2+} content in the presence and absence of ATP in all experiments. The term "Ca^{2+} uptake" is used as a synonym for Ca^{2+} content throughout the manuscript. Free Ca^{2+} concentrations were calculated with a computer program using the true proton, Ca^{2+} and Mg^{2+} dissociation of ATP, EDTA and EGTA. The resulting polynomial equations were solved as described previously [55].

Protein and Enzyme Determination

For protein determination of plasma membrane and rough endoplasmic reticulum the "Fluorescamin" method was used [59]. Only negligible interference of Percoll with this test was observed. The protein content of the rough endoplasmic reticulum fraction was corrected by using a blank Percoll gradient without vesicles.

For ribonucleic acid (RNA) determination, 50 to 100 μg of protein were treated with 10% ice-cold TCA in the presence of 0.1% BSA for 15 min. The resulting pellet was washed three times with 5% TCA and RNA was hydrolyzed according to Mejbaum et al. [34]. The procedure was continued by the method of Hatcher et al. [16] and the RNA content was calculated using RNA standards.

(Na^+,K^+)-ATPase activity was determined according to the method of Scharschmidt et al. [44] with the final reaction medium modified to 2.5 mmol/liter ATP and 2 mmol/liter phosphoenolpyruvate (PEP). The ouabain-suppressible fraction of total ATPase activity was determined in the same sample by adding ouabain directly to the cuvette to a final concentration of 1.5 mmol/liter.

Alkaline phosphatase activity was measured as the rate of hydrolysis of *p*-nitrophenylphosphate using a Merck test kit (no. 3344).

NADPH- and NADH cytochrome *c* reductase activities were measured by the procedure of Sottocasa et al. [51].

Cytochrome *c* oxidase activity was determined in a 30 mmol/liter phosphate buffer at pH 7.4 and 37°C, containing 0.8 mmol/liter cytochrome *c* previously reduced by 20 mmol/liter sodium dithionite. Samples were preincubated with 0.0015% Triton X.

In all enzyme determinations as described above, samples were preincubated for 5 to 10 min at 37°C and the reaction was started by addition of the substrate. Enzyme activities were measured kinetically using a Beckman spectrophotometer (Model 25) and a Beckman recorder (Model 24-25ACC).

For determination of adenylate cyclase activity, 5 to 200 μg of membrane protein were incubated in 200 μl of a medium as described [37] that was modified to 3 mmol/liter PEP and 0.15 mg pyruvate kinase. Samples were preincubated for 5 min at 30°C in the presence and absence of the octapeptide of cholecystokinine (CCK-OP) at a concentration of 10^{-7} mol/liter and the reaction was started by addition of K_2ATP to a final concentration of 1 mmol/liter. The reaction was stopped by placing the tubes in a boiling water bath for 3 min. To remove the protein, samples were centrifuged for 3 min in an Eppendorf table centrifuge. The supernatants were immediately analyzed for the cyclic AMP produced, using an Amersham test kit (TRK 432, Amersham, Braunschweig, W. Germany). The radioactivity was quantitated as described.

Electron Microscopy

For electron microscopical demonstration of plasma membranes the sample was fixed as a pellet after the last preparation step. The ice-cold fixative consisted of 5% (vol/vol) glutardialdehyde in 0.1 mol/liter cacodylate buffer, pH 7.4. Samples were postfixed with 1% (wt/vol) osmic acid in cacodylate buffer, dehydrated with alcohol and embedded in Spurr's resin [52]. Rough endoplasmic reticulum vesicles were fixed in suspension and then treated as described [3]. Thin sections were stained with uranylacetate and lead citrate and were examined in a Philips 300 electron microscope.

Results

Identification of Plasma Membranes

Membrane Separation

To purify plasma membranes from rat pancreatic acinar cells a new methodological approach was

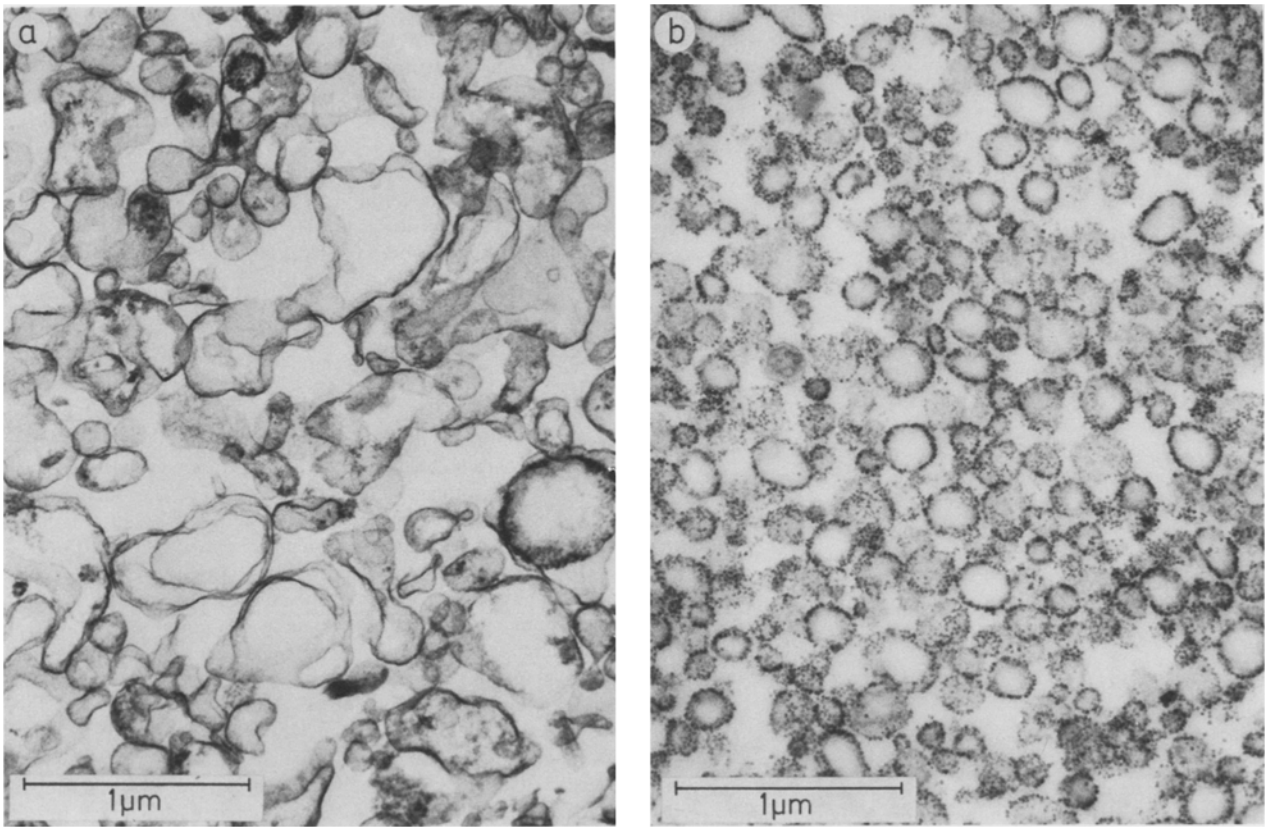


Fig. 2. Electron micrographs of purified plasma membrane vesicles (*a*) and purified rough endoplasmic reticulum vesicles (*b*) from rat pancreatic acinar cells. Plasma membrane vesicles were prepared for electron-microscopical demonstration as described in Materials and Methods. Rough endoplasmic reticulum vesicles were treated as described previously [3]

used. The precipitation with $MgCl_2$ is based on a principle that is different from isopycnic density centrifugation employed in previous fractionation procedures [35, 36, 43, 56]. The precipitation with $MgCl_2$ has so far been applied for the preparation of brush-border membranes of kidney proximal tubule [4] and small intestine [17]. In this study however, precipitation with $MgCl_2$ has been used to prepare a plasma membrane fraction which was mainly enriched in basolateral plasma membrane markers. For purification of the rough endoplasmic reticulum an isopycnic Percoll gradient was applied as described previously [3].

Morphological Studies

To estimate the composition of the final plasma membrane fraction we used electron microscopy as well as the measurement of marker enzyme activities (*see below*). For electron-microscopical demonstration of plasma membrane vesicles, the final pellet obtained from the isolation procedure was used as described (Fig. 1*a*). This allowed to roughly estimate the contamination of this fraction with other structures that would be mainly rough endoplasmic reticulum and also mitochondria and zymogen granules. One typical micrograph of the plasma membrane fraction is presented in Fig. 2*a*. More than 80% of the membranes are smooth, whereas membranes coated with ribosomes originating from the rough endoplasmic reticulum were minimized. Almost no mitochondria and zymogens were detectable in the plasma membrane fraction. We know that pancreatic acinar cells possess only a hardly detectable amount of intracellular smooth membranes [3, 60]. Moreover the marker enzyme activity for the smooth endoplasmic reticulum was reduced by about sixfold in the final membrane fraction. This suggests that nearly all smooth membranes obtained in this fraction derived from plasma membrane. The vesicle diameter of plasma membranes ranged from 0.1 to 0.6 μm.

The rough endoplasmic reticulum membranes obtained from Percoll gradient consisted of vesicles with a diameter of 0.08 to 0.3 μm, which were almost entirely coated with ribosomes (Fig. 2*b*). Since neither smooth membranes nor other organ-

Table 1. Enzyme activities and enrichment factors of plasma membranes and rough endoplasmic reticulum[a]

		Total homogenate	Plasma membranes	Rough endoplasmic reticulum
$(Na^+ + K^+)$-ATPase	Spec. act.	24.6 ± 2.1 (7)	1060.2 ± 72.7 (7)	1.4 ± 0.4 (7)
	Enrichment	1	43.1	0.06
	Recovery	100%	5.61%	0.048%
AP	Spec. act.	28.8 ± 3.4 (5)	347.8 ± 37 (5)	2.9 ± 1.6 (5)
	Enrichment	1	12.1	0.1
	Recovery	100%	1.57%	0.08%
RNA	Spec. act.	176 ± 8 (7)	60 ± 10 (7)	552 ± 23 (5)
	Enrichment	1	0.34	3.14
	Recovery	100%	0.04%	2.51%
NADPH cytochrome *c* reductase	Spec. act.	5.1 ± 0.6 (5)	0.8 ± 0.5 (5)	159 ± 7.4 (5)*
	Enrichment	1	0.16	1.71
	Recovery	100%	0.02%	1.37%
Cytochrome *c* oxidase	Spec. act.	124 ± 34 (5)	3 ± 2 (5)	3.7 ± 1.3 (5)
	Enrichment	1	0.02	0.03
	Recovery	100%	0.0026%	0.024%
Adenylate cyclase (basal)	Spec. act.	1.99 ± 0.78 (4)	88.75 ± 31.79 (4)	—
	Enrichment	1	44.6	—
	Recovery	100%	8.8%	—
Adenylate cyclase (CCK-OP-stimulated)	Spec. act.	10.82 ± 0.91 (4)	458.44 ± 49.5 (4)	—
	Enrichment	1	42	—
	Recovery	100%	7.3%	—
Protein[o]	Yield	295 ± 37 (7)	0.39 ± 0.07 (7)	2.2 ± 0.4 (7)
	Recovery	100%	0.13%	0.8%

[a] The total homogenate as starting material is compared with the purified plasma membrane or with the purified rough endoplasmic reticulum fraction. Specific enzyme activities are expressed in nmol substrate split per min and mg protein for the (Na^+,K^+)-ATPase, alkaline phosphatase, NADPH cytochrome *c* reductase and cytochrome *c* oxidase. The specific activities of the basal and CCK-OP-stimulated adenylate cyclase are given in pmol substrate split per min and mg protein. The RNA content is given in μg per mg protein and the protein yield in mg. The values are means ± SE; the number of preparations is given in parentheses. Enrichment is expressed as specific activity divided by the specific activity of the total homogenate. (* In this preparation the NADH cytochrome *c* reductase activity was measured.)

elles such as mitochondria or zymogens could be detected, these thin-section micrographs represent a highly purified fraction of rough endoplasmic reticulum [3].

Enzymatic Characterization

As enzyme markers for the plasma membrane, the (Na^+,K^+)-ATPase activity, the adenylate cyclase activity and the alkaline phosphatase activity (AP), were measured. The amount of rough endoplasmic reticulum was estimated by the RNA content and the NADPH cytochrome *c* reductase activity was determined for both rough and smooth endoplasmic reticulum. The mitochondrial content was checked by the cytochrome *c* oxidase activity.

In order to prove the morphological and functional viability of the plasma membranes after collagenase digestion the adenylate cyclase activity in response to stimulation by the terminal octapeptide of cholecystokinine (CCK-OP) was used. CCK-OP in a concentration of 10^{-7} mol/liter stimulated the basal adenylate cyclase activity in the homogenate by 5.4-fold and in the plasma membrane fraction by 5.2-fold (Table 1). The stimulation factors were very similar in the homogenate and in the plasma membrane fraction, indicating that the hormone receptors of plasma membranes are functionally intact after exposure to digestion and homogenization of the cells and that they maintained their ability to react to hormone stimulation during the isolation procedure.

In the final membrane fraction the markers for the plasma membrane (Na^+,K^+)-ATPase activity, basal adenylate cyclase activity, CCK-OP-stimulated adenylate cyclase activity and alkaline phosphatase activity, were enriched by 43-fold, 45-fold, 42-fold and 12-fold, respectively, as compared to the total homogenate. The alkaline phosphatase, which was less enriched than the other marker enzymes, is considered to be localized at the small luminal side of the cell and also in intracellular structures [26, 48]. The marker for the rough endoplasmic reticulum RNA and the NADPH cytochrome *c* reductase activity, which is present in

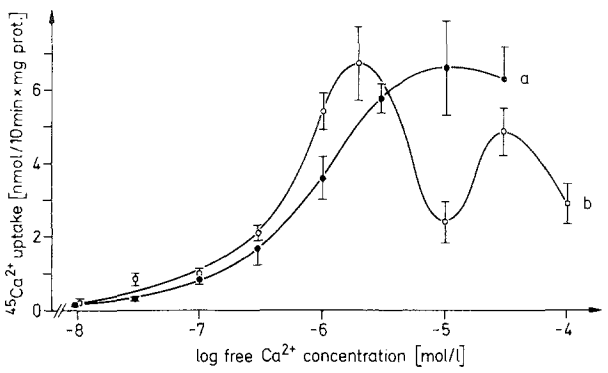


Fig. 3. Dependence of calcium uptake into plasma membranes (*a*) and rough endoplasmic reticulum (*b*) on the free Ca^{2+} concentration. The values represent means ± SE of five preparations for (*a*, ●) and eight preparations for (*b*, ○). Highest specific $^{45}Ca^{2+}$ uptake after 10 min was 6.6 ± 1.3 nmol/mg protein in (*a*) and 6.7 ± 1.0 nmol/mg protein in (*b*). The medium-free Ca^{2+} concentrations were buffered with EGTA/ATP, or EDTA/ATP, or ATP using association constants from reference [49]. The free Mg^{2+} concentration was kept constant at 0.3 mmol/liter. To minimize the source of error the calcium concentrations were adjusted exclusively with $^{45}CaCl_2$ and checked with a Ca^{2+}-specific electrode for each tracer batch as described [55]. All experiments were performed with the same solutions which were stored at −25°C between the experiments

both rough and smooth endoplasmic reticulum, were reduced by threefold and sixfold, respectively, as compared to the total homogenate. The mitochondrial marker cytochrome *c* oxidase was decreased by 50-fold in the final plasma membrane fraction (Table 1).

The enzymatic characterization of rough endoplasmic reticulum has been described in detail previously [3]. Briefly, the RNA, marker for the rough endoplasmic reticulum, was enriched by 3.2-fold, and the NADH cytochrome *c* reductase activity was enriched by 1.7-fold as compared to the total homogenate. The markers for the plasma membrane (Na^+,K^+)-ATPase activity and alkaline phosphatase activity were decreased 17-fold and 10-fold, respectively, in the final fraction. The mitochondrial marker cytochrome *c* oxidase was decreased by 33-fold (Table 1).

The data of marker enzyme activities show that both preparations were of a remarkably high purity. (Na^+,K^+)-ATPase activity, the typical marker for plasma membranes, was distributed 720 : 1 between both preparations and the RNA, typical for rough endoplasmic reticulum, was distributed 1 : 9.3. This clear separation of both structures allowed us to differentiate between the characteristics of two calcium transport systems located in both plasma membrane and rough endoplasmic reticulum.

Characterization of Calcium Transport

Calcium Dependency

To obtain reliable results for the dependence of calcium transport on the free Ca^{2+} concentration into plasma membranes and rough endoplasmic reticulum the desired free Ca^{2+} concentrations were precisely adjusted: All experiments for both preparations were performed with identical solutions that were stored at −25°C between the uptake measurements. The total calcium concentrations were supplied exclusively by $^{45}CaCl_2$ and the free Ca^{2+} concentrations were checked by a Ca^{2+}-selective electrode for each tracer batch [55]. The free Ca^{2+} concentrations 10^{-8} and 3×10^{-8} mol/liter were buffered with EGTA and between 10^{-7} and 3×10^{-6} mol/liter with EDTA.

In plasma membranes measurable calcium uptake slightly increased between 10^{-8} and 10^{-7} mol/liter free Ca^{2+} concentration. A considerable increase of calcium uptake was found at concentrations higher than 10^{-7} mol/liter which reached a maximum at 10^{-5} mol/liter free Ca^{2+} concentration. Half-maximal uptake was observed at 8.8×10^{-7} mol/liter (Fig. 3, curve *a*). The absolute amount of calcium uptake at 10^{-5} mol/liter free Ca^{2+} concentration in five measurements was 6.6 ± 1.3 nmol/mg protein after 10 min. At optimal conditions, i.e. at 10^{-5} mol/liter free Ca^{2+} concentration, 3 mmol/liter free Mg^{2+} concentration, 5 mmol/liter ATP and pH 7, the specific calcium storage capacity was even higher and about 30 nmol/mg protein after 10 min.

For the rough endoplasmic reticulum a similar calcium-dependence curve was obtained between 10^{-8} and 10^{-7} mol/liter free Ca^{2+} concentration. Then calcium uptake increased at a lower concentration than in plasma membranes and reached its maximum at 2×10^{-6} mol/liter free Ca^{2+} concentration with a half-maximal uptake at 5.4×10^{-7} mol/liter free Ca^{2+} (Fig. 3, curve *b*). The specific calcium uptake at 2×10^{-6} mol/liter free Ca^{2+} concentration in eight measurements was 6.7 ± 1.0 nmol/mg protein after 10 min. When calcium uptake into plasma membranes was measured under optimal conditions, as described above, the specific calcium storage capacity was about four times greater than measured for the rough endoplasmic reticulum under optimal conditions, i.e. at 2 μmol/liter free Ca^{2+} and 0.2 mmol/liter free Mg^{2+} concentration, 1 mmol/liter ATP and pH 7. However, when the volume of the vesicles is calculated from the different diameters, the different Ca^{2+} storage capacities per mg protein

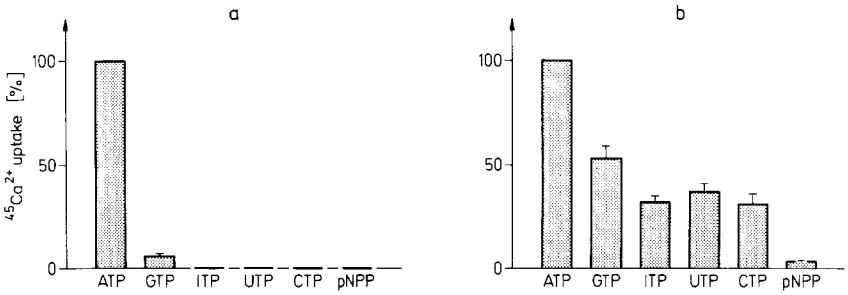


Fig. 4. Dependence of calcium uptake into plasma membranes (*a*) and rough endoplasmic reticulum (*b*) on adenosinetriphosphate (ATP), guanosinetriphosphate (GTP), inosinetriphosphate (ITP), uridinetriphosphate (UTP), cytidinetriphosphate (CTP) or *p*-nitrophenylphosphate (*p*NPP). The final substrate concentration was 1 mmol/liter. Substrate stocks were adjusted to pH 7 with Tris. The columns represent means ± SE of three preparations. Uptake is expressed in % of the uptake in the presence of ATP. 100% were equivalent to a specific uptake of 6.0 ± 1.1 nmol/mg protein in (*a*) and 5.7 ± 0.7 nmol/mg protein in (*b*) after 20 min. The medium-free Ca^{2+} concentration was kept constant at 5 μmol/liter and was not corrected for the different substrates as their association constants for Ca^{2+} and Mg^{2+} were very similar [49]. The free Mg^{2+} concentration was kept constant at 0.3 mmol/liter

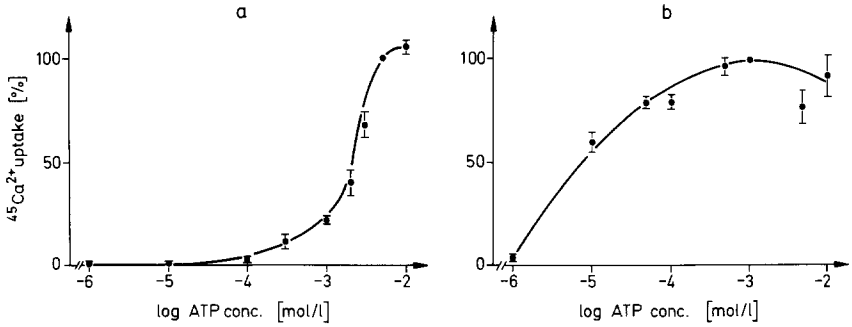


Fig. 5. Dependence of calcium uptake into plasma membranes (*a*) and rough endoplasmic reticulum (*b*) on the ATP concentration of the incubation medium. The values represent means ± SE of five preparations. Calcium uptake is expressed in % of the highest uptake; 100% were equivalent to a specific uptake of 6.7 ± 1.4 nmol/mg protein in (*a*) and 4.2 ± 0.4 nmol/mg protein in (*b*) after 20 min. The medium-free calcium concentration was kept constant at 3 μmol/liter with EDTA and ATP using the association constants with Ca^{2+} from reference [49]. The free Mg^{2+} concentration was kept constant at 0.3 mmol/liter. The incubation medium contained additionally 5 mmol/liter creatine phosphate and 5 U/ml creatine kinase. The Ca^{2+} concentrations were exclusively adjusted with $^{45}CaCl_2$ and checked with a Ca^{2+} specific electrode for each tracer batch as described [55]. All experiments were performed with the same solutions which were stored at −25°C between the experiments

in both preparations could be fully explained by the greater vesicle volume of plasma membranes.

Dependence of Ca^{2+} Uptake on ATP and Other Substrates

Calcium uptake into plasma membranes was dependent on ATP under the employed conditions, i.e. KCl at both sides of the membrane. The specificity for ATP at a concentration of 1 mmol/liter was very high. No uptake was observed with ITP, UTP, CTP and *p*NPP at this concentration (Fig. 4*a*). In the presence of 1 mmol/liter GTP little calcium uptake of about 6.0% as compared with ATP was observed, which could be fully explained by a little cross contamination of plasma membranes with rough endoplasmic reticulum. Also when the substrate concentration was raised to 5 mmol/liter the specificity for ATP remained unaltered (*data not shown*). In the rough endoplasmic reticulum (Fig. 4*b*) calcium uptake was maximal with ATP and decreased in the sequence GTP < UTP < ITP < CTP. No uptake was measured with *p*NPP.

The dependence of calcium uptake into plasma membranes on the ATP concentration is shown in Fig. 5*a*. Ca^{2+} uptake became measurable at about 0.1 mmol/liter ATP and steeply increased at about 1 mmol/liter ATP to reach a plateau at about 5 mmol/liter ATP. Half-maximal Ca^{2+} uptake was observed at about 2 mmol/liter ATP. Figure 5*b* shows the dependence of calcium uptake on the ATP concentration for the rough endoplasmic reticulum. Measurable calcium uptake was observed at about 1

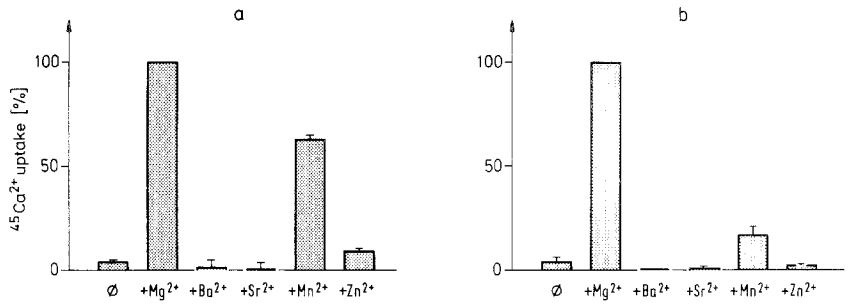


Fig. 6. Dependence of calcium uptake into plasma membranes (*a*) and rough endoplasmic reticulum (*b*) on various divalent cations. Mg^{2+} was replaced by either no divalent cation, Ba^{2+}, Sr^{2+}, Mn^{2+} or Zn^{2+}. The columns represent means ± SE of five preparations (three preparations for Ba^{2+} and Sr^{2+}), calcium uptake is expressed in % of the uptake in the presence of Mg^{2+}. 100% were equivalent to a specific uptake of 5.9 ± 1.2 nmol/mg protein in (*a*) and 6.1 ± 0.9 nmol/mg protein in (*b*) after 20 min. The medium-free Ca^{2+} concentration was kept constant at 5 μmol/liter and the free concentration of the divalent cations at 0.3 mmol/liter using the association constants with ATP from reference [49]. The differences in the relative calcium uptake between (*a*) and (*b*) for Mn^{2+} and Zn^{2+} were highly significant with $P < 0.001$, as calculated by *t*-statistics for unpaired values

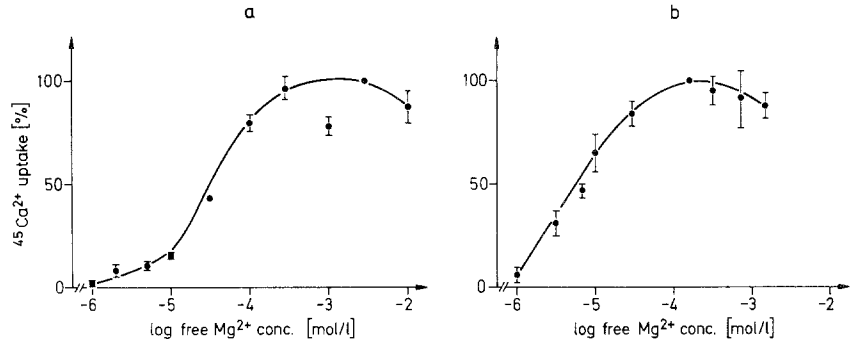


Fig. 7. Dependence of calcium uptake into plasma membranes (*a*) and rough endoplasmic reticulum (*b*) on the free Mg^{2+} concentration. The values represent means ± SE of four preparations. Uptake is expressed in % of the highest uptake. 100% were equivalent to a specific uptake of 6.8 ± 0.8 nmol/mg protein in (*a*) and 4.4 ± 0.4 nmol/mg protein in (*b*) after 20 min. The free Ca^{2+} concentration was kept constant at 3 μmol/liter. The desired free Mg^{2+} concentrations were buffered with EDTA using the association constants from reference [49]. The Ca^{2+} concentrations were exclusively adjusted with $^{45}CaCl_2$ and were checked with a Ca^{2+}-specific electrode for each tracer batch as described [55]. All experiments were performed with the same solutions which were stored at −25°C between the experiments

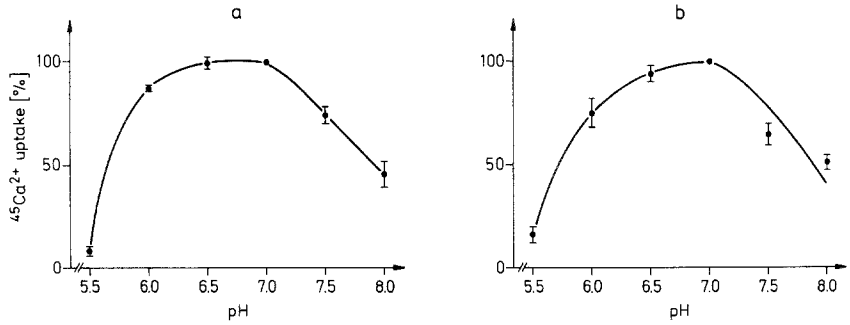


Fig. 8. Dependence of calcium uptake into plasma membranes (*a*) and rough endoplasmic reticulum (*b*) on the pH of the incubation medium. The values are means ± SE of four preparations. Calcium uptake is expressed in % of the highest uptake. 100% were equivalent to a specific uptake of 6.8 ± 1.0 nmol/mg protein in (*a*) and 7.1 ± 0.5 nmol/mg protein in (*b*). The free Ca^{2+} concentration was kept constant at 10 μmol/liter and the free Mg^{2+} concentration at 0.3 mmol/liter using the association constants with ATP [49] and their dependence on the pH. All uptake measurements were performed with the same solutions which were stored at −25°C. The Ca^{2+} concentrations were exclusively adjusted with $^{45}CaCl_2$. The $^{45}CaCl_2$ stock had been checked before by a Ca^{2+}-specific electrode as described [55]

μmol/liter ATP and was optimal at about 1 mmol/liter ATP. Half-maximal uptake was found at 10 μmol/liter ATP. Calcium uptake slightly decreased at higher ATP concentrations.

Dependence of Ca^{2+} Uptake on Mg^{2+} and Other Divalent Cations

Calcium uptake into plasma membranes was highest in the presence of Mg^{2+}. Without Mg^{2+} no calcium uptake was observed (Fig. 6*a*). Mg^{2+} could partially be substituted by Mn^{2+} and Zn^{2+}. Relative calcium uptake was 63.0 ± 2.1% and 10.7 ± 1.6%, respectively, as compared with Mg^{2+}. Ba^{2+} and Sr^{2+} could not replace Mg^{2+} for calcium uptake (Fig. 6*a*). Calcium uptake into rough endoplasmic reticulum was also highest in the presence of Mg^{2+} (Fig. 6*b*). Without divalent cations no Ca^{2+} uptake was measured. Mn^{2+} could replace Mg^{2+} to a small extent, i.e. 20.3 ± 4.1% as compared with Mg^{2+} (Fig. 6*b*). No Ca^{2+} uptake was observed when Mg^{2+} was replaced by either Zn^{2+}, Ba^{2+}, or Sr^{2+} (Fig. 6*b*). The differences between Ca^{2+} uptake into vesicles from plasma membrane and rough endoplasmic reticulum in the presence of Mn^{2+} and Zn^{2+} were statistically significant ($P < 0.001$ for both).

The dependence of calcium uptake into plasma membranes on the free Mg^{2+} concentration is shown in Fig. 7*a*. Measurable Ca^{2+} uptake slightly increased between 1 and 10 μmol/liter free Mg^{2+} and steeply increased between 10 and 100 μmol/liter free Mg^{2+} to reach a plateau between 0.3 and 10 mmol/liter free Mg^{2+}. Ca^{2+} uptake was maximal at 3 mmol/liter and half-maximal at 30 μmol/liter free Mg^{2+} concentration. Figure 7*b* shows the dependence of calcium uptake into rough endoplasmic reticulum on the free Mg^{2+} concentration. Measurable Ca^{2+} uptake was observed at about 1 μmol/liter free Mg^{2+} and was maximal at 0.2 mmol/liter free Mg^{2+} concentration. Half-maximal Ca^{2+} uptake was measured at about 8 μmol/liter free Mg^{2+}. Ca^{2+} uptake decreased at higher free Mg^{2+} concentrations. The free Mg^{2+} and Ca^{2+} concentrations were buffered with EDTA and the free Ca^{2+} concentration was kept at 3 μmol/liter.

pH Dependence of Ca^{2+} Uptake

Ca^{2+} uptake into plasma membranes was dependent on the pH of the incubation medium. The pH was optimal between 6.5 and 7.0 (Fig. 8*a*). A very similar pH dependence curve for Ca^{2+} uptake was obtained for the rough endoplasmic reticulum which had its pH optimum also between 6.5 and 7.0 (Fig. 8*b*).

Table 2. Dependence of calcium uptake into plasma membranes and rough endoplasmic reticulum on monovalent cations[a]

	Plasma membranes	Rough endoplasmic reticulum
KCl	100 (7)	100 (4)
RbCl	79.1 ± 9.2 (5)	103.2 ± 5.1 (4)
NaCl	67.5 ± 7.9 (6)	95.0 ± 4.6 (4)
LiCl	17.6 ± 3.8 (4)	45.5 ± 2.8 (4)
Choline Cl	15.2 ± 3.8 (6)	7.2 ± 3.0 (4)

[a] 130 mmol/liter K^+ were isosmotically replaced by either Rb^+, Na^+, Li^+ or choline$^+$ in a Cl^- medium. The values represent means ± SE. The number of preparations is given in parentheses. Calcium uptake after 20 min in the presence of K^+ was set as 100% and was equivalent to a specific uptake of 7.4 ± 0.6 nmol/mg protein for plasma membranes and 4.2 ± 0.8 nmol/mg protein for rough endoplasmic reticulum. The free Ca^{2+} concentration was kept constant at 3 μmol/liter and the free Mg^{2+} concentration at 0.3 mmol/liter as described.

Dependence of Ca^{2+} Uptake on Monovalent Cations and Different Anions

To gain further insight into the mechanism and the characteristics of calcium transport of plasma membranes the influence of monovalent cations and different permeable and impermeable anions was investigated. Ca^{2+} uptake was maximal in the presence of K^+. When K^+ was isosmotically replaced by either Rb^+, Na^+, Li^+ or choline$^+$, Ca^{2+} uptake decreased in the sequence $Rb^+ < Na^+ < Li^+ <$ choline$^+$ (Table 2). The sequence for the monovalent cation dependence of Ca^{2+} uptake in this study is similar as it has been described for the plasma membrane permeabilities for these cations from electrophysiological studies [31, 40].

When Cl^- was isosmotically replaced by either Br^-, I^-, SCN^-, NO_3^-, isethionate$^-$, gluconate$^-$, cyclamate$^-$, SO_4^{2-} or glutarate^{2-}, Ca^{2+} uptake into plasma membrane vesicles was not significantly changed in the presence of Br^- or I^- (Table 3). Other monovalent anions decreased Ca^{2+} uptake in the sequence $SCN^- < NO_3^- <$ isethionate$^-$ < cyclamate$^-$ < gluconate$^-$ (Table 3). Lowest Ca^{2+} uptake into plasma membranes was found in the presence of the divalent anions SO_4^{2-} and glutarate^{2-} (Table 3). The anion dependence of Ca^{2+} uptake into plasma membrane vesicles exhibits characteristics of a lyophilic anion series with some specificity for halides as Cl^-, Br^- and I^-.

The monovalent cation and the anion dependence of calcium transport into rough endoplasmic reticulum have been described in detail previously [3], and as shown in Tables 2 and 3 were different from those of the plasma membrane. The same cation and anion sequences were found for Ca^{2+} up-

Table 3. Dependence of calcium uptake into plasma membranes and rough endoplasmic reticulum on different permeable and impermeable anions[a]

	Plasma membranes	Rough endoplasmic reticulum
KCL	100 (7)	100 (4)
KBr	105.7 ± 2.5 (4)	71.1 ± 6.0 (4)
KI	98.1 ± 14.3 (4)	30.9 ± 5.0 (4)
KSCN	85.0 ± 7.0 (5)	20.3 ± 2.0 (4)
KNO_3	68.6 ± 7.2 (4)	43.7 ± 4.6 (4)
K-gluconate	46.4 ± 14.0 (4)	55.2 ± 8.9 (4)
K-cyclamate	53.3 ± 13.4 (4)	22.2 ± 1.8 (4)
K-isethionate	60.2 (2)	—
K_2SO_4	40.3 ± 4.3 (7)	50.8 ± 9.2 (4)
K_2-glutarate	39.0 (2)	—

[a] 130 mmol/liter Cl^- were isosmotically replaced by either Br^-, I^-, SCN^-, NO_3^-, gluconate$^-$, cyclamate$^-$, or isethionate$^-$. In the case of divalent anions 130 mmol/liter KCl were isosmotically replaced by either 65 mmol/liter K_2SO_4, or K_2glutarate + 65 mmol/liter mannitol. The values represent means ± SE. The number of preparations is given in parentheses. Calcium uptake after 20 min in the presence of Cl^- was set as 100% and was equivalent to a specific uptake of 7.2 ± 0.8 nmol/mg protein for plasma membranes and 4.2 ± 0.8 nmol/mg protein for rough endoplasmic reticulum. The free Ca^{2+} concentration was kept at 3 μmol/liter and the free Mg^{2+} concentration at 0.3 mmol/liter as described.

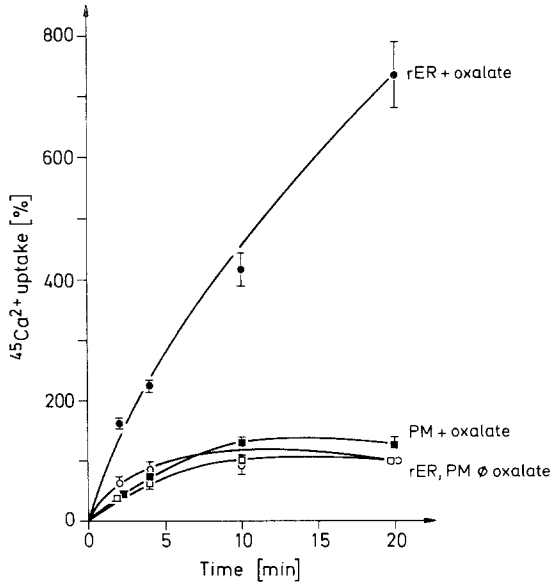


Fig. 9. Effect of oxalate on Ca^{2+} uptake into plasma membranes and rough endoplasmic reticulum. 30 mmol/liter KCl were isosmotically replaced by 20 mmol/liter K_2oxalate. The points represent means ± SE of three preparations. Calcium uptake into plasma membranes or rough endoplasmic reticulum after 20 min in the absence of oxalate was set as 100%. 100% were equivalent to a specific Ca^{2+} uptake of 2.2 ± 0.4 nmol/mg protein for plasma membranes and 2.3 ± 0.2 nmol/mg protein for rough endoplasmic reticulum. The free Ca^{2+} concentration was kept constant at 0.5 μmol/liter and the free Mg^{2+} concentration at 0.3 mmol/liter using EDTA or oxalate with the association constants from reference [49]

take of the sarcoplasmic reticulum [10, 15, 32, 33, 57, 58] and for the endoplasmic reticulum of other tissues [21, 38, 39, 61].

Effect of Oxalate on Ca^{2+} Uptake

The stimulation of Ca^{2+} uptake into rough endoplasmic reticulum by oxalate is a characteristic feature of this structure [1, 3, 23, 38, 39, 61]. It is due to the permeability of the membrane to oxalate and the Ca^{2+}-precipitating effect of oxalate [1, 60]. Since plasma membranes are impermeable to oxalate, Ca^{2+} uptake into this structure cannot be stimulated [39, 61].

When 30 mmol/liter KCl were replaced by 20 mmol/liter K_2oxalate, Ca^{2+} uptake into rough endoplasmic reticulum was increased by 7.3-fold and Ca^{2+} uptake into plasma membranes by only 1.3-fold as compared to the control (Fig. 9). These results provide another indication for the purity of the plasma membrane fraction.

Mechanism of Calcium Transport

Additive Effect of Cation and Anion Replacement on Ca^{2+} Uptake

To obtain information about the mechanism of Ca^{2+} transport we investigated whether the effects on Ca^{2+} uptake were additive when cations and anions were replaced simultaneously. If Ca^{2+} uptake occurs electrogenically it would be determined by the membrane permeabilities for other ions present in the incubation medium which would be needed for charge compensation. With respect to the results shown in Tables 2 and 3, we tested the additive effect of K^+ replacement by Na^+ or choline$^+$ and of Cl^- replacement by SO_4^{2-}. As shown in Fig. 10, in both preparations Ca^{2+} uptake was lower when Cl^- was exchanged by SO_4^{2-}. When K^+ was replaced by choline$^+$, Ca^{2+} uptake was reduced to the level obtained in the absence of ions, i.e. in the presence of mannitol, irrespective of the presence of anions (Fig. 10).

Effects of Electrical Potential Differences on Ca^{2+} Uptake

To decide whether Ca^{2+} transport into plasma membrane vesicles was electrogenic or electroneutral we investigated the influence of diffusion potentials on ATP-promoted Ca^{2+} uptake. With regard to the cation and anion dependence of Ca^{2+} uptake (Tables 2 and 3) we chose K^+ and SCN^- as fairly permeable ions to generate a diffusion potential and Li^+ or choline$^+$ and SO_4^{2-} as relatively impermeable

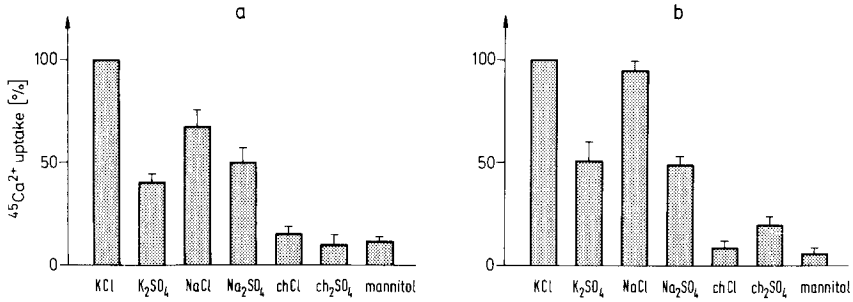


Fig. 10. Effect of simultaneous replacement of cations and anions on calcium uptake in plasma membranes (*a*) and rough endoplasmic reticulum (*b*). 130 mmol/liter KCl were replaced isosmotically by either 130 mmol/liter NaCl or cholineCl, or 65 mmol/liter K_2SO_4, Na_2SO_4 or $choline_2SO_4$. The solutions with SO_4^{2-} contained additionally 65 mmol/liter mannitol to maintain the osmotic pressure. The columns represent means ± SE of five to seven preparations. Ca^{2+} uptake after 20 min in the presence of KCl was set as 100% and was equivalent to a specific uptake of 7.2 ± 1.2 nmol/mg protein in (*a*) and 5.9 ± 0.5 nmol/mg protein in (*b*). The free Ca^{2+} concentration was kept constant at 3 μmol/liter and the free Mg^{2+} concentration at 0.3 mmol/liter as described

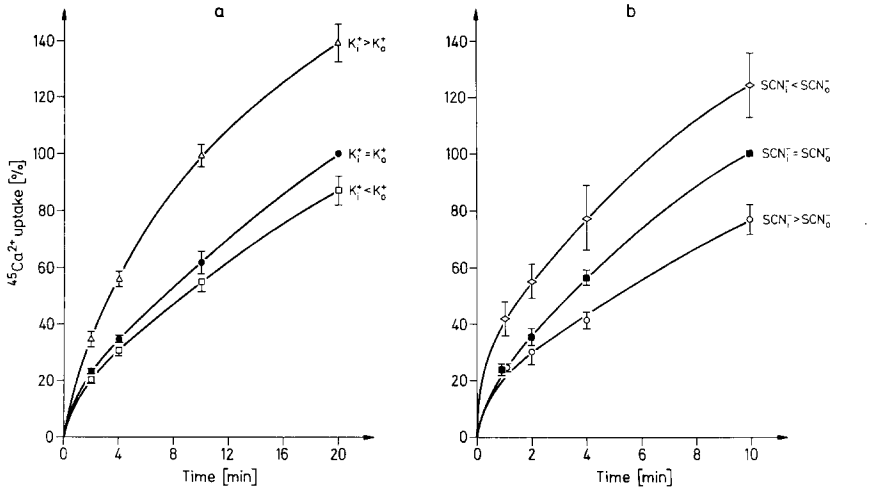


Fig. 11. Effect of electrical potential differences on Ca^{2+} uptake into plasma membranes. An electrical diffusion potential was created by either a K^+ gradient (*a*) or an SCN^- gradient (*b*) across the vesicle membrane. *a*: Vesicles were preloaded for 1 hr at 25°C with either 100 mmol/liter K_2SO_4 (△, ●) or 100 mmol/liter $choline_2SO_4$ (□) and diluted 33-fold into 100 mmol/liter K_2SO_4 (●, □) or 100 mmol/liter $choline_2SO_4$ (△). *b*: Vesicles were preloaded for 1 hr at 25°C with either 100 mmol/liter Li_2SO_4 (■, ◇) or 200 mmol/liter LiSCN (○) and diluted 33-fold into 100 mmol/liter Li_2SO_4 (■, ○) or 200 mmol/liter LiSCN (◇). The preloading media contained additionally 100 mmol/liter mannitol when SO_4^{2-} was present, 30 mmol/liter HEPES, pH 7 with Tris, and 2.6 mmol/liter Mg-gluconate. The uptake media contained also 100 mmol/liter mannitol when SO_4^{2-} was present and additionally in mmol/liter: 30 HEPES, pH 7 with Tris, 2.6 Mg-gluconate, 3 Tris-ATP, and 0.016 $^{45}CaCl_2$. The free Ca^{2+} and Mg^{2+} concentrations were 3 μmol/liter and 0.3 mmol/liter, respectively, and calculated as described. The points represent means ± SE of four (*a*) or three (*b*) preparations. Ca^{2+} uptake is expressed in %, Ca^{2+} uptake after 20 min (*a*), or 10 min (*b*), without any diffusion potential was set as 100%, and was equivalent to a specific uptake of 3.9 ± 0.7 nmol/mg protein in (*a*) and 2.7 ± 0.6 nmol/mg protein in (*b*)

ions. K_2SO_4 or Li_2SO_4 preloaded vesicles were diluted into a $choline_2SO_4$ or LiSCN medium, respectively, to create an inside-negative diffusion potential. $Choline_2SO_4$ or LiSCN preloaded vesicles were diluted into a K_2SO_4 or Li_2SO_4 medium, respectively, to create an inside-positive diffusion potential. As shown in Fig. 11*a* Ca^{2+} uptake was stimulated about 39% by an outward-directed K^+ gradient creating an inside-negative K^+ diffusion potential and was inhibited about 13% with an inward-directed K^+ gradient. These effects could not be further enhanced by addition of 2×10^{-6} mol/liter valinomycin (*data not shown*). This indicates that the plasma membranes were highly permeable for K^+. In the presence of an inward-directed SCN^- gradient creating an inside-negative diffusion potential Ca^{2+} uptake was stimulated about 24% and was inhibited about 23% with an outward-directed SCN^- gradient (Fig. 11*b*).

Discussion

We have recently demonstrated ATP-dependent Ca^{2+} uptake into rough endoplasmic reticulum of cells with permeabilized plasma membranes [3, 60]

and into isolated purified rough endoplasmic reticulum vesicles [3]. Recently, we have also shown that the rough endoplasmic reticulum is able to regulate the cytosolic-free Ca^{2+} concentration to 4×10^{-7} mol/liter [3, 55]. Since in intact cells plasma membrane-bound Ca^{2+} transport mechanisms should determine the steady-state Ca^{2+} concentration at rest and at stimulation, it would be important to get insight on how the plasma membrane can influence the intracellular-free Ca^{2+} concentration.

However, lacking successful fractionation methods, previous investigators were not able to differentiate between Ca^{2+} transport located either in the plasma membrane or in the rough endoplasmic reticulum. We now report Ca^{2+} transport into purified plasma membrane vesicles which gives clear evidence for another Ca^{2+} transport system, with characteristics different from those of the rough endoplasmic reticulum.

Evidence for the existence of two different Ca^{2+} transport systems in one cell type which are located in the plasma membrane and in the rough endoplasmic reticulum, respectively, has been presented for the skeletal muscle, heart muscle, smooth muscle, liver, kidney, in islet cells, adipocytes, axons and others [5, 6, 11, 15, 20, 28, 38, 39, 61].

Preparation of Plasma Membranes

A new approach to purify plasma membranes has been used in this study. Precipitation with $MgCl_2$ has so far been applied for the preparation of brush-border membranes from kidney proximal tubule [4] and small intestine [17]. In this study $MgCl_2$ precipitation was used to prepare a plasma membrane fraction containing mainly basolateral plasma membranes. The typical markers for this structure (Na^+,K^+)-ATPase, basal- and hormone-stimulated adenylate cyclase were enriched by 43-, 45-, and 42-fold, respectively, compared to the starting material. These enrichment factors indicate a purity greater than described so far for plasma membranes of pancreatic acinar cells [29, 35, 36, 56].

Electron-microscopical investigation (Fig. 2*a*) showed that 80 to 90% of the membranes in the final fraction were smooth. Rat pancreatic acinar cells contain only a negligible amount of intracellular smooth membranes which cannot be differentiated by electron microscopy of cell membranes [60]. Together with the activity of the marker enzyme for the smooth endoplasmic reticulum, NADPH cytochrome *c* reductase which was decreased by sixfold in the final plasma membrane pellet, this suggests that nearly all smooth membranes in the final fraction derived from the plasma membrane. From the enrichment and the percentage of smooth membranes in the final fraction we can estimate that the plasma membrane accounts for about 2% of the cell protein.

Since the purity of the plasma membrane fraction itself does not guarantee functional and morphological viability, we used as criteria for biological activity the response of the adenylate cyclase activity to hormone stimulation. The basal adenylate cyclase activity in the homogenate was stimulated by 5.4-fold and in the purified plasma membrane fraction by 5.2-fold with 10^{-7} mol/liter CCK-OP. This indicates that the plasma membranes were functionally intact after collagenase digestion and that they remained intact during the whole isolation procedure. As another criteria for intactness, the plasma membrane vesicles were capable of maintaining ion gradients for more than 20 min (Fig. 11).

Thus the quality and the purity of this preparation allowed us to characterize the plasma membrane-located Ca^{2+} transport system. Further we could clearly differentiate this Ca^{2+} transport system in the plasma membrane from that located in the rough endoplasmic reticulum.

Characterization of Ca^{2+} Transport

Since the affinity of the transport system for Ca^{2+} is important for the regulation of the cytosolic Ca^{2+} level we have studied the dependence of Ca^{2+} transport into plasma membranes on the free Ca^{2+} concentration. The characteristics were compared with those of the rough endoplasmic reticulum. Ca^{2+} uptake into plasma membranes significantly increased between 10^{-7} and 3×10^{-6} mol/liter free Ca^{2+} to reach a plateau between 3×10^{-6} and 3×10^{-5} mol/liter free Ca^{2+} concentration. Half-maximal and maximal Ca^{2+} uptake were observed at 8.8×10^{-7} and 10^{-5} mol/liter free Ca^{2+}, respectively (Fig. 3, curve *a*), and were similar as described previously [29]. In contrast, the dependence of Ca^{2+} uptake into rough endoplasmic reticulum on the free Ca^{2+} concentration showed a different pattern (Fig. 3, curve *b*). Above 10^{-7} mol/liter free Ca^{2+} uptake into rough endoplasmic reticulum increased steeper to be half-maximal at 5.4×10^{-7} mol/liter free Ca^{2+} and to reach the maximum earlier than the plasma membrane at 2×10^{-6} mol/liter free Ca^{2+}. The values for half-maximal Ca^{2+} uptake of both structures were not significantly different, i.e. 8.8×10^{-7} and 5.4×10^{-7} mol/liter free Ca^{2+}. In cells, whose plasma membrane had been permeabilized to ATP and Ca^{2+} by washing the cells in a nominally Ca^{2+}-free solution, the rough endoplasmic reticulum has been shown to regulate the free Ca^{2+} concentration to 4×10^{-7} in the incubation medium, that could be

regarded as "extended cytosol" [3, 55]. It is likely that the plasma membrane regulates to the same Ca^{2+} concentration or even to a lower level. If the plasma membrane were not able to keep the cytosolic Ca^{2+} concentration at 4×10^{-7} mol/liter or lower, permanent Ca^{2+} influx should exceed the storage capacity of the rough endoplasmic reticulum.

At concentrations higher than 2×10^{-6} mol/liter free Ca^{2+}, uptake into the rough endoplasmic reticulum decreased strongly to have a minimum at 10^{-5} mol/liter free Ca^{2+}, the concentration at which the plasma membrane showed maximal Ca^{2+} uptake (Fig. 3). We have described this phenomenon that Ca^{2+} uptake into rough endoplasmic reticulum is inhibited at higher concentrations before [3] and it seems to be a characteristic feature of this structure. Regarding the regulation of the cytosolic Ca^{2+} level as a possible physiological function of the rough endoplasmic in pancreatic acinar cells, such a characteristic appears to be reasonable. During stimulation of enzyme secretion, increased cytosolic Ca^{2+} concentration [14, 24, 42, 45, 46, 53, 54] would suppress the calcium-sequestering activity of the rough endoplasmic reticulum and thus the cytosolic Ca^{2+} level could stay high.

As shown in Figs. 5 and 7 Ca^{2+} uptake into plasma membranes and rough endoplasmic reticulum was dependent on ATP and Mg^{2+}. This agrees with the results of other tissues and suggests the involvement of a Ca^{2+} + Mg^{2+}-dependent ATPase [30]. The ATP and Mg^{2+} concentrations for half-maximal and maximal Ca^{2+} uptake were significantly different for plasma membranes and rough endoplasmic reticulum (Figs. 5 and 7). The similarity between the ATP- and Mg^{2+}-dependence curves of the rough endoplasmic reticulum suggests that MgATP is used as substrate for Ca^{2+} transport [25], whereas the high K_m value of ATP for Ca^{2+} transport in the plasma membrane does not suggest this. Further it was observed that ATP-promoted Ca^{2+} uptake could not be facilitated by other substrates for the plasma membrane [29], but by several other substrates in the rough endoplasmic reticulum (Fig. 4). Both structures also behaved differently when Mg^{2+} was replaced by other divalent cations. Whereas considerable Ca^{2+} transport into plasma membranes was measured with Mn^{2+} and little uptake with Zn^{2+}, the rough endoplasmic reticulum showed significantly less uptake with Mn^{2+} and none with Zn^{2+} (Fig. 6). Mn^{2+} could partially replace Mg^{2+} in ATP-dependent Ca^{2+} transport in the sarcoplasmic reticulum [9]. This is used as an indirect evidence that Mg^{2+} is not countertransported for Ca^{2+}. The pH dependence curves of Ca^{2+} uptake were very similar for both structures (Fig. 8). This seems reasonable since both structures are in contact with the same cytosol, the optimal pH values found, agree with intracellular pH measurements in pancreatic acinar cells [19].

In order to obtain further information about the Ca^{2+} transport mechanisms in both plasma membrane and rough endoplasmic reticulum we have investigated the influence of different cations and anions on Ca^{2+} uptake. If we assume that Ca^{2+} transport occurs electrogenically, charges moved by Ca^{2+} would raise an electrical potential across the membrane. Then Ca^{2+} uptake could be enhanced by compensating the positive charge of Ca^{2+} by either countermovement of a cation or movement of an anion into the same direction. This agrees with the finding that the resting plasma membrane is more permeable for K^+ than Na^+ [31, 40]. In the presence of K^+, which is the best permeable cation, the highest Ca^{2+} uptake was observed. Addition of valinomycin, which increases the membrane permeability for K^+ did not further increase Ca^{2+} uptake suggesting a high permeability of our plasma membrane preparation for K^+. Correspondingly in the presence of the less-permeable Na^+ cation, Ca^{2+} uptake was lower and it was lowest with the quite impermeable cations Li^+ and choline$^+$ (Table 2). If the hypothesis of an electrogenic Ca^{2+} transport mechanism were right, we would also expect a stimulatory effect of permeable anions, and moreover additive effect of simultaneous cation and anion replacement.

The anion sequence for stimulating Ca^{2+} uptake into plasma membranes cannot be explained as easily as for the monovalent cations. The results also suggest some specificity for halides (Table 3). However, in the presence of the less-permeable anions SO_4^{2-} and glutarate^{2-}, the lowest Ca^{2+} uptake was measured (Table 3). Moreover, when Cl^- was replaced by SO_4^{2-} or glutarate^{2-} in the presence of different monovalent cations it was found that the inhibitory effects on Ca^{2+} uptake were additive (Fig. 10). To obtain further information about the Ca^{2+} transport mechanism in the plasma membrane we have investigated the influence of a transmembranal diffusion potential on Ca^{2+} uptake. We choose K^+ or SCN^- as permeable ions together with SO_4^{2-} or Li^+ to create a diffusion potential. ATP-dependent Ca^{2+} uptake was enhanced by an inside-negative diffusion potential created by an outward-directed K^+ gradient as well as by an inward-directed SCN^- gradient (Fig. 11). An inside-positive diffusion potential created by an inward-directed K^+ gradient inhibited Ca^{2+} uptake (Fig. 11). Since the plasma membrane vesicles were highly permeable for K^+, this result makes the existence of an electroneutral Ca^{2+}/K^+ exchange sys-

tem very unlikely which could mimic the effect of an inside-negative K^+ diffusion potential created by an outward-directed K^+ gradient. Ca^{2+} uptake into plasma membrane vesicles was also inhibited by an inside-positive diffusion potential created by an outward-directed SCN^- gradient (Fig. 11) which again makes unlikely an electroneutral Ca^{2+}-SCN^- cotransport and supports an electrogenic Ca^{2+} transport mechanism. However, an electrogenic Ca^{2+}/K^+ exchange mechanism or an electrogenic Ca^{2+}-anion cotransport mechanism could not be excluded by these experiments. Ca^{2+} uptake was not affected by proton gradients across the membrane and also the protonophore CFCCP had no effect on ATP-dependent Ca^{2+} transport under proton gradient conditions. These data therefore do not favor a Ca^{2+}/H^+ exchange mechanism as described for the sarcoplasmic reticulum, the liver plasma membrane and the erythrocyte [7, 21, 28, 50].

For the rough endoplasmic reticulum we failed to elucidate the Ca^{2+} transport mechanism by using the same experimental conditions. A transmembranal K^+, Na^+, H^+ and Cl^- gradient did not affect Ca^{2+} uptake. This suggests that the rough endoplasmic reticulum is very permeable for these ions and the transport mechanism is hard to investigate by these methods. Further preliminary experiments showed that the ATPase activity in Triton X-solubilized vesicles of rough endoplasmic reticulum was dependent on monovalent cations and anions in similar sequence as Ca^{2+} uptake into vesicles of this structure. This could suggest a direct effect of cations and anions on the Ca^{2+} transport in rough endoplasmic reticulum, such as coupling of these ions to the transport system, or direct effects on the Ca^{2+}-ATPase intermediate.

Conclusion

By using a purified plasma membrane fraction of pancreatic acinar cells with negligible contamination by rough endoplasmic reticulum, Ca^{2+} uptake into this structure could be demonstrated. The characteristics of Ca^{2+} uptake into this highly purified plasma membrane fraction could be clearly differentiated from those of Ca^{2+} uptake into rough endoplasmic reticulum of these cells [3]. Ca^{2+} transport across the plasma membrane occurs electrogenically. It is suggested that this Ca^{2+} transport system is involved in the regulation of the cytosolic-free Ca^{2+} level together with the previously investigated rough endoplasmic reticulum [3, 55].

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References

1. Agostini, B., Hasselbach, W. 1971. Electron cytochemistry of calcium uptake in the fragmented sarcoplasmic reticulum. *Histochemistry* **28**:55–67
2. Amsterdam, A., Jamieson, J.D. 1972. Structural and functional characterization of isolated pancreatic exocrine cells. *Proc. Natl. Acad. Sci. USA* **69**:3028–3032
3. Bayerdörffer, E., Streb, H., Eckhardt, L., Haase, W., Schulz, I. 1984. Characterization of calcium uptake into rough endoplasmic reticulum of rat pancreas. *J. Membrane Biol.* **81**:69–82
4. Booth, A.G., Kenny, A.J. 1974. A rapid method for the preparation of microvilli from rabbit kidney. *Biochem. J.* **142**:575–581
5. Bruns, D.E., McDonald, J.M., Jarett, L. 1976. Energy-dependent calcium transport in endoplasmic reticulum of adipocytes. *J. Biol. Chem.* **251**:7191–7197
6. Carafoli, E., Zurini, M. 1982. The Ca^{2+}-pumping ATPase of plasma membranes. *Biochim. Biophys. Acta* **683**:279–301
7. Carafoli, E., Zurini, M., Niggli, V., Krebs, J. 1982. The calcium-transporting ATPase of erythrocytes. *Ann. N.Y. Acad. Sci.* **402**:304–326
8. Case, R.M., Clausen, T. 1973. The relationship between calcium exchange and enzyme secretion in the isolated rat pancreas. *J. Physiol. (London)* **235**:75–102
9. Chiesi, M., Inesi, G. 1980. Adenosine 5-triphosphate-dependent fluxes of manganese and hydrogen ions in sarcoplasmic reticulum vesicles. *Biochemistry* **19**:2912–2918
10. Chu, A., Tate, C.A., Bick, R.J., Van Winkle, W.B., Entman, M.L. 1983. Anion effects on in vitro sarcoplasmic reticulum function. *J. Biol. Chem.* **258**:1656–1664
11. Colca, J.R., McDonald, J.M., Kotagal, N., Patke, Ch., Fink, C.J., Greider, M.H., Lacy, P.E., McDaniel, M.L. 1982. Active calcium uptake by islet-cell endoplasmic reticulum. *J. Biol. Chem.* **257**:7223–7228
12. Dormer, R.L., Williams, J.A. 1981. Secretagogue-induced changes in subcellular Ca^{2+} distribution in isolated pancreatic acini. *Am. J. Physiol.* **240**:G130–G140
13. Eimerl, S., Savion, N., Heichal, O., Selinger, Z. 1974. Induction of enzyme secretion in rat pancreatic slices using the ionophore A-23187 and calcium. *J. Biol. Chem.* **249**:3991–3993
14. Gardner, J.D., Conlon, T.P., Klaeveman, H.L., Adams, T.D., Ondetti, M.A. 1975. Action of cholecystokinin and cholinergic agents on calcium transport in isolated pancreatic acinar cells. *J. Clin. Invest.* **56**:366–375
15. Hasselbach, W. 1974. Sarcoplasmic ATPase. In: The Enzymes. P.D. Boyer, editor. Vol. 10, pp. 431–467. Academic, New York
16. Hatcher, D.W., Goldstein, G. 1969. Improved methods for determination of RNA and DNA. *Anal. Biochem.* **31**:42–50
17. Hauser, H., Howell, K., Dawson, R.M.C., Bowyer, D.E. 1980. Rabbit small intestine brush border membrane preparation and lipid composition. *Biochim. Biophys. Acta* **602**:567–577
18. Heisler, S., Fast, D., Tenenhouse, A. 1972. Role of calcium and cyclic AMP in protein secretion from rat exocrine pancreas. *Biochim. Biophys. Acta* **279**:561–572
19. Hellmessen, W., Christian, A.L., Fasold, H., Schulz, I. 1985. Coupled Na^+/H^+-ion exchange in isolated acinar cells from the exocrine rat pancreas. *Am. J. Physiol. (in press)*
20. Henkart, P.H., Reese, T.S., Brinkley, F.J., Jr. 1978. Endoplasmic reticulum sequesters calcium in the squid giant axon. *Science* **202**:88–89

21. Hill, T.L., Inesi, G. 1982. Equilibrium cooperative binding of calcium and protons by sarcoplasmic reticulum ATPase. *Proc. Natl. Acad. Sci. USA* **79:**3978–3982
22. Hokin, L.E. 1966. Effects of calcium omission on acetylcholine-stimulated amylase secretion and phospholipid synthesis in pigeon pancreas slices. *Biochim. Biophys. Acta* **115:**219–221
23. Immelmann, A., Söling, H.-D. 1983. ATP-dependent calcium sequestration and calcium/ATP stoichiometry in isolated microsomes from guinea pig parotid glands. *FEBS* **162:**406–410
24. Iwatsuki, N., Petersen, O.H. 1977. Acetylcholine-like effects of intracellular calcium application in pancreatic acinar cells. *Nature (London)* **268:**147–149
25. Kanazawa, T., Boyer, P.D. 1973. Occurrence and characteristics of a rapid exchange of phosphate oxygens catalyzed by sarcoplasmic reticulum vesicles. *J. Biol. Chem.* **248:**3163–3172
26. Kaplan, M.M. 1972. Progress in hepatology: Alkaline phosphatase. *Gastroenterology* **62:**452–468
27. Kondo, S., Schulz, I. 1976. Calcium ion uptake in isolated pancreas cells induced by secretagogues. *Biochim. Biophys. Acta* **419:**76–92
28. Kraus-Friedmann, N., Biber, J., Murer, H., Carafoli, E. 1982. Calcium uptake in isolated hepatic plasma-membrane vesicles. *Eur. J. Biochem.* **129:**7–12
29. Kribben, A., Tyrakowski, T., Schulz, I. 1983. Characterization of Mg-ATP-dependent Ca^{2+} transport in cat pancreatic microsomes. *Am. J. Physiol.* **244:**G480–G490
30. Lambert, M., Christophe, J. 1978. Characterization of (Mg,Ca)-ATPase activity in rat pancreatic plasma membranes. *Eur. J. Biochem.* **91:**485–492
31. Matthews, E.K., Petersen, O.H. 1973. Pancreatic acinar cells: Ionic dependence of the membrane potential and acetylcholine-induced depolarization. *J. Physiol. (London)* **231:**283–295
32. Meissner, G. 1981. Calcium transport and monovalent cation and proton fluxes in sarcoplasmic reticulum vesicles. *J. Biol. Chem.* **256:**636–643
33. Meissner, G., McKinley, D. 1982. Permeability of canine cardiac sarcoplasmic reticulum vesicles to K^+, Na^+, H^+ and Cl^-. *J. Biol. Chem.* **257:**7704–7711
34. Mejbaum, W. 1939. Über die Bestimmung kleiner Pentosemengen insbesondere in Derivaten der Adenylsäure. *Z. Physiol. Chem.* **258:**117–120
35. Meldolesi, J., Jamieson, J.D., Palade, G.E. 1971. Composition of cellular membranes in the pancreas of the guinea pig. I. Isolation of membrane fractions. *J. Cell Biol.* **49:**109–129
36. Milutinovic, S., Sachs, G., Haase, W., Schulz, I. 1977. Studies on isolated subcellular components of cat pancreas. I. Isolation and enzymatic characterization. *J. Membrane Biol.* **36:**253–279
37. Milutinoviç, S., Schulz, I., Rosselin, G. 1976. The interaction of secretin with pancreatic membranes. *Biochim. Biophys. Acta* **436:**113–127
38. Moore, L., Chen, T.S., Knapp, H.R., Jr., Landon, E.J. 1975. Energy-dependent calcium sequestration activity in rat liver microsomes. *J. Biol. Chem.* **250:**4562–4568
39. Moore, L., Fitzpatrick, D.F., Chen, T.S., Landon, E.J. 1974. Calcium pump activity of the renal plasma membrane and renal microsomes. *Biochim. Biophys. Acta* **345:**405–418
40. Nishiyama, A., Petersen, O.H. 1975. Pancreatic acinar cells: Ionic dependence of acetylcholine-induced membrane potential and resistance change. *J. Physiol. (London)* **244:**431–465
41. Petersen, O.H., Iwatsuki, N. 1978. The role of calcium in pancreatic acinar cell stimulus-secretion coupling: An electrophysiological approach. *Ann. N.Y. Acad. Sci.* **307:**599–617
42. Renckens, B.A.M., Schrijen, J.J., Swarts, H.G.P., De Pont, J.J.H.H.M., Bonting, S.L. 1978. Role of calcium in exocrine pancreatic secretion. IV. Calcium movements in isolated acinar cells of rabbit pancreas. *Biochim. Biophys. Acta* **544:**338–350
43. Rosenzweig, S.A., Miller, L.J., Jamieson, J.D. 1983. Identification and localization of cholecystokinin-binding sites on rat pancreatic plasma membranes and acinar cells: A biochemical and autoradiographic study. *J. Cell Biol.* **96:**1288–1297
44. Scharschmidt, B.F., Keeffe, E.B., Blankenship, N.M., Ockner, R.K., 1979. Validation of a recording spectrophotometric method for measurement of membrane-associated Mg- and NaK-ATPase activity. *J. Lab. Clin. Med.* **93:**790–799
45. Schulz, I. 1980. Messenger role of calcium in function of pancreatic acinar cells. *Am. J. Physiol.* **239:**G335–G347
46. Schulz, I., Kimura, T., Wakasugi, H., Haase, W., Kribben, A. 1981. Analysis of Ca^{2+} fluxes and Ca^{2+} pools in pancreatic acini. *Philos. Trans. R. Soc. London B* **296:**105–113
47. Schulz, I., Wakasugi, H., Stolze, H., Kribben, A., Haase, W. 1981. Analysis of Ca^{2+} fluxes and their relation to enzyme secretion in dispersed pancreatic acinar cells. *Fed. Proc.* **40:**2503–2510
48. Shields, H.M., Bair, F.A., Bates, M.L., Yedlin, S.T., Alpers, D.H. 1982. Localization of immunreactive alkaline phosphatase in the rat small intestine at the light microscopic level by immuncytochemistry. *Gastroenterology* **82:**39–45
49. Sillen, L.G. 1971. Stability Constants of Metal-Ion Complexes. (Special publication No. 25, supplement No. 1 to special publication No. 17), 2nd ed., Chemical Society, London
50. Smallwood, J.I., Waisman, D.M., Lafreniere, D., Rasmussen, H. 1983. Evidence that the erythrocyte calcium pump catalyzes a $Ca^{2+}:nH^+$ exchange. *J. Biol. Chem.* **258:**11092–11097
51. Sottocasa, G.L., Kuylenstierna, B., Ernster, L., Bergstrand, A. 1967. An electron-transport system associated with the outer membrane of liver mitochondria. *J. Cell Biol.* **32:**415–436
52. Spurr, A.R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* **26:**31–43
53. Stolze, H., Schulz, I. 1980. Effect of atropine, ouabain, antimycin A, and A23187 on "trigger Ca^{2+} pool" in exocrine pancreas. *Am. J. Physiol.* **238:**G338–G348
54. Streb, H., Irvine, R.F., Berridge, M.J., Schulz, I. 1983. Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol-1,4,5-trisphosphate. *Nature (London)* **306:**67–69
55. Streb, H., Schulz, I. 1983. Regulation of cytosolic free Ca^{2+} concentration as studied in leaky isolated acinar cells of rat pancreas. *Am. J. Physiol.* **245:**G347–G357
56. Tartakoff, A.M., Jamieson, J.D. 1974. Subcellular fractionation of the pancreas. *Methods Enzymol.* **31:**41–59
57. The, R., Hasselbach, W. 1972. The modification of the reconstituted sarcoplasmic ATPase by monovalent cations. *Eur. J. Biochem.* **30:**318–324
58. The, R., Hasselbach, W. 1975. The action of chaotropic anions on the sarcoplasmic calcium pump. *Eur. J. Biochem.* **53:**105–113

59. Udenfriend, S., Stein, S., Böhlen, P., Dairman, W., Lerngruber, W., Weigele, M. 1972. Fluorescamine: A reagent for assay of amino acids, peptides, proteins, primary amines in the picomole range. *Science* **178:**871–872
60. Wakasugi, H., Kimura, T., Haase, W., Kribben, A., Kaufmann, R., Schulz, I. 1982. Calcium uptake into acini from rat pancreas: Evidence for intracellular ATP-dependent calcium sequestration. *J. Membrane Biol.* **65:**205–220
61. Wibo, M., Morel, N., Godfraind, T. 1981. Differentiation of Ca^{2+} pumps linked to plasma membrane and endoplasmic reticulum in the microsomal fraction from intestinal smooth muscle. *Biochim. Biophys. Acta* **649:**651–660

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