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Characterization of Calcium Uptake into Rough Endoplamic Reticulum of Rat Pancreas

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Summary. ATP-dependent Ca^{2+} uptake into isolated pancreatic acinar cells with permeabilized plasma membranes, as well as into isolated endoplasmic reticulum prepared from these cells, was measured using a Ca^{2+}-specific electrode and $^{45}Ca^{2+}$. Endoplasmic reticulum was purified on an isopycnic Percoll gradient and characterized by marker enzyme distribution. When compared to the total homogenate, the typical marker for the rough endoplasmic reticulum RNA was enriched threefold and the typical marker for the plasma membrane $Na^+,K^+(Mg^{2+})$ATPase was decreased 20-fold. When different fractions of the Percoll gradient were compared, $^{45}Ca^{2+}$ uptake correlated with the RNA content and not with the $Na^+,K^+(Mg^{2+})$ATPase activity. The characteristics of nonmitochondrial Ca^{2+} uptake into leaky isolated cells and $^{45}Ca^{2+}$ uptake into isolated endoplasmic reticulum were very similar: Calcium uptake was maximal at 0.3 and 0.2 mmol/liter free Mg^{2+}, at 1 and 1 mmol/liter ATP, at pH 6.0 and 6.5, and free Ca^{2+} concentration of 2 and 2 μmol/liter, respectively. Calcium uptake decreased at higher free Ca^{2+} concentration. $^{45}Ca^{2+}$ uptake was dependent on monovalent cations ($Rb^+ > K^+ > Na^+ > Li^+ > choline^+$) and different anions ($Cl^- > Br^- > SO_4^{2-} > NO_3^- > I^- > cyclamate^- > SCN^-$) in both preparations. Twenty mmol/liter oxalate enhanced $^{45}Ca^{2+}$ uptake in permeabilized cells 10-fold and in vesicles of endoplasmic reticulum, fivefold. Calcium oxalate precipitates in the endoplasmic reticulum of both preparations could be demonstrated by electron microscopy. The nonmitochondrial Ca^{2+} pool in permeabilized cells characterized in this study has been previously shown to regulate the cytosolic free Ca^{2+} concentration to 0.4 μmol/liter. Our results provide firm evidence that the endoplasmic reticulum plays an important role in the regulation of the cytosolic free Ca^{2+} concentration in pancreatic acinar cells.

Key Words Ca^{2+} transport · Ca^{2+} pools · permeabilized cells · rough endoplasmic reticulum · pancreatic acinar cells

Introduction

Evidence suggests that the intracellular free calcium concentration is an important factor in the sequence of events leading to enzyme secretion in pancreatic acinar cells [4, 14, 15, 19, 21, 24, 28, 29, 43, 47, 49, 55]. At least three structures are considered to influence the cytosolic calcium concentration: plasma membrane [30, 43, 55], mitochondria [5, 6, 10, 12] and one or more not yet clearly identified nonmitochondrial structures [12, 33, 44, 46]. Attempts to determine these nonmitochondrial structures in leaky acinar cells [33, 57, 61], as well as in isolated microsomal fractions of pancreatic acinar cells [34, 44, 46], suggest that the endoplasmic reticulum is involved in the regulation of the cytosolic free calcium concentration. The main evidence for this conclusion was based on the presence of calcium oxalate precipitates in the rough endoplasmic reticulum [46, 61] and not on Ca^{2+} uptake studies in purified membrane fractions of the endoplasmic reticulum.

This study provides further characterization of the Ca^{2+} uptake mechanism into purified membrane vesicles of rough endoplasmic reticulum. Additionally, it compares Ca^{2+} uptake characteristics in isolated endoplasmic reticulum with those in its *in vivo* arrangement in permeabilized cells.

Materials and Methods

Materials

All reagents were of analytical grade. Percoll was obtained from Pharmacia, Uppsala, Sweden. Ethyleneglycol-bis-(β-aminoethylether)-N,N-tetraacetic acid (EGTA), ethylene-diamine-tetra-acetic acid (EDTA); nitrilo-triacetic acid (NTA), adenosine 5-triphosphate (ATP, as Mg- and K_2-salt), phosphocreatine (sodium salt), oxalic acid and collagenase (No. 2139) were from Sigma, München, W. Germany. cytochrome *c*, lactatedehydrogenase (LDH), pyruvate kinase (PK), creatine kinase (CK), phosphoenolpyruvate (PEP), NADH, RNA and trypsin inhibitor from soybean were from Boehringer, Mannheim, W. Germany. Oligomycin, antimycin A, Triton X, L-histidine, sodium dodecyl sulfate (SDS) and bovine serum albumin were from Serva, Heidelberg, W. Germany. Sodium azide and sodium dithionite were from Merck, Darmstadt, W, Germany. The calcium ionophore A23187 was obtained from Calbiochem, Giessen, W. Germany. $^{45}CaCl_2$ (4 to 50 Ci/g) and ATP_γ-^{32}P (tetra/triethylammonium salt,

1000 to 3000 Ci/mmol) were from New England Nuclear, Dreieich, W. Germany. Ca^{2+}-selective electrode membranes containing the neutral carrier N,N'-di((11-ethoxycarbonyl) undecyl)-N,N',4,5-tetramethyl-3,6-dioxaoctane amide were purchased from Glasbläserei W. Möller, Zürich, Switzerland.

Preparation of Isolated Acinar Cells and Membrane Fractions

Rat pancreatic acinar cells were prepared as described by Streb and Schulz [2, 57]. Briefly, pancreatic tissue from 4 to 6 rats was digested in a collagenase containing Krebs-Ringer's solution for approximately 75 min at 37°C. Single cells were obtained by washing the tissue with an EDTA-containing solution subsequent to the initial 15 min of collagenase digestion. To permeabilize the plasma membrane of the isolated cells, they were washed three times with a nominal calcium-free solution containing, in mmol/liter: KCl 135, HEPES 10, $MgCl_2$ 1, pH 7.4 adjusted with Tris.

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 12 to 18 ml of MB in a tight-fitting glass/Teflon potter by 50 strokes at 900 rpm. The homogenate was centrifuged for 12 min at 1,000 × *g* and the resulting pellet, containing unbroken cells, was homogenized again in 4 to 6 ml of MB by 50 strokes. It was then combined with the first supernatant and centrifuged for 15 min at 11,000 × *g* and the resulting supernatant for 15 min at 27,000 × *g*. The pellet of the last centrifugation step was taken up in 4 to 6 ml of its supernatant and 2 ml of this suspension were mixed with 11% Percoll (wt/vol) in a solution containing, in mmol/liter: 280 mannitol, 5 HEPES, 5 KCl, 0.5 $MgCl_2$, 0.5 benzamidine, pH 7.4 adjusted with Tris. The resulting density was 1.035 g/cm³. A gradient of densities from 1.020 to 1.060 g/cm³ was generated by spinning the tubes at 41,000 × *g* for 40 min in a Beckman ultracentrifuge (Model L3-50) using a 23.5° fixed-angle rotor. The centrifuge brake was not used in order to avoid any vibrations which might have destroyed the gradient. One-ml fractions were collected from the top of the gradient. Purified endoplasmic reticulum (*see* Results) was localized in fractions VI through VIII, i.e. in the densest fractions, with an average density of 1.055 g/cm³. The fractions were diluted fivefold with a slightly modified MB containing 240 mmol/liter mannitol. They were then spun down for 15 min at 45,000 × *g*. Vesicles were immediately used for the experiment.

Measurement of Calcium Uptake

Ca^{2+} Electrode Measurements

Ca^{2+}-specific macroelectrodes were built using the neutral carrier ETH 1001 in PVC and calibrated in EDTA buffered, nitrilotriacetic acid buffered and in unbuffered solutions as previously described [57]. To determine Ca^{2+} uptake, permeabilized cells (about 2 to 5 mg protein/ml) were added to 3 ml of a solution containing, in mmol/liter: 120 KCl, 25 HEPES, 6 $MgCl_2$, 5 K_2 ATP, 10 creatine phosphate, 10 U/ml creatine kinase, 0.01 antimycin A, 0.005 oligomycin, pH 7.4 adjusted with KOH. The decrease in medium-free Ca^{2+} concentration due to cellular Ca^{2+} uptake was measured at 25°C with the Ca^{2+}-specific electrode as previously described [57].

$^{45}Ca^{2+}$ Flux Measurements

Permeabilized cells (2.5 to 4.5 mg protein) were preincubated at 25°C for 15 min in 5 ml of a solution containing, in mmol/liter: 130 KCl, 25 HEPES, 5 creatine phosphate, 10 U/ml creatine kinase, 0.01 antimycin A, 0.005 oligomycin, 0.02 $CaCl_2$ and 1 μCi/ml of $^{45}CaCl_2$. Uptake was initiated by addition of MgATP to a final concentration of 5 mmol/liter (free Ca^{2+} concentration = 5 μmol/liter). At given time points, triplicate samples were filtrated rapidly through uni-pore polycarbonate membranes (pore size 3 μm, Bio-Rad, Richmond, Va.). Filters were washed with 4 ml ice-cold solution containing, in mmol/liter: 140 KCl, 10 HEPES, 1 $MgCl_2$, 0.1 $LaCl_3$, pH 7.4 adjusted with KOH. The radioactivity was quantitated using Pico-Fluor™15 (Packard Instrument Company, Illinois) in a Mark 111 Liquid Scintillation System, Model 6880 (Searle Analytic, Des Plaines, Illinois).

$^{45}Ca^{2+}$ uptake into vesicles was measured as described for cells with the following modifications: the incubation mixture contained 0.1 to 0.2 mg of protein in a total volume of 1 ml. Antimycin A and oligomycin were replaced by 5 mmol/liter NaN_3 and the ATP regenerating system was omitted. The MgATP concentration was 1 mmol/liter and the $CaCl_2$ concentration 0.01 (free Ca^{2+} concentration = 4 μmol/liter). Vesicles were filtered through cellulose nitrate filters (pore size 0.65 μm, Sartorius, Göttingen, W. Germany).

Measurement of Ca^{2+}-Stimulated Mg^{2+}-Dependent ATPase

The activity of the Ca^{2+}-stimulated Mg^{2+}-dependent ATPase was measured using 30 to 50 μg of membrane protein in 200 μl of a solution containing, in mmol/liter: 130 KCl, 25 HEPES, 5 NaN_3, 1.5 ouabain, 0.001 A23187, pH 7.4 adjusted with Tris. The samples were preincubated for 5 min at 25°C and the reaction was started by addition of ATP mixed with ^{32}P-ATP to a final concentration of 1 mmol/liter and 5 μCi/ml, respectively. The reaction was terminated after 10 min by addition of SDS to a final concentration of 1.2%. The extraction of inorganic phosphate was performed according to the method of Seals et al. [52]. Radioactivity was quantitated as described above.

Protein and Enzyme Determination

For determination of cell protein, samples were precipitated in a volume of 1 ml with ice-cold 10% trichloroacetic acid and dissolved in 0.1 mol/liter NaOH and processed according to Lowry et al. [32] using bovine serum albumin as a standard.

For protein determination in isolated vesicles, the Fluorescamin method [60] was used since only small interference of Percoll with the test was observed. The protein content of the membrane fractions was corrected by using a blank Percoll gradient without vesicles.

For RNA determination, 200 μg of tissue 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 [38]. The procedure was continued by the method of Hatcher and Goldstein [18] and the RNA content was calculated using RNA standards (Boehringer).

$Na^+,K^+(Mg^{2+})$ATPase activity was determined according to the method of Scharschmidt et al. [48] with the final reaction medium modified to 2.5 mmol/liter ATP and 2 mmol/liter 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.

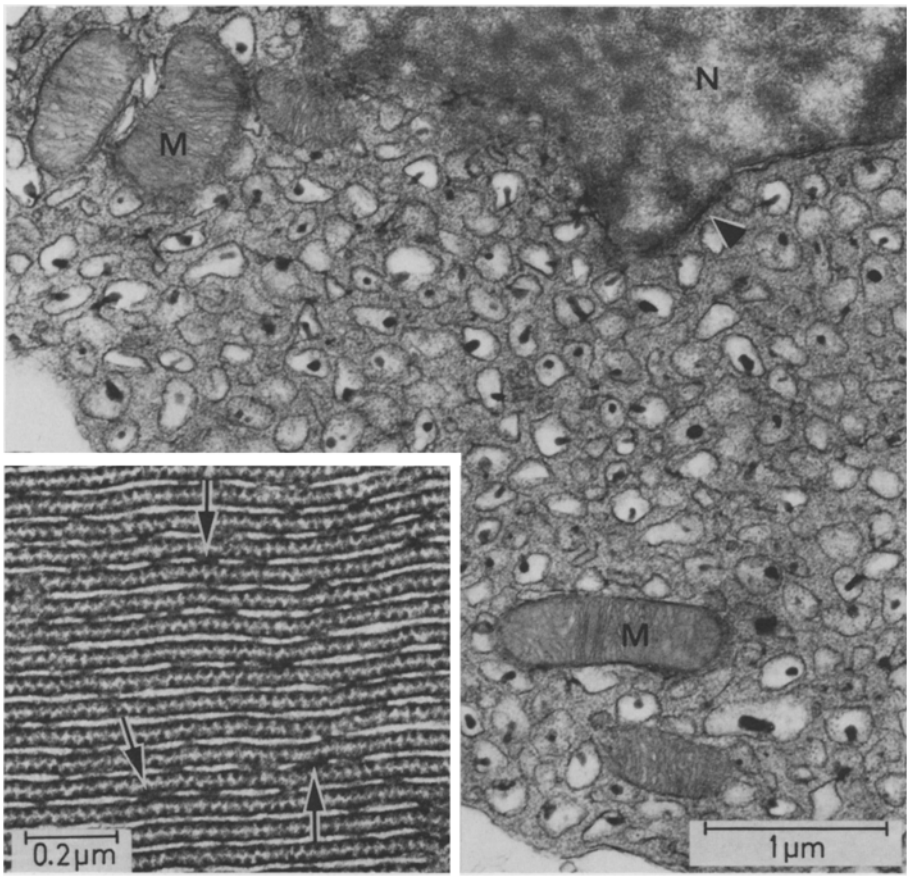

Fig. 1. Electron micrograph of isolated permeabilized acinar cells after incubation for calcium uptake in the presence of calcium, ATP and oxalate. Opaque precipitates can be seen in the vesiculated rough endoplasmic reticulum, in the envelope (arrowhead) of the nucleus (*N*), and in the mitochondria (*M*). The inset shows a part of lamellar rough endoplasmic reticulum with precipitates in the lumen (arrows)

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

NADH-cytochrome *c* reductase activity was measured by the procedure of Sottocasa et al. [53].

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.

Samples were preincubated in all enzyme determinations 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).

Electron Microscopy

For electron microscopic demonstration of calcium uptake into isolated pancreatic acinar cells and into their isolated rough endoplasmic reticulum, the oxalate precipitation method was used [1, 35]. Incubation of permeabilized cells or endoplasmic reticulum vesicles was performed in the respective media as described except that the total calcium concentration was raised to 100 µmol/liter in the vesicle experiments. Following an incubation of 20 min for cells and 60 min for vesicles, calcium uptake was stopped by addition of an equal volume of ice-cold fixative which consisted of 5% (vol/vol) glutaraldehyde (Serva, Heidelberg, W. Germany) in 0.1 mol/liter sodium cacodylate buffer, pH 7.4. The suspensions were centrifuged and parts of the pellets were enclosed in agar. Samples were postfixed with 1% (wt/vol) osmic acid in cacodylate buffer, dehydrated with alcohol and embedded in Spurr's resin [54]. All steps except the agar step were performed in the cold. Solutions were supplemented with 20 mmol/liter K_2oxalate to prevent calcium precipitates from dissolving. The embedded material was sectioned in water in the presence of oxalate. Uranylacetate and lead citrate stained or unstained sections were examined in a Philips 300 electron microscope.

Results

Localization of Calcium Transport

Morphological Studies

To localize the site of calcium uptake by electron microscopy, precipitation of calcium with oxalate was used [1, 35].

When isolated acinar cells were incubated in a medium containing calcium, ATP and oxalate, dark deposits were detected predominantly in the lumen of rough endoplasmic reticulum (Fig. 1, inset). Some precipitates were also seen in the perinuclear membrane (arrowhead) and in the mitochondria. In isolated cells the endoplasmic reticulum appeared

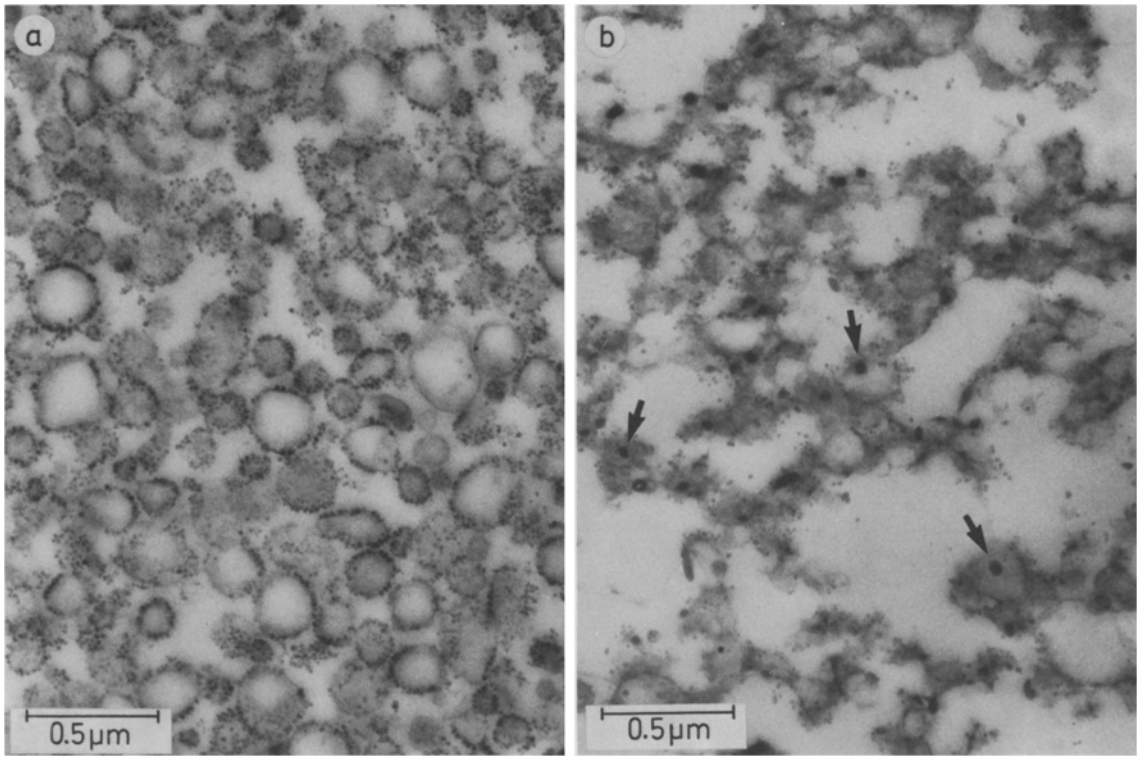

Fig. 2. (*a*) Representative micrograph of isolated endoplasmic reticulum vesicles fixed before incubation for calcium uptake. The ribosomes on the membranes indicate that the membrane vesicles originate from rough endoplasmic reticulum. (*b*) Representative micrograph of unstained rough endoplasmic reticulum vesicles after incubation for calcium uptake in the presence of calcium, ATP and oxalate. Despite the low contrast of the unstained section most precipitates can be seen to be localized inside the vesicles (arrows). During the incubation most of the membranes have lost their ribosomes. The small dots are due to Percoll particles.

in two forms. In one form the endoplasmic reticulum was organized as flat cisternae, which is the typical form in intact exocrine pancreatic tissue cells. The other form was the result of transformation of flat cisternae into vesicles. Deposits were observed in both forms of the rough endoplasmic reticulum (Fig. 1, and Fig. 1 inset). In the presence of ATP, precipitates were found in about 60 to 80% of the isolated cells. In the absence of ATP, precipitates were only seen in about 10% of the cells. Similarly, no precipitates were found in the absence of either Ca^{2+} or oxalate or in the presence of the Ca^{2+} ionophore A23187 (1 µmol/liter).

Membrane fractions VI through VIII from the Percoll gradient used for Ca^{2+} uptake studies consisted of vesicles with a diameter of 0.08 to 0.3 µm, which were almost entirely coated with ribosomes. Since neither smooth membranes nor other organelles (mitochondria or zymogen granules) could be detected, these thin-section micrographs suggest that we are dealing with a highly purified fraction of rough endoplasmic reticulum vesicles (Fig. 2*a*).

Also in these vesicles electron-dense precipitates of variable size were observed when Ca^{2+} uptake was performed in the presence of oxalate (Fig. 2*b*). Since precipitates may disappear, especially when the sections were stained, only micrographs from unstained sections are presented. The exact localization of precipitates was sometimes difficult to determine, because of the low contrast of membranes in unstained sections. However, most precipitates were clearly localized inside the vesicles.

Correlation with Marker Enzymes

To purify endoplasmic reticulum, a 27,000 × *g* pellet was used as starting material. This pellet was selected as it was already enriched in endoplasmic reticulum as judged by electron microscopy and marker enzyme distribution (Table). Further purification was achieved by isopycnic centrifugation of this pellet on a Percoll gradient. Markers of rough endoplasmic reticulum, RNA and the NADH-dependent cytochrome *c* reductase activity, were enriched by 3.1-fold and 1.7-fold, respectively, as

Table. Enzyme activities and enrichment factors of different preparation steps of rough endoplasmic reticulum vesicles[a]

		Total homogenate	27,000 × *g* (differential centrifugation)	Fractions III–IV (Percoll gradient)	Fractions VI–VIII (Percoll gradient)
$(Na^+ + K^+)$-ATPase	spec. act.	22.9 ± 1.6 (14)	8.9 ± 1.1 (14)	43.1 ± 2.1 (7)	1.4 ± 0.4 (7)
	enrichment	1	0.38	1.88	0.06
AP	spec. act.	31.6 ± 2.2 (10)	18.4 ± 0.9 (10)	109 ± 3.1 (5)	3.3 ± 1.6 (5)
	enrichment	1	0.58	3.45	0.1
RNA	spec. act.	147 ± 12 (13)	285 ± 21 (13)	353 ± 22 (5)	461 ± 23 (5)
	enrichment	1	1.94	2.4	3.14
NADH cytochrome *c* reductase	spec. act.	93 ± 7.3 (10)	91 ± 7.4 (10)	107 ± 6.3 (5)	159 ± 7.4 (5)
	enrichment	1	0.98	1.15	1.71
Cytochrome *c* oxidase	spec. act.	148 ± 38 (13)	6.2 ± 0.9 (13)	18.8 ± 2.2 (5)	4.4 ± 1.3 (5)
	enrichment	1	0.04	0.13	0.03

[a] The 27,000 × *g* fraction obtained by differential centrifugation was separated into lighter (fraction III–IV) and denser membranes (fraction VI–VIII). Fractions VI to VIII were used for calcium uptake studies. Specific enzyme activities are expressed in nmol substrate split per min and mg protein. The RNA content is given in μg per mg protein. The values are means ± SE; the number of determinations is given in parentheses. Enrichment is expressed as specific activity divided by specific activity of the total homogenate.

compared to the total homogenate. Since a large part of the intracellular protein in pancreatic acinar cells originates from rough endoplasmic reticulum (Fig. 1), a threefold enrichment of the RNA produced essentially a pure fraction of rough endoplasmic reticulum (Fig. 2*a*). The activities of $Na^+,K^+(Mg^{2+})$ATPase and alkaline phosphatase (AP), marker enzymes of the plasma membrane, were decreased by 17-fold and 10-fold, respectively. The mitochondrial marker cytochrome *c* oxidase, was decreased by 33-fold as compared to the total homogenate (Table).

Since in this purified fraction of endoplasmic reticulum there was still some contamination with plasma membranes (Table), nonmitochondrial Ca^{2+} uptake can take place into endoplasmic reticulum and into inside-out plasma membrane vesicles. To discriminate between both, $^{45}Ca^{2+}$ uptake and its stimulation by 20 mmol/liter oxalate in two different fractions of the Percoll gradient (fraction III–IV and VI–VIII) was correlated with the activity of the $Na^+,K^+(Mg^{2+})$ATPase, a marker enzyme for the plasma membrane, and with the RNA content, a marker of the rough endoplasmic reticulum. $^{45}Ca^{2+}$ uptake as measured in both fractions did not correlate with the $Na^+,K^+(Mg^{2+})$ATPase activity (correlation factor $r = 0.03$). The correlation of $^{45}Ca^{2+}$ uptake with the RNA marker of the rough endoplasmic reticulum was $r = 0.6$, whereas $^{45}Ca^{2+}$ uptake as stimulated by 20 mmol/liter oxalate correlated with the RNA by $r = 0.82$ (7 membrane preparations, Fig. 3). These results show that in these fractions of purified rough endoplasmic reticulum calcium uptake into plasma membrane vesicles can be neglected.

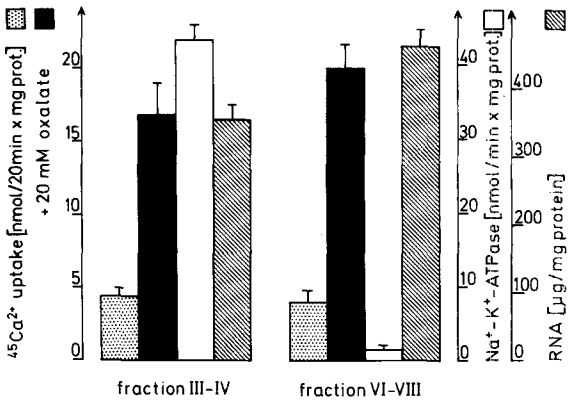

Fig. 3. Correlation of $^{45}Ca^{2+}$ uptake and its stimulation by 20 mmol/liter oxalate with $Na^+,K^+(Mg^{2+})$ATPase activity and the RNA content in fraction III–IV and fraction VI–VIII of the Percoll gradient. The bars represent the means of 7 experiments ± SE

Characterization of Calcium Transport

Ca^{2+} Dependence

To examine the dependence of the rate of nonmitochondrial Ca^{2+} uptake on free Ca^{2+} concentration in leaky cells, Ca^{2+} uptake was determined in the presence of 20 mmol/liter oxalate by measuring the decrease in free Ca^{2+} concentration of the surrounding incubation medium with a Ca^{2+}-specific electrode (Fig. 4*a*, inset), similar to that previously described [57]. Oxalate was added so that inside the endoplasmic reticulum the free Ca^{2+} concentration was maintained constant by formation of insoluble calcium oxalate [1, 35]. Conditions were chosen such

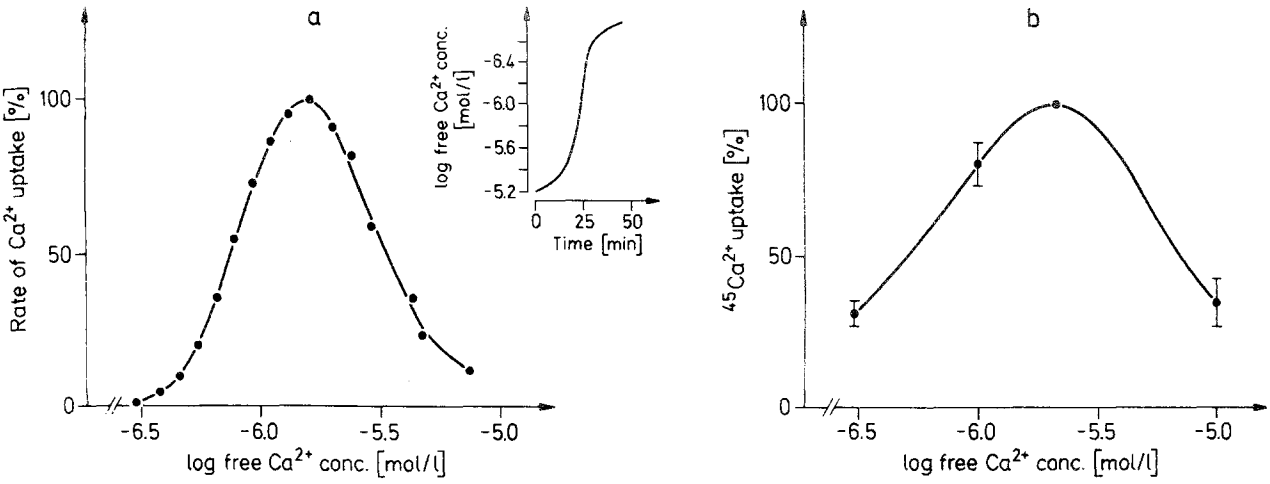

Fig. 4. Dependence of calcium uptake on the medium-free Ca^{2+} concentration. The experiments were performed as described in Results. For permeabilized cells one typical (from $n = 6$) curve of the calcium uptake rate is shown (*a*). The inset shows the original trace of calcium uptake against time as measured with the calcium-specific electrode, on which the calculation of the calcium uptake rate was based. 100% were equivalent to 8 ± 1.2 nmol/mg protein per min. The values in (*b*) represent the means of $^{45}Ca^{2+}$ uptake after 20 min of 4 experiments ± SE performed with rough endoplasmic reticulum vesicles. When the first 2 min were considered, a similar curve was obtained. ATP-dependent calcium uptake is expressed in % of the highest uptake, 100% were equivalent to a specific uptake of 5.1 ± 0.3 nmol/mg protein per 20 min

that uptake ceased because the free Ca^{2+} concentration in the medium became too low and reached the steady-state level, and not because the capacity of the endoplasmic reticulum was exceeded. At free Ca^{2+} concentrations of 0.4 to 10 μmol/liter the rate of Ca^{2+} uptake was calculated from the hand-drawn tangent to the Ca^{2+} uptake curve.

The results of a typical experiment are shown in Fig. 4*a*. The rate of Ca^{2+} uptake increased with free Ca^{2+} concentration to reach a maximum at a free Ca^{2+} concentration of 2.2 ± 0.3 μmol/liter ($n = 6$) with a maximal rate of 8.0 ± 1.2 nmol/mg protein × min. Half-maximal uptake was observed at a concentration of 0.59 ± 0.06 μmol/liter. At Ca^{2+} concentrations exceeding 2 to 3 μmol/liter the rate of uptake decreased sharply to reach a very low value at 10 μmol/liter.

This method has several advantages: the free Ca^{2+} concentration can be determined precisely, no Ca^{2+} buffering agents like EGTA are necessary, and a large number of different Ca^{2+} concentrations can be tested in a single experiment. The results are, however, based on the assumption that the free Ca^{2+} concentration inside the endoplasmic reticulum is kept constant during Ca^{2+} uptake due to formation of calcium oxalate.

To avoid a methodological error, a completely different method was used to study the dependence of Ca^{2+} uptake into endoplasmic reticulum vesicles on the free Ca^{2+} concentration. $^{45}Ca^{2+}$ uptake per 20 min was measured at different free Ca^{2+} concentrations buffered with EDTA. As shown in Fig. 4*b*, results similar to those with cells were obtained: maximal uptake at a value of 2 μmol/liter, half-maximal uptake at 0.5 μmol/liter, and again a very sharp decrease at higher free Ca^{2+} concentrations. In

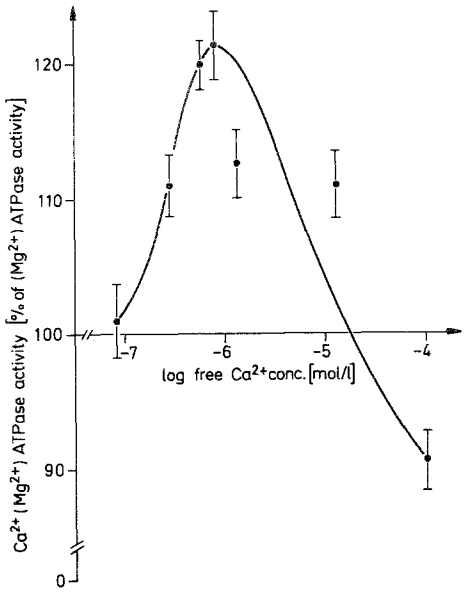

Fig. 5. Dependence of Ca^{2+}(Mg^{2+})ATPase activity on the medium-free calcium concentration in rough endoplasmic reticulum vesicles. The values represent means of 4 experiments ± SE. The basal Mg^{2+}-dependent ATPase activity was set as 100%

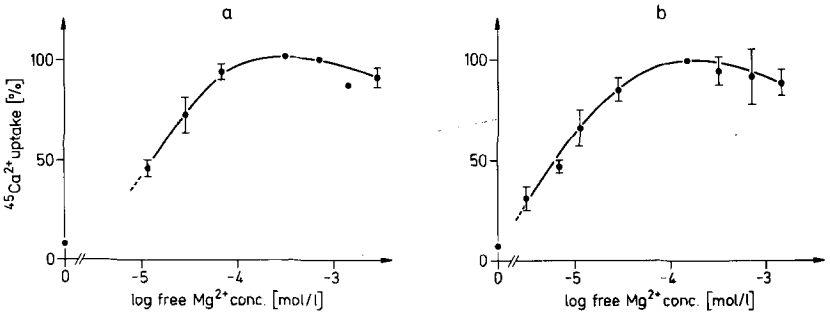

Fig. 6. Dependence of nonmitochondrial $^{45}Ca^{2+}$ uptake into permeabilized cells (*a*) and into rough endoplasmic reticulum vesicles (*b*) on the medium-free Mg^{2+} concentration. The values represent means of 4 experiments ± SE; points without SE are the means of 2 experiments. Calcium uptake is expressed in % of the highest uptake; 100% were equivalent to a specific uptake of 4.4 ± 0.4 nmol/mg protein (vesicles) and 1.4 ± 0.1 nmol/mg protein (cells) per 20 min. The medium-free calcium concentration was kept at 5 μmol/liter in (*a*) and at 4 μmol/liter in (*b*) as described in Results. Before incubation for calcium uptake vesicles were washed once with a 0.1 mmol/liter EDTA containing mannitol buffer

principle, the same result was obtained when the amount of Ca^{2+} uptake in the first 2 min (first time point) was considered. Absolute amounts of Ca^{2+} uptake into vesicles were 4.2 nmol/mg protein in 20 min under the usually employed conditions of 4 μmol/liter free calcium, 0.3 mmol/liter free magnesium, 1 mmol/liter ATP and pH 7.4. Under optimal conditions, i.e., 2 μmol/liter free calcium, 0.2 mmol/liter magnesium, 1 mmol/liter ATP and pH 7.0, Ca^{2+} uptake was even higher and about 8 nmol/mg protein in 20 min.

The dependence of the activity of the Ca^{2+}-stimulated Mg^{2+}-dependent ATPase in vesicles on free Ca^{2+} concentration was next determined. This enzyme could not be determined with sufficient accuracy in leaky cells due to the much higher amount of basal ATP splitting. The same pattern for stimulation of the $Ca^{2+}(Mg^{2+})$ATPase activity was obtained as was observed for Ca^{2+} uptake: maximal activity at 0.8 μmol/liter, half-maximal activity at 0.3 μmol/liter, and a sharp decrease at higher free Ca^{2+} concentration (Fig. 5). The absolute amount of $Ca^{2+}(Mg^{2+})$ATPase activity was 9.1 ± 1.7 nmol/min/mg protein substrate split.

Mg^{2+}, pH and ATP Dependence

Nonmitochondrial $^{45}Ca^{2+}$ uptake into permeabilized cells and $^{45}Ca^{2+}$ uptake into rough endoplasmic reticulum vesicles were dependent on magnesium. No uptake at all could be detected in the absence of Mg^{2+}. Measurable calcium uptake was observed at a free Mg^{2+} concentration of about 1 μmol/liter and was maximal at 0.3 mmol/liter for cells and at 0.2 mmol/liter for vesicles. Calcium uptake slightly decreased at higher concentrations in both preparations (Fig. 6). The medium-free calcium concentration was kept constant with 5 mmol/liter ATP in cells and 1 mmol/liter ATP plus 1 mmol/liter EDTA in vesicles.

The dependence of nonmitochondrial $^{45}Ca^{2+}$ uptake into permeabilized cells on the pH showed a maximum at pH 6. In vesicles $^{45}Ca^{2+}$ uptake showed a plateau between pH 6 and 7 with a maximum at pH 7 (Fig. 7).

Both nonmitochondrial $^{45}Ca^{2+}$ uptake into permeabilized cells and $^{45}Ca^{2+}$ uptake into rough endoplasmic reticulum vesicles were dependent on ATP. Measurable calcium uptake was observed at about 1 μmol/liter ATP in both preparations and was maximal at 1 mmol/liter ATP for both preparations. Calcium uptake slightly decreased at higher ATP concentrations (Fig. 8).

Monovalent Cations

Ca^{2+} uptake is dependent upon monovalent cations in the endoplasmic reticulum of several tissues [11, 22, 26, 39, 40, 62], as well as in the sarcoplasmic reticulum [13, 17, 25, 36, 37, 58]. We have therefore investigated the influence of different cations such as Rb^+, K^+, Na^+, Li^+ and choline$^+$ on nonmitochondrial calcium uptake into leaky isolated cells and in isolated endoplasmic reticulum vesicles prepared from these cells. K^+ was isosmotically replaced by either Rb^+, Na^+, Li^+ or choline$^+$ and $^{45}Ca^{2+}$ uptake was measured as described in Materials and Methods. Maximal $^{45}Ca^{2+}$ uptake after 20 min of incubation occurred in the presence of Rb^+ and was reduced by replacing it in the sequence: $K^+ < Na^+ < Li^+ <$ choline$^+$ in permeabilized cells as well as in endoplasmic reticulum vesicles (Fig. 9).

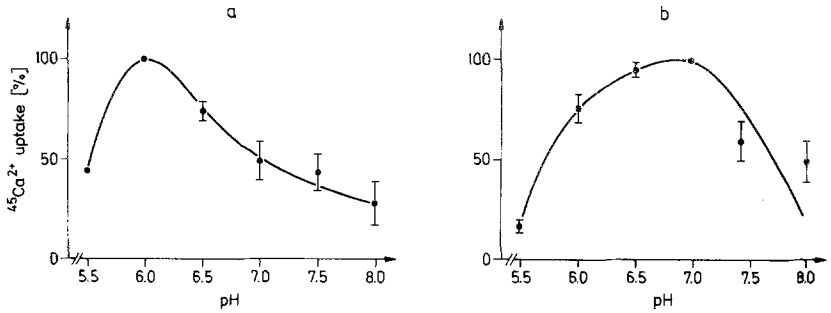

Fig. 7. Dependence of nonmitochondrial $^{45}Ca^{2+}$ uptake into permeabilized cells (*a*) and into rough endoplasmic reticulum vesicles (*b*) on the pH of the incubation medium. The values represent means of 4 experiments ± SE; points without SE are the means of 2 experiments. Calcium uptake is expressed in % of the highest uptake; 100% were equivalent to a specific uptake of 7.1 ± 0.5 nmol/mg protein (vesicles) and 3.5 ± 0.7 nmol/mg protein (cells) per 20 min. The medium-free calcium concentration was kept constant as described

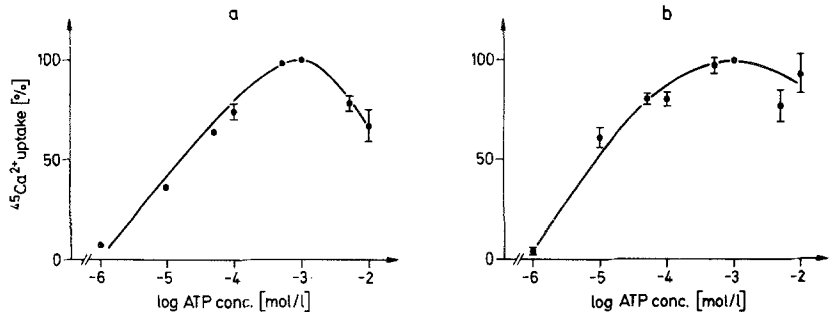

Fig. 8. Dependence of nonmitochondrial $^{45}Ca^{2+}$ uptake into permeabilized cells (*a*) and into rough endoplasmic reticulum vesicles (*b*) on the ATP concentration. The values represent means of 4 experiments ± SE; points without SE are the means of 2 experiments. Calcium uptake is expressed in % of the highest uptake; 100% were equivalent to a specific uptake of 4.2 ± 0.4 nmol/mg protein (vesicles) and 2.0 ± 0.5 nmol/mg protein (cells) per 20 min. The medium-free calcium concentration was kept constant as described

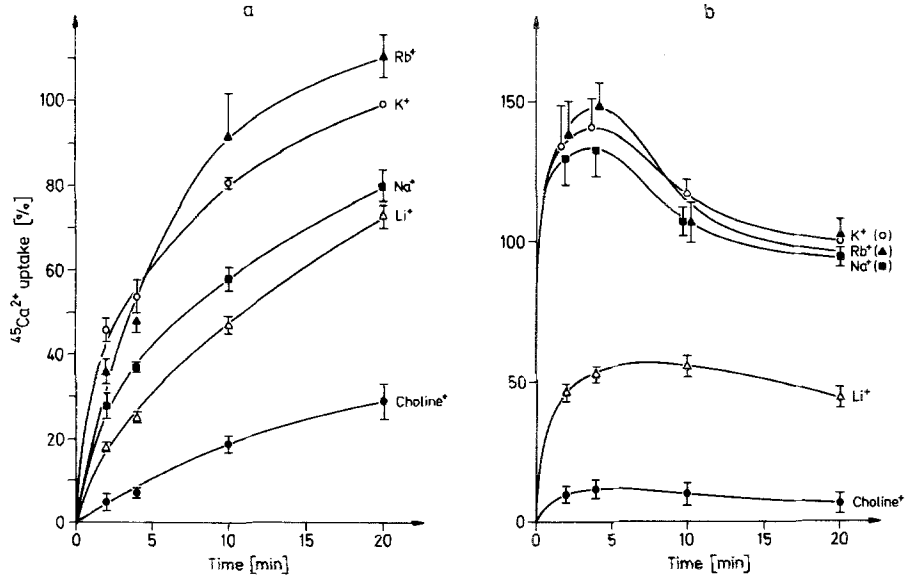

Fig. 9. Dependence of nonmitochondrial $^{45}Ca^{2+}$ uptake into permeabilized cells (*a*) and into rough endoplasmic reticulum vesicles (*b*) on monovalent cations. 130 mmol/liter K^+ (○) were isosmotically replaced by either Rb^+ (▲), Na^+ (■), Li^+ (△) or $choline^+$ (●). The values represent the means of 4 experiments ± SE. Calcium uptake after 20 min in the presence of K^+ was set as 100% and was equivalent to a specific uptake of 4.2 ± 0.8 nmol/mg protein (vesicles) and 1.0 ± 0.1 nmol/mg protein (cells)

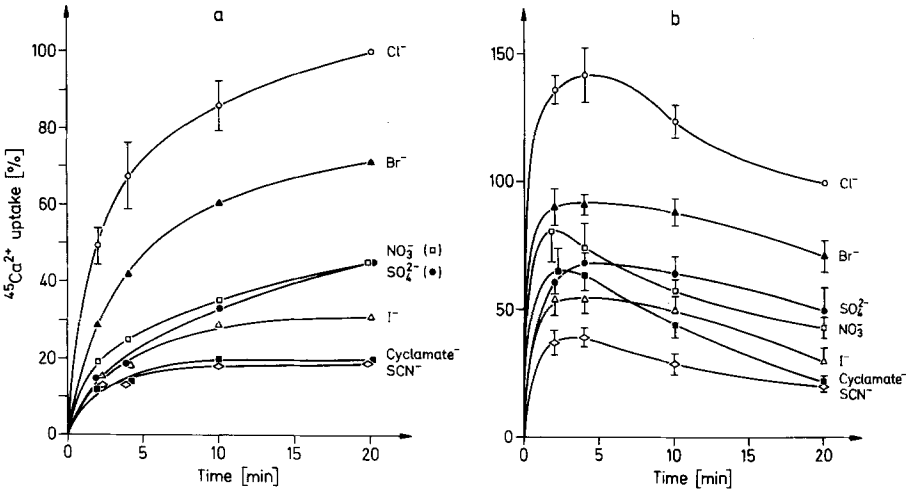

Fig. 10. Dependence of nonmitochondrial $^{45}Ca^{2+}$ uptake into permeabilized cells (*a*) and into rough endoplasmic reticulum vesicles (*b*) on different permeable and impermeable anions. 130 mmol/liter Cl^- (○) were isosmotically replaced by either Br^- (▲), NO_3^- (□), SO_4^{2-} (●), I^- (△), cyclamate$^-$ (■) or SCN^- (◇). The values represent the means of 4 experiments ± SE; points without standard error are the means of two experiments. Calcium uptake after 20 min in the presence of Cl^- was set as 100% and was equivalent to a specific uptake of 4.2 ± 0.8 nmol/mg protein (vesicles) and 1.4 ± 0.2 nmol/mg protein (cells)

Since $^{45}Ca^{2+}$ uptake was not significantly different in the presence of either Rb^+, K^+ or Na^+ in vesicle studies, the rate of calcium uptake was enhanced using the trapping agent oxalate. Thirty mmol/liter of NaCl and KCl were replaced by 20 mmol/liter K_2oxalate and Na_2oxalate, respectively. Under these conditions the $^{45}Ca^{2+}$ uptake in the presence of Na^+ was only 70% of the uptake with K^+ (*data not shown*).

Anions

In the sarcoplasmic reticulum, calcium uptake also depends upon anions [7–9, 59]. We therefore tested the effect of different anions on calcium uptake in permeabilized cells and endoplasmic reticulum vesicles. Membranes are fairly permeable for Cl^-, Br^-, NO_3^- and SCN^-, whereas I^-, SO_4^{2-} and cyclamate hardly penetrate through membranes. Maximum $^{45}Ca^{2+}$ uptake was found in the presence of Cl^- and isosmotic replacement decreased calcium uptake in the sequence: $Br^- < SO_4^{2-} < NO_3^- < I^- <$ cyclamate$^- < SCN^-$ (Fig. 10).

Calcium-Precipitating Anions

Calcium uptake into sarcoplasmic reticulum [1] and endoplasmic reticulum of various tissues [11, 16, 20, 22, 26, 33, 35, 39, 44, 46, 62] can be stimulated by oxalate. This is a characteristic feature of the endoplasmic reticulum, in contrast to calcium uptake into plasma membrane vesicles [30, 62], due to the permeability of the endoplasmic reticulum to oxalate. With increasing concentrations of calcium inside the vesicles during uptake, oxalate forms insoluble precipitates with calcium which keeps the intravesicular free calcium concentration low and thus leads to a constant calcium uptake for a longer period of time.

In the present study, 30 mmol/liter KCl was replaced by 20 mmol/liter K_2oxalate, which increased nonmitochondrial $^{45}Ca^{2+}$ uptake in permeabilized cells 10-fold and $^{45}Ca^{2+}$ uptake into endoplasmic reticulum vesicles fivefold as compared to the control without oxalate. The more often employed oxalate concentration of 5 mmol/liter stimulated $^{45}Ca^{2+}$ uptake into vesicles only by 1.7-fold. In addition, in leaky cells 130 mmol/liter Cl^- was replaced by 110 mmol/liter $H_2PO_4^-/HPO_4^{2-}$, which increased $^{45}Ca^{2+}$ uptake 3.2-fold (Fig. 11).

Discussion

ATP-dependent calcium uptake into microsomal fractions of pancreatic acinar cells in the presence of mitochondrial inhibitors has been previously observed by several authors [34, 44, 46]. In some of these studies calcium uptake was attributed to the endoplasmic reticulum. However, the evidence that this Ca^{2+} uptake occurred into endoplasmic reticulum was rather weak and was mainly based on the stimulatory effect of oxalate [44]. Ca^{2+} uptake into

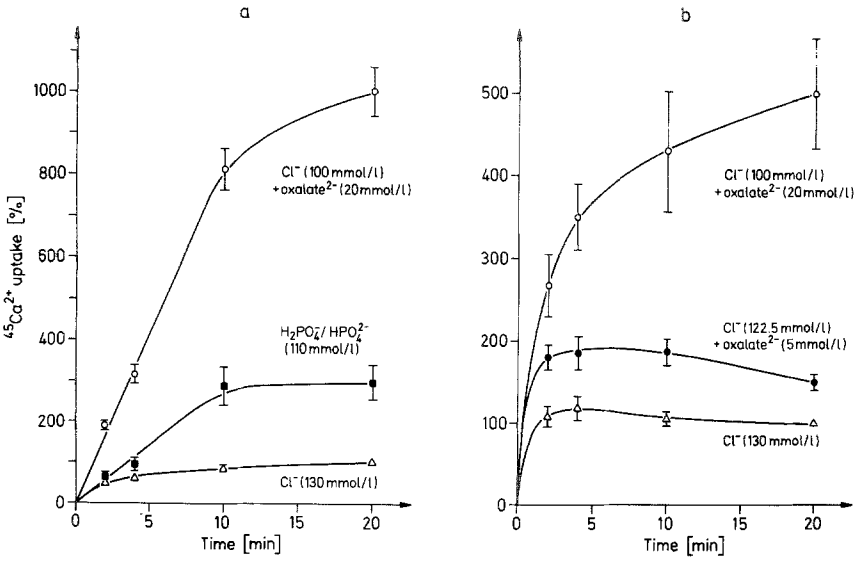

Fig. 11. Stimulation of nonmitochondrial $^{45}Ca^{2+}$ uptake into permeabilized cells (*a*) and into rough endoplasmic reticulum vesicles by calcium-precipitating anions. 7.5 mmol/liter KCl were replaced by 5 mmol/liter K_2oxalate (●). 30 mmol/liter KCl were replaced by 20 mmol/liter K_2oxalate (○). 130 mmol/liter KCl were replaced by 110 mmol/liter $KH_2PO_4^-/K_2HPO_4^{2-}$ (■). Calcium uptake after 20 min in the presence of 130 mmol/liter KCl was set as 100% (△), and was equivalent to a specific uptake of 4.2 ± 0.8 nmol/mg protein (vesicles) and 1.2 ± 0.2 nmol/mg protein (cells). The values are the means of 4 experiments ± SE

inside-out plasma membrane vesicles as previously described [30] could not be excluded.

In the studies of Lucas et al. [34], no distinction between plasma membrane and endoplasmic reticulum was attempted. Microsomal Ca^{2+} uptake in "heavy microsomes" observed by Ponnappa et al. [44] correlated with the plasma membrane marker $Na^+,K^+(Mg^{2+})$ATPase activity and not with the RNA content. The absence of Ca^{2+} uptake into membranes enriched in $Na^+,K^+(Mg^{2+})$ATPase activity, which were prepared by a completely different method in the same study, was presented as evidence that Ca^{2+} transport did not occur in plasma membranes. However, since this latter preparation consisted of membrane "sheets," this observation obviously does not exclude Ca^{2+} uptake into plasma membrane vesicles of the former preparation.

Preissler and Williams [46] employed the same impure membrane fraction as described by Ponnappa et al. [44] and showed ATP- and oxalate-dependent electron-dense precipitates that were localized both inside and outside the vesicles. Treatment of these vesicles with puromycin to remove ribosomes increased the specific Ca^{2+} uptake. This led the authors to conclude that Ca^{2+} uptake occurred into rough endoplasmic reticulum. Since removal of nonspecific proteins would increase the specific activities of all enzymes in the same fraction, this cannot be accepted as evidence to localize calcium transport.

More convincing evidence suggesting Ca^{2+} uptake into the endoplasmic reticulum was obtained by using isolated pancreatic acini with saponin-permeabilized plasma membranes. Wakasugi et al. [61] could distinguish mitochondrial and nonmitochondrial intracellular calcium pools, which was later confirmed by Lucas et al. [33]. Electron-dense precipitates in the presence of ATP and oxalate were localized clearly inside the rough endoplasmic reticulum and the presence of calcium in these precipitates was identified by laser microprobe mass analysis [61].

In studies using isolated cells with permeabilized plasma membranes, Streb and Schulz [57] have recently shown that intracellular nonmitochondrial calcium uptake regulates the free Ca^{2+} concentration of the surrounding incubation medium to 0.4 μmol/liter. This suggested an important role of this structure for the regulation of the cytosolic Ca^{2+} concentration.

The aim of the present study therefore was first to demonstrate ATP-dependent Ca^{2+} uptake into an isolated endoplasmic reticulum fraction sufficiently purified to exclude uptake into inside-out plasma membrane vesicles and secondly to identify the morphological equivalent of the nonmitochondrial intracellular pool [57] by comparison to an isolated fraction.

Localization of Ca^{2+} Uptake

A new approach to purify rough endoplasmic reticulum was used in the present study. The self-generating isopycnic Percoll gradient separates membranes deriving from different structures according to their densities [42]. Rough endoplasmic reticulum fully coated with ribosomes (Fig. 2*a*) could clearly be separated from plasma membranes. Al-

though we could prepare purified rough endoplasmic membranes, we were not able to prepare purified plasma membranes in the same preparation step, possibly since rough endoplasmic reticulum membranes that had lost their ribosomes moved together with the plasma membranes in the Percoll gradient. The purification of rough endoplasmic reticulum was proven by marker enzyme distribution. RNA, the marker of the rough endoplasmic reticulum was enriched by about threefold and the $Na^+,K^+(Mg^{2+})$ATPase as marker of the plasma membrane was decreased by about 20-fold (Table). Since pancreatic acinar cells contain much rough endoplasmic reticulum (Fig. 1) a threefold enrichment of the RNA produced an almost pure membrane fraction (Fig. 2*a*). As there was still some contamination with plasma membranes present in the purified endoplasmic reticulum fraction from the Percoll gradient, this fraction (VI–VIII) that was usually employed in this study, was compared with another fraction (III–IV) from the Percoll gradient containing significantly more plasma membranes (Table). There was no correlation of $^{45}Ca^{2+}$ uptake with the plasma membrane marker enzyme activity. This does not generally exclude Ca^{2+} uptake into plasma membrane vesicles for which clear evidence has been presented [30], but it does show that it is of a negligible amount in the purified fraction used.

Electron micrographs demonstrated that the membrane fraction used for $^{45}Ca^{2+}$ uptake in this work consisted of 98% vesicles coated with ribosomes (Fig. 2*a*). The very rare smooth membranes present in this fraction hardly fit to a specific calcium storage capacity of about 8 nmol/mg protein under optimal conditions. Furthermore, calcium oxalate precipitates could be demonstrated in these isolated rough endoplasmic reticulum vesicles by electron microscopy (Fig. 2*b*). Also in our study precipitates were observed both inside and outside of the vesicles. Therefore this result should only be considered in addition to our other data rather than as conclusive proof in itself.

Characterization of Ca^{2+} Uptake

The characteristics of calcium uptake into this purified rough endoplasmic reticulum fraction were compared with the characteristics of nonmitochondrial calcium uptake into cells with permeabilized plasma membranes [57]. In general, the characteristics of Ca^{2+} uptake in both preparations were very similar and comparable to results from endoplasmic reticulum of other tissues.

Nonmitochondrial calcium uptake in permeabilized cells and in isolated rough endoplasmic reticulum vesicles was measurable at micromolar concentrations and its maximum was found at a free Ca^{2+} concentration of 2 and 2 μmol/liter, respectively. Calcium uptake decreased at higher free calcium concentrations (Fig. 4). Increase in cell permeability due to washing with solutions containing low Ca^{2+} concentration was not reversible upon readdition of Ca^{2+} [57]. Decrease of Ca^{2+} uptake into permeabilized cells at higher Ca^{2+} concentration therefore cannot be due to resealing of the cells, but rather reflects Ca^{2+} uptake into rough endoplasmic reticulum as demonstrated in the isolated fraction (Fig. 4). The Ca^{2+}-stimulated Mg^{2+}-dependent ATPase activity in isolated rough endoplasmic reticulum vesicles was dependent on the free Ca^{2+} concentration in a similar fashion as calcium uptake and was maximal at 0.8 μmol/liter free calcium (Fig. 5). The values for half-maximal calcium uptake into cells (0.6 μmol/liter) and isolated rough endoplasmic reticulum vesicles (0.5 μmol/liter) are in the range of the intracellular free calcium concentration as estimated by O'Doherty et al. [41] and Streb and Schulz [57]. This suggests that the intracellular free Ca^{2+} concentration can be optimally regulated by the rough endoplasmic reticulum in its *in vivo* arrangement in cells. The finding that calcium uptake and $Ca^{2+}(Mg^{2+})$ATPase activity decreased at higher free Ca^{2+} concentrations seems to be an individual characteristic of rough endoplasmic reticulum in pancreatic acinar cells. It is in contrast to characteristics of Ca^{2+} uptake and $Ca^{2+}(Mg^{2+})$ATPase activity of the plasma membrane [30, 31], which did not decrease at higher free calcium concentrations. Probably our results can only be detected in an endoplasmic reticulum fraction with negligible plasma membrane contamination. Moreover, regarding the regulation of the cytosolic-free calcium level as a possible physiological function of the rough endoplasmic reticulum in pancreatic acinar cells, such a characteristic appears to be reasonable. During stimulation of enzyme secretion, increased cytosolic calcium concentration [15, 24, 47, 49–51, 55, 56] would suppress the calcium sequestering activity of the rough endoplasmic reticulum. Whether calcium release from an intracellular pool as described by Streb et al. [56] involves the rough endoplasmic reticulum as isolated in this study or occurs from a different structure cannot yet be decided.

As shown in Fig. 8, calcium uptake into rough endoplasmic reticulum was dependent on ATP. Ca^{2+} uptake could be measured at micromolar ATP concentrations and was maximal at 1 mmol/liter ATP in both preparations. This agrees with the characteristics of other tissues [17].

It has been clearly demonstrated in studies using the widely investigated sarcoplasmic reticulum

that magnesium plays an important role in the dephosphorylation step of the Ca^{2+} transport cycle [23] and several other tissues also showed magnesium dependence of microsomal calcium transport [3, 11, 22, 39, 40]. Calcium uptake was measurable at micromolar magnesium concentrations and was maximal at a free Mg^{2+} concentration of 0.2 mmol/liter for vesicles and 0.3 mmol/liter for cells, equivalent to a total magnesium concentration of 0.8 and 1 mmol/liter, respectively (Fig. 6). This agrees with the model of magnesium being a cosubstrate for the Ca-ATPase operating at a 1 : 1 ratio together with ATP as described for the sarcoplasmic reticulum Ca-ATPase [27].

A discrepancy was found for the pH dependence of calcium uptake between both preparations with an optimum of pH 6 in permeabilized cells and pH 7 in isolated rough endoplasmic reticulum vesicles (Fig. 7). One explanation for this difference might be the influence of the intracellular structures additionally present in permeabilized cells. Nevertheless, the optimal pH for the rough endoplasmic reticulum vesicles agrees with the knowledge on the intracellular pH of pancreatic acinar cells[1] and with that in other cells [45].

The influence of monovalent cations on calcium uptake was similar in our preparation as reported for the better investigated sarcoplasmic reticulum [13, 17, 25, 36, 37, 58], as well as other tissues [11, 22, 26, 39, 40, 62].

The anion dependence of calcium uptake into endoplasmic reticulum vesicles cannot be simply explained by the different permeabilities of the endoplasmic membrane for the investigated anions according to a lyophilic anion series. Thus our results suggest a specific involvement of anions in calcium uptake. The anion specificity might be explained either by anion-selective channels to accompany electrogenic calcium uptake or by coupled Ca^{2+}-anion transport [8, 59].

Conclusion

By using a purified microsomal preparation with negligible plasma membrane contamination, calcium uptake into rough endoplasmic reticulum of pancreatic acinar tissue could be demonstrated unambiguously. The characteristics of calcium uptake into this highly purified rough endoplasmic reticulum fraction were very similar to the characteristics of calcium uptake into a nonmitochondrial pool in permeabilized acinar cells. As this nonmitochondrial pool was recently shown to buffer the cytosolic-free calcium concentration [57], we conclude that the rough endoplasmic reticulum is an important site for the regulation of the free calcium level in pancreatic acinar cells.

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