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Na^+/Ca^{2+} Countertransport in Plasma Membrane of Rat Pancreatic Acinar Cells

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Summary. The presence of a coupled Na^+/Ca^{2+} exchange system has been demonstrated in plasma membrane vesicles from rat pancreatic acinar cells. Na^+/Ca^{2+} exchange was investigated by measuring $^{45}Ca^{2+}$ uptake and $^{45}Ca^{2+}$ efflux in the presence of sodium gradients and at different electrical potential differences across the membrane (= $\Delta\varphi$) in the presence of sodium. Plasma membranes were prepared by a $MgCl_2$ precipitation method and characterized by marker enzyme distribution. When compared to the total homogenate, the typical marker for the plasma membrane, ($Na^+ + K^+$)-ATPase was enriched by 23-fold. Markers for the endoplasmic reticulum, such as RNA and NADPH cytochrome *c* reductase, as well as for mitochondria, the cytochrome *c* oxidase, were reduced by twofold, threefold and 10-fold, respectively. For the Na^+/Ca^{2+} countertransport system, the Ca^{2+} uptake after 1 min of incubation was half-maximal at 0.62 μmol/liter Ca^{2+} and at 20 mmol/liter Na^+ concentration and maximal at 10 μmol/liter Ca^{2+} and 150 mmol/liter Na^+ concentration, respectively. When Na^+ was replaced by Li^+, maximal Ca^{2+} uptake was 75% as compared to that in the presence of Na^+. Amiloride (10^{-3} mol/liter) at 200 mmol/liter Na^+ did not inhibit Na^+/Ca^{2+} countertransport, whereas at low Na^+ concentration (25 mmol/liter) amiloride exhibited dose-dependent inhibition to be 62% at 10^{-2} mol/liter. CFCCP (10^{-5} mol/liter) did not influence Na^+/Ca^{2+} countertransport. Monensin inhibited dose dependently; at a concentration of 5×10^{-6} mol/liter inhibition was 80%. A SCN^- or K^+ diffusion potential (= $\Delta\varphi$), being positive at the vesicle inside, stimulated calcium uptake in the presence of sodium suggesting that Na^+/Ca^{2+} countertransport operates electrogenically, i.e. with a stoichiometry higher than 2 Na^+ for 1 Ca^{2+}. In the absence of Na^+, $\Delta\varphi$ did not promote Ca^{2+} uptake. We conclude that in addition to ATP-dependent Ca^{2+} outward transport as characterized previously (E. Bayerdörffer, L. Eckhardt, W. Haase & I. Schulz, 1985, *J. Membrane Biol.* **84**:45–60) the Na^+/Ca^{2+} countertransport system, as characterized in this study, represents a second transport system for the extrusion of calcium from the cell. Furthermore, the high affinity for calcium suggests that this system might participate in the regulation of the cytosolic free Ca^{2+} level.

Key Words Na^+/Ca^{2+} countertransport · plasma membrane · pancreatic acinar cells · amiloride

Introduction

Evidence for the operation of a Na^+/Ca^{2+} countertransport system to extrude Ca^{2+} from pancreatic acinar cells was obtained from $^{45}Ca^{2+}$ flux experiments with isolated cells [42]. These tracer exchange studies at zero net flux conditions showed that following hormonal stimulation, $^{45}Ca^{2+}$ extrusion from the cell was inhibited in the presence of 2 mmol/liter ouabain or when Na^+ was replaced by K^+ in the extracellular medium. However, direct demonstration of a Na^+/Ca^{2+} countertransport system in a vesicular plasma membrane preparation of pancreatic acinar cells was lacking.

In the present study we give evidence for the operation of a Na^+/Ca^{2+} countertransport system in plasma membranes of pancreatic acinar cells which were prepared by a recently described isolation procedure [3] that has been slightly modified. As in other tissues such as heart muscle [32, 33], the squid giant axon [2], brain synaptosomes [6, 10], kidney proximal tubule [11, 45], erythrocytes [27], smooth muscle [12], small intestine epithelia [9, 18], liver [20] and lymphocytes [44], extrusion of Ca^{2+} from pancreatic acinar cells is performed by the cooperation of a Ca^{2+} ATPase and a Na^+/Ca^{2+} countertransport system.

Materials and Methods

Materials

All reagents were of analytical grade. Collagenase (from clostridium histolyticum) type III was obtained from Worthington, Freehold, N.J. Ethyleneglycol-bis-(β-amino-ethylether)-N,N-tetraacetic acid (EGTA), ethylene-diamine-tetraacetic acid (EDTA), adenosine 5-triphosphate (ATP), phosphocreatine (sodium salt), the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (CFCCP) and benzamidine were bought from Sigma, München, W. Germany. Cytochrome *c*, lactatedehydrogenase (LDH), pyruvate kinase (PK), creatine kinase (CK), phosphoenolpyruvate (PEP), nicotine adenine dinucleotide (NADH), ribonucleic acid (RNA), trypsin inhibitor (from soybean) and valinomycin were purchased from Boehringer, Mannheim, W. Germany and Triton X-100 and bovine serum albumin from Serva, Heidelberg, W. Germany. Sodium dithionite

was from Merck, Darmstadt, W. Germany. The Ca^{2+} ionophore A23187 and the Na^+ ionophore monensin were obtained from Calbiochem, Giessen, W. Germany. Amiloride was purchased from Sharp and Dohme, München, W. Germany. $^{45}CaCl_2$ (4 to 50 Ci/g) and $^{85}SrCl_2$ were bought from New England Nuclear, Dreieich, W. Germany.

Preparation of Acinar Cells

Rat pancreatic acinar cells were prepared as described previously [1, 43] with the following modifications. Pancreatic tissue from six rats was digested for 15 min in a collagenase (108 U/ml)-containing Krebs-Ringer's solution, followed by a washing step with a 1 mmol/liter EDTA-containing solution for 5 min. Single cells were then obtained by a second collagenase digestion (162 U/ml) for 60 min.

Preparation of Plasma Membranes

Preparation of a fraction enriched in plasma membranes was in principle performed as described recently [3], except that the last precipitation step with $MgCl_2$ was omitted. In brief: 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 ml of MB in a tight-fitting motor-driven glass/Teflon® Potter (Braun, Melsungen, W. Germany) by 50 strokes at 900 rpm. The final Mg^{2+} concentration during the precipitation and separation steps was 11 mmol/liter. Two low-speed centrifugations to remove the aggregated material alternated with two high-speed centrifugations to collect the material from the plasma membrane-enriched supernatants. The first precipitation was repeated once to raise the final protein yield. The centrifugation steps were performed in a JS 7.5 rotor in a Beckman J-21C centrifuge for the first low-speed centrifugation and in a SW 27 rotor (or SW 50 rotor for small volumes) in a Beckman L3-50 ultracentrifuge.

Measurement of $^{45}Ca^{2+}$ Uptake in Plasma Membrane vesicles

In general 80 to 120 μg of membrane protein were preincubated for 60 min at 25°C in 12.5 μl of an incubation medium containing in mmol/liter: 100 Na_2SO_4, 33 K_2SO_4, 30 HEPES, pH 7 adjusted with Tris. In addition this incubation medium contained 0.01 $^{45}CaCl_2$, when indicated in the legends to the figures. Vesicles were then diluted by 40-fold into a medium containing in mmol/liter: 100 $choline_2SO_4$, 33 K_2SO_4, 30 HEPES, pH 7 adjusted with Tris, 0.01 $^{45}CaCl_2$, 0.002 valinomycin. Variations in preincubation or in the composition of the uptake media are described in detail in the legends to the figures. The amount of radioactivity varied from 12 to 16 μCi/ml according to the specific activity.

Following preincubation and dilution of vesicles into the incubation medium, samples were taken in duplicates at 20 sec and at 80 sec of incubation. $^{45}Ca^{2+}$ accumulation between 20 and 80 sec of incubation was used to quantify $^{45}Ca^{2+}$ uptake.

Samples were then 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 5 ml 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).

Measurement of $^{45}Ca^{2+}$ Efflux from Plasma Membrane Vesicles

To obtain optimal filling of vesicles with $^{45}Ca^{2+}$, they were preincubated in the presence of MgATP. 100 to 150 μg of membrane protein were preincubated for 40 min at 25°C in 20 μl of an incubation medium containing in mmol/liter: 100 $choline_2SO_4$, 33 K_2SO_4, 30 HEPES, pH 7 adjusted with Tris, 0.9 $CaCl_2$, 0.1 $^{45}CaCl_2$, 10 MgATP. The vesicles were then diluted 40-fold into a medium containing in mmol/liter: 100 Na_2SO_4, 33 K_2O_4, 30 HEPES, pH 7 adjusted with Tris, 0.002 valinomycin, 0.5 vanadate, 0.2 EDTA. All variations of the efflux media are given in the legend to the figure. The amount of radioactivity in the preincubation medium varied from 120 to 160 μCi/ml. At given time points aliquots were taken in duplicates and treated as described above.

Protein and Enzyme Determination

Protein determination was made according to Lowry et al. [23] using bovine serum albumin (BSA) as a standard.

Determination of ribonucleic acid (RNA) was made according to the methods of Mejbaum [24], and Hatcher and Goldstein [15] and the RNA content was calculated using RNA standards.

$(Na^+ + K^+)$-ATPase activity was determined according to the method of Scharschmidt et al. [34] with the final reaction medium modified to 2.5 mmol/liter ATP and 2 mmol/liter PEP. The ouabain suppressible fraction of the 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 cytochrome *c* reductase activity was measured by the procedure of Sottocasa et al. [41].

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-100.

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).

Electron Microscopy

The electron-microscopical demonstration of plasma membranes was performed as described recently [3, 4]. In brief: plasma membranes were fixed as a pellet with glutardialdehyde in the last preparation step, post-fixed with 1% osmic acid, dehydrated with alcohol and embedded in Spurr's resin. Thin sections were stained with uranylacetate and lead citrate and were examined in a Philips 300 electron microscope.

Results

Characterization of Plasma Membranes

Membrane Preparation

To isolate plasma membranes from rat pancreatic acinar cells a methodological approach was used that has been described recently [3]. To investigate a Na^+/Ca^{2+} countertransport system, the modifications designed in this study had been necessary to shorten the isolation procedure as much as possible to preserve the relative impermeability of plasma membranes for sodium.

Enzymatic Characterization

As enzyme markers for the plasma membrane, the $(Na^+ + K^+)$-ATPase activity and the alkaline phosphatase activity (AP) were measured. In the final membrane fraction $(Na^+ + K^+)$-ATPase activity and alkaline phosphatase activity were enriched by 23-fold and fivefold, respectively, as compared with the total homogenate. The alkaline phosphatase activity which was less enriched than the $(Na^+ + K^+)$-ATPase activity, is considered to be localized at the small luminal side of the cell and also in intracellular structures [19, 37]. The amount of rough endoplasmic reticulum was estimated by the RNA content, and the NADPH cytochrome *c* reductase activity was determined for quantitation of both rough and smooth endoplasmic reticulum. In the final plasma membrane fraction both RNA and the NADPH cytochrome *c* reductase activity were decreased by twofold and threefold, respectively, as compared to the total homogenate. The mitochondrial content was monitored by the cytochrome *c* oxidase activity which was decreased by 10-fold in the final plasma membrane fraction (Table 1).

Morphological Studies

To estimate the composition of the plasma membrane-enriched fraction we also used electron microscopy. For the electron-microscopical demonstration of plasma membrane vesicles, the final membrane pellet obtained from the isolation procedure was treated as described above (*see* Materials and Methods, ref. 3). This allowed us to roughly estimate the contamination of the plasma membrane fraction with other structures such as rough endoplasmic reticulum, zymogen granules and mitochondria. One typical micrograph of the plasma membrane fraction is presented in Fig. 1. About 50% of the membranes are smooth; membranes

Table 1. Enzyme activities and enrichment factors in the total homogenate and the plasma membrane-enriched fraction[a]

		Total homogenate	Plasma membranes
$(Na^+ + K^+)$-ATPase	spec. act.	24.6 ± 2.1 (7)	568.3 ± 61.5 (7)
	enrichment	1	23.1
	recovery	100%	11.4%
AP	spec. act.	28.8 ± 3.4 (5)	147.7 ± 12.6 (5)
	enrichment	1	5.13
	recovery	100%	2.5%
RNA	spec. act.	176 ± 8 (7)	89 ± 19 (7)
	enrichment	1	0.51
	recovery	100%	0.25%
NADPH cytochrome *c* reductase	spec. act.	5.1 ± 0.6 (5)	2.04 ± 0.08 (5)
	enrichment	1	0.4
	recovery	100%	0.2%
Cytochrome *c* oxidase	spec. act.	124 ± 11 (5)	12 ± 2.5 (5)
	enrichment	1	0.1
	recovery	100%	0.05%
Protein	yield	295 ± 37 (7)	1.46 ± 0.1 (7)
	recovery	100%	0.49%

[a] The total homogenate as starting material is compared with the plasma membrane-enriched fraction. Specific enzyme activities are expressed in nmol substrate split per min and mg protein for the $(Na^+ + K^+)$-ATPase, alkaline phosphatase (AP), NADPH cytochrome *c* reductase and cytochrome *c* oxidase. 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.

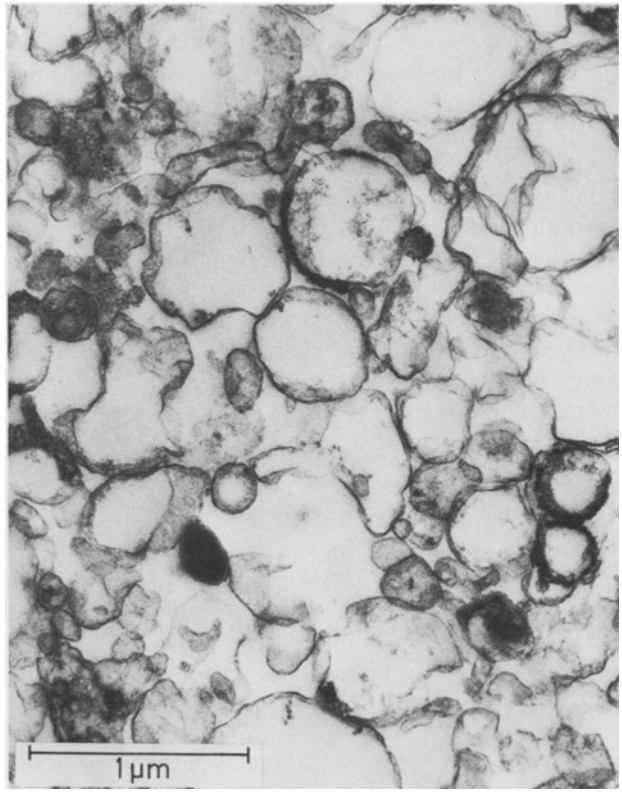

Fig. 1. Electron micrograph of plasma membrane vesicles derived from rat pancreatic acinar cells. Vesicles were prepared for electron microscopical demonstration as described in Materials and Methods and previously [3, 4]

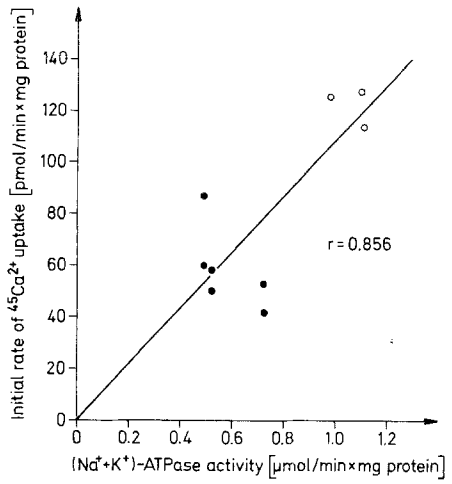

Fig. 2. Correlation of the initial sodium-driven $^{45}Ca^{2+}$ uptake rate with the plasma membrane marker $(Na^+ + K^+)$-ATPase. The plasma membrane-enriched fraction as described in Results (●) was compared with a highly purified plasma membrane fraction (○) which has been described previously [3]. Individual data for $^{45}Ca^{2+}$ uptake as measured during 1 min of incubation are plotted against the $(Na^+ + K^+)$-ATPase activity of the same preparation. The points are the results of three preparation pairs. The mean values for $^{45}Ca^{2+}$ uptake were 62.3 ± 13.7 (●), or 122.5 ± 4.2 (○) pmol/min and mg protein. r = correlation coefficient

coated with ribosomes originating from the rough endoplasmic reticulum are the other part of the membrane fraction. Since we know that pancreatic acinar cells possess only a small amount of intracellular smooth membranes [47] and the marker for smooth endoplasmic reticulum was reduced by about threefold (the marker for plasma membranes however was enriched by 23-fold), we assume that nearly all smooth membranes in the final membrane fraction derive from the plasma membrane. The diameter of the plasma membrane vesicles ranged from 0.1 to 0.6 µm.

Localization of the Na^+/Ca^{2+} Countertransport System

Since only about 50% of the vesicles in the final membrane fraction originate from the plasma membrane it seemed to be necessary to prove the localization of the investigated Na^+/Ca^{2+} countertransport system. We therefore correlated the activity of the plasma membrane marker $(Na^+ + K^+)$-ATPase with sodium gradient-driven $^{45}Ca^{2+}$ uptake into plasma membrane vesicles. We also used a purer plasma membrane fraction that was prepared on the same day from the same starting material as described recently [3]. As shown in Fig. 2, sodium gradient-driven $^{45}Ca^{2+}$ uptake during 1 min of incubation correlated very well with the $(Na^+ + K^+)$-ATPase activity of the individual preparation. The correlation was $r = 0.856$ from three preparation pairs.

Characterization of Na^+/Ca^{2+} Countertransport

Calcium Binding and Passive Ca^{2+} Influx

To determine the amount of $^{45}Ca^{2+}$ binding to the plasma membrane vesicles we used two different approaches. Sodium gradient-driven $^{45}Ca^{2+}$ uptake was studied in plasma membrane vesicles which were preincubated for 60 min in the presence or absence of 10^{-5} mol/liter $^{45}CaCl_2$. In vesicles preincubated for 60 min $^{45}Ca^{2+}$ binding had reached equilibrium. The calcium uptake curves as shown in Fig. 3 (right panel) indicate that after preincubation of the vesicles in the presence of $^{45}CaCl_2$ further $^{45}Ca^{2+}$ influx into the vesicles was only observed in the presence of a vesicle outwardly directed sodium gradient. In the absence of this sodium gradient no change of the vesicular $^{45}Ca^{2+}$ content was observed. We also tried to assess the amount of $^{45}Ca^{2+}$ binding to the plasma membrane fraction by using Triton X-100 to disrupt the vesicle membrane. The Triton X-100 concentration of 0.015% was the lowest concentration which abolished ATP-promoted $^{45}Ca^{2+}$ uptake in the same membrane fraction (*data not shown*). Since different composition of the incubation media might influence Ca^{2+} binding, it was determined in all uptake media used in this study. During 20 min of incubation binding of $^{45}Ca^{2+}$ to disrupted membrane vesicles reached between 50 and 60% of $^{45}Ca^{2+}$ content in intact vesicles in the absence of a sodium gradient.

Sodium Gradient-Driven Calcium Uptake

To investigate the presence of a Na^+/Ca^{2+} countertransport system in plasma membrane vesicles $^{45}Ca^{2+}$ uptake was measured with or without an outwardly directed sodium gradient. To check possible differences of $^{45}Ca^{2+}$ binding and passive influx in different incubation media, controls were performed with equal sodium or choline concentrations at both sides of the vesicle membrane (Fig. 3). The time course of $^{45}Ca^{2+}$ uptake shows that $^{45}Ca^{2+}$ uptake without sodium gradient reached a plateau after 20 min, whereas $^{45}Ca^{2+}$ uptake in the presence of an outwardly directed sodium gradient still continued after 20 min. The maximal sodium-driven $^{45}Ca^{2+}$ accumulation during 20 min was 311 ± 57 pmol/mg in not pre-equilibrated vesicles and 363 ±

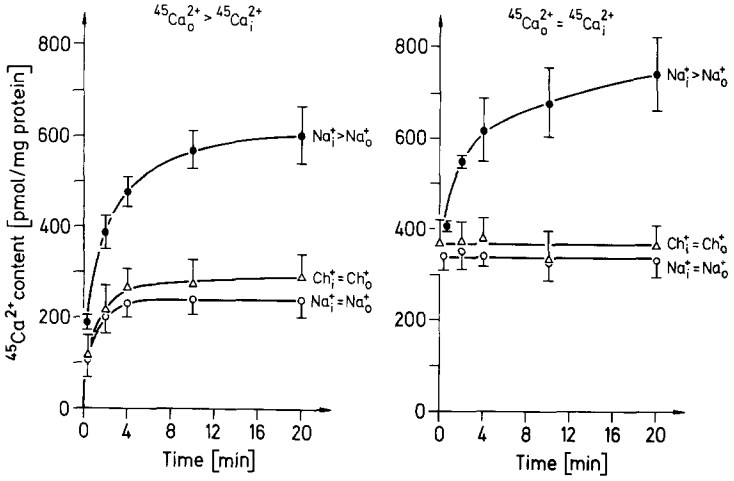

Fig. 3. Time-dependent $^{45}Ca^{2+}$ uptake in the presence or absence of an outwardly oriented sodium gradient. Vesicles were preincubated for 60 min in the presence of 100 mmol/liter Na_2SO_4 (○, ●) or 100 mmol/liter $choline_2SO_4$ (△) in the absence (left panel) or in the presence (right panel) of 10^{-5} mol/liter $^{45}Ca^{2+}$, and were diluted by 40-fold into a medium containing 100 mmol/liter Na_2SO_4 (○) or 100 mmol/liter $choline_2SO_4$ (△, ●) and 10^{-5} mol/liter $^{45}Ca^{2+}$. All measurements were performed in the presence of 66 mmol/liter K^+ and 2×10^{-6} mol/liter valinomycin. The values represent means ± SE of nine preparations (left panel) and three preparations (right panel). Ch = choline

78 pmol/mg protein in vesicles that were pre-equilibrated with $^{45}CaCl_2$ (Fig. 3). Sodium-driven $^{45}Ca^{2+}$ uptake as measured after 1 min of incubation was 62.3 ± 13.7 pmol/protein × min.

Sodium-Induced Calcium Efflux

Experimental conditions were chosen to demonstrate that the Na^+/Ca^{2+} countertransport system operates symmetrically. $^{45}Ca^{2+}$ efflux was therefore measured with or without an inwardly directed sodium gradient. To load the plasma membrane vesicles sufficiently with $^{45}Ca^{2+}$, they were preincubated in the presence of 10 mmol/liter MgATP and 1 mmol/liter $^{45}Ca^{2+}$ to allow MgATP-driven Ca^{2+} uptake in inside-out vesicles [3]. At this high Ca^{2+} concentration it can be assumed that mainly plasma membrane vesicles were loaded with Ca^{2+} since Ca^{2+} uptake into endoplasmic reticulum is nearly abolished at this high Ca^{2+} concentration [3, 4]. Vesicles were then diluted 40-fold into a medium that contained 5×10^{-4} mol/liter ortho-vanadate to inhibit the $Ca^{2+}(Mg^{2+})$-ATPase [21] and the MgATP concentration was diluted from 10 to 0.25 mmol/liter, a concentration at which ATP-promoted Ca^{2+} uptake was dramatically decreased [3]. Furthermore 0.2 mmol/liter EDTA were added to buffer the outside Ca^{2+} concentration to 10^{-7} mol/liter free Ca^{2+}. The time course of $^{45}Ca^{2+}$ release is shown in Fig. 4. The amount of $^{45}Ca^{2+}$ that could be released by an inwardly directed sodium gradient during 20 min was 252 ± 27 pmol/mg protein and was in the same range as sodium-driven $^{45}Ca^{2+}$ uptake. Ca^{2+} and Na^+ concentrations for maximal and half-maximal $^{45}Ca^{2+}$ uptake (*see below*) were therefore estimated assuming a symmetric behavior of the transport system. As in $^{45}Ca^{2+}$ uptake measurements (*see below*) lithium could replace sodium by 60% (Fig. 4).

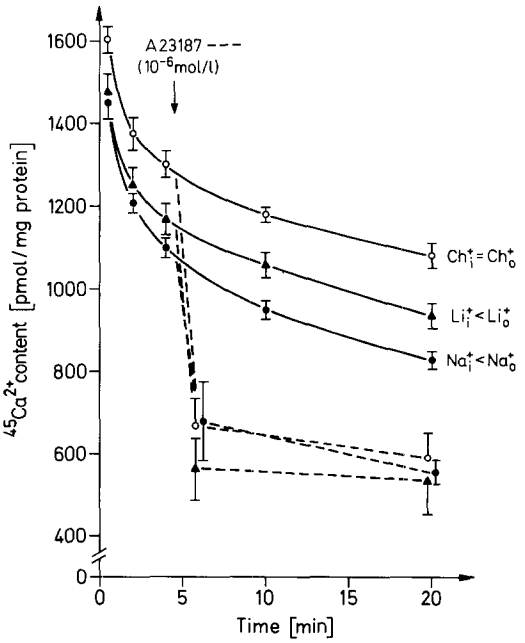

Fig. 4. Time-dependent $^{45}Ca^{2+}$ efflux in the presence or absence of inwardly oriented sodium or lithium gradients. Vesicles were preincubated for 40 min in the presence of 100 mmol/liter $choline_2SO_4$, 0.9 mmol/liter $CaCl_2$, 0.1 mmol/liter $^{45}CaCl_2$ and 10 mmol/liter MgATP. They were diluted by 40-fold into a medium containing 100 mmol/liter $choline_2SO_4$ (○), or 100 mmol/liter Na_2SO_4 (●), or 100 mmol/liter Li_2SO_4 (▲) and 0.2 mmol/liter EDTA and 0.5 mmol/liter vanadate. The Ca^{2+} ionophore A23187 was added to a final concentration of 10^{-6} mol/liter as indicated by the arrow. All measurements were performed in the presence of 66 mmol/liter K^+ at both sides of the membranes and 2×10^{-6} mol/liter valinomycin. The values are means ± SE of five preparations. Ch = choline

Cation Specificity of Na^+/Ca^{2+} Countertransport

To investigate the cation specificity of the Na^+/Ca^{2+} countertransport system, sodium was isosmotically replaced by lithium, caesium or rubidium and cal-

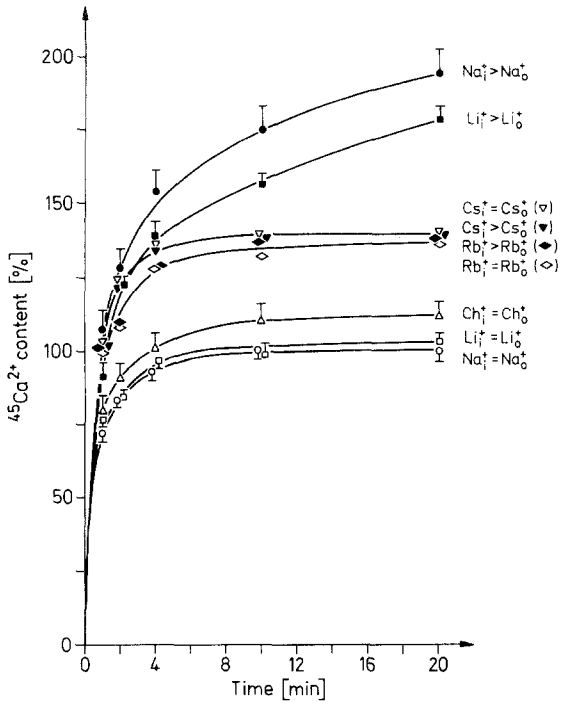

Fig. 5. Specificity for monovalent cations of the Na^+/Ca^{2+} countertransport system. Vesicles were preincubated for 60 min in the presence of 100 mmol/liter Na_2SO_4 (○, ●), or 100 mmol/liter Li_2SO_4 (□, ■), or 100 mmol/liter Cs_2SO_4 (▽, ▼) or 100 mmol/liter Rb_2SO_4 (◇, ◆) or 100 mmol/liter $choline_2SO_4$ (△). Then the vesicles were diluted by 40-fold into a medium containing 100 mmol/liter $choline_2SO_4$ (●, ■, ▼, ◆, △) or 100 mmol/liter Na_2SO_4 (○), or 100 mmol/liter Li_2SO_4 (□), or 100 mmol/liter Cs_2SO_4 (▽), or 100 mmol/liter Rb_2SO_4 (◇) and 10^{-5} mmol/liter $^{45}CaCl_2$. All measurements were performed in the presence of 66 mmol/liter K^+ at both sides of the membrane and 2×10^{-6} mol/liter valinomycin. The values are given in % of $^{45}Ca^{2+}$ uptake after 20 min with equal Na^+ concentrations at both sides of the vesicle membrane. The values are means ± SE of five preparations. Points without SE are the mean of two preparations. Ch = choline

cium was replaced by strontium. Since all measurements were performed in the presence of K^+ and valinomycin (*see* Materials and Methods), potassium was not used to replace sodium. As shown in Fig. 5, lithium could replace sodium by 76%, as in the Ca^{2+} efflux experiments (Fig. 4). No significant caesium or rubidium gradient-driven $^{45}Ca^{2+}$ uptake was measured (Fig. 6). $^{85}Sr^{2+}$ was used in a concentration of 10^{-5} mol/liter to test the specificity for calcium. The amount of sodium-driven $^{85}Sr^{2+}$ uptake was 32% (n = 2) as compared with sodium-driven $^{45}Ca^{2+}$ uptake (*data not shown*).

Ca^{2+} Concentration Dependence of Na^+/Ca^{2+} Countertransport

To investigate the affinity of the Na^+/Ca^{2+} countertransport system to Ca^{2+} the dependence of so-

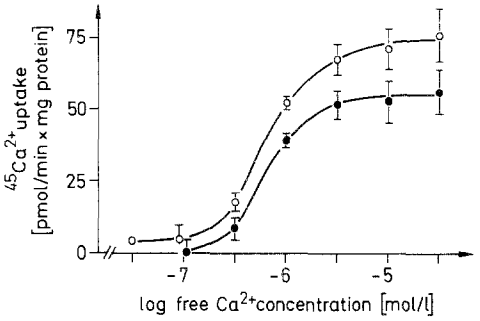

Fig. 6. Dependence of $^{45}Ca^{2+}$ uptake by the Na^+/Ca^{2+} countertransport system on calcium concentration. Vesicles were preincubated for 60 min in the presence of 100 mmol/liter Na_2SO_4 and the indicated $^{45}CaCl_2$ concentration. They were diluted by 40-fold into a medium containing 100 mmol/liter $choline_2SO_4$ and the same $^{45}CaCl_2$ concentration. To calculate sodium-driven $^{45}Ca^{2+}$ uptake, either $^{45}Ca^{2+}$ uptake in the presence of equal sodium (○) or equal choline (●) concentrations at both sides of the membrane was subtracted from total vesicular $^{45}Ca^{2+}$ content. All measurements were performed in the presence of 66 mmol/liter K^+ at both sides of the membrane and 2×10^{-6} mol/liter valinomycin. The free Ca^{2+} concentrations were buffered with EGTA for 3×10^{-8} mol/liter and EDTA for 10^{-7} mol/liter in the presence of 1 mmol/liter Mg^{2+} using the true proton, Ca^{2+} and Mg^{2+} dissociation constants of EGTA and EDTA [14, 29, 39]. The free Ca^{2+} concentrations at 10^{-5} and 3×10^{-5} mol/liter were unbuffered. To minimize the source of error the calcium concentrations were adjusted exclusively with $^{45}CaCl_2$ and each tracer batch was checked with a Ca^{2+}-specific electrode as described previously [43]. Furthermore all experiments were performed with identical solutions which were stored at −25°C between the experiments. The values are means ± SE of three preparations

dium-driven Ca^{2+} uptake on the free Ca^{2+} concentration was investigated. The indicated free Ca^{2+} concentrations were adjusted in the following way to obtain reliable results: All experiments were performed with identical solutions that were stored at −25°C between the uptake measurements. Total calcium concentrations were adjusted by $^{45}CaCl_2$ exclusively and the total Ca^{2+} concentration of each tracer batch was checked by a Ca^{2+}-selective electrode [43]. The free Ca^{2+} concentrations were buffered in the presence of 1 mmol/liter Mg^{2+} with 0.1 mmol/liter EGTA to adjust 3×10^{-8} mol/liter free Ca^{2+}; with 0.5, 0.25, 0.1 and 0.03 mmol/liter EDTA to adjust 10^{-7}, 3×10^{-7} and 3×10^{-6} mol/liter free Ca^{2+}, respectively, using the true proton, Ca^{2+} and Mg^{2+} dissociation constants of EGTA and EDTA [29, 39]. The free Ca^{2+} concentrations 10^{-5} and 3×10^{-5} mol/liter were unbuffered. Two controls without an outwardly directed sodium gradient were performed with either equal choline or equal sodium concentrations at both sides of the vesicle membrane (Fig. 6, closed or open dots). Sodium-driven $^{45}Ca^{2+}$ uptake after 1 min of incubation was measurable at free Ca^{2+} concentrations higher than 10^{-7} mol/liter, was maximal at 10^{-5} and half-maxi-

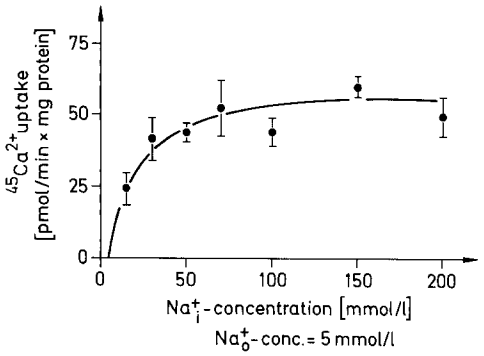

Fig. 7. Dependence of the Na^+/Ca^{2+} countertransport system for vesicular $^{45}Ca^{2+}$ content after 1 min of $^{45}Ca^{2+}$ uptake on different Na^+ concentrations. Vesicles were preincubated for 60 min in a 100 mmol/liter $choline_2SO_4$ solution where choline was partly replaced by the indicated sodium concentration and 10^{-5} mol/liter $^{45}CaCl_2$. They were diluted by 40-fold into a medium containing 100 mmol/liter $choline_2SO_4$ and 10^{-5} mol/liter $^{45}CaCl_2$. The Na^+ concentration at the outside of the vesicle was kept at 5 mmol/liter. To calculate sodium-driven $^{45}Ca^{2+}$ uptake, $^{45}Ca^{2+}$ uptake in the presence of equal choline concentrations at both sides of the membrane was subtracted from total vesicular $^{45}Ca^{2+}$ content. All measurements were performed in the presence of 66 mmol/liter K^+ at both sides of the membrane and 2×10^{-6} mol/liter valinomycin. The values are means ± SE from three preparations

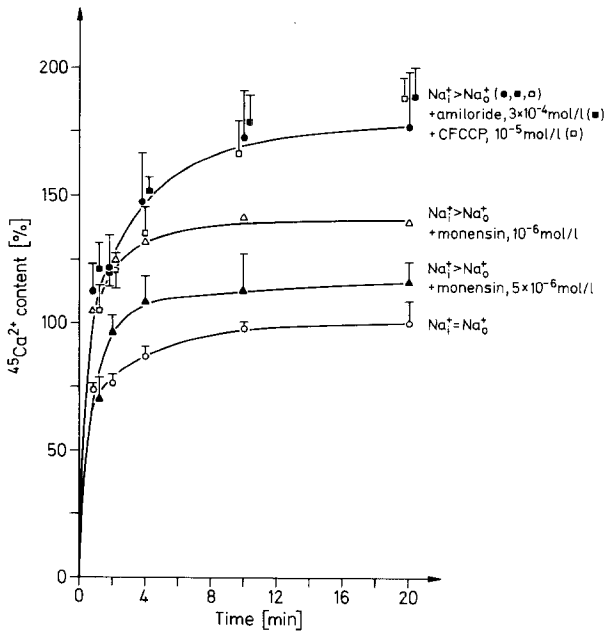

Fig. 8. Effect of monensin, CFCCP and amiloride on the Na^+/Ca^{2+} countertransport system. Vesicles were preincubated for 60 min in 100 mmol/liter Na_2SO_4 and were diluted by 40-fold into a medium containing 100 mmol/liter Na_2SO_4 (○) or 100 $choline_2SO_4$ (●) and 10^{-5} mol/liter $^{45}CaCl_2$. When the vesicles were diluted into a choline sulfate medium it contained in addition 10^{-5} mol/liter CFCCP (□), or 3×10^{-4} mol/liter amiloride (■), or 10^{-6} mol/liter monensin (△), or 5×10^{-6} mol/liter monensin (▲). All measurements were performed in the presence of 66 mmol/liter K^+ at both sides of the membrane and 2×10^{-6} mol/liter valinomycin. The values are given in % of $^{45}Ca^{2+}$ uptake after 20 min with equal Na^+ concentrations at both sides of the vesicle membrane. The values are means ± SE of three preparations. Points without SE are the mean of two preparations

mal at 6.2×10^{-7} mol/liter free Ca^{2+} concentration (Fig. 6).

Na^+ Concentration Dependence of Na^+/Ca^{2+} Countertransport

Similarly the affinity for Na^+ to the Na^+/Ca^{2+} countertransport system was investigated. In a 100 mmol/liter $choline_2SO_4$-containing medium for preincubation of the plasma membrane vesicles, $choline_2SO_4$ was partially replaced by either 7.5, 15, 25, 35, or 50, or totally by 100 mmol/liter Na_2SO_4. Sodium-driven $^{45}Ca^{2+}$ uptake as measured after 1 min of incubation was measurable at a Na^+ gradient of 15 mmol/liter at the inside of the vesicles with 5 mmol/liter Na^+ at the outside. It was maximal at about 150 and half-maximal at about 20 mmol/liter Na^+ concentration at the inside with 5 mmol/liter at the outside of the vesicle (Fig. 7).

Effect of Inhibitors on Na^+/Ca^{2+} Countertransport

The effect of monensin, a sodium-specific ionophore, was tested on sodium-driven $^{45}Ca^{2+}$ uptake. With 10^{-6} mol/liter monensin present in the uptake medium $^{45}Ca^{2+}$ uptake after 20 min of incubation was decreased by 40% and with 5×10^{-6} mol/liter monensin by 80% (Fig. 8). Furthermore, the effects of amiloride and the protonophore CFCCP were checked to exclude the possibility that apparent sodium-driven Ca^{2+} uptake was due to the presence of both coupled Na^+/H^+- and Ca^{2+}/H^+ systems in the plasma membrane. Whereas the latter has not been found, Na^+/H^+ exchange is present in pancreatic plasma membrane [17]. As shown in Fig. 8, amiloride, which inhibited Na^+/H^+ exchange in pancreatic acinar cells in a concentration of 10^{-4} mol/liter [17], did not significantly inhibit Na^+/Ca^{2+} exchange in a concentration of 3×10^{-4} mol/liter. However, it has been described that amiloride exerts its inhibitory effect on Na^+/Ca^{2+} exchange at higher concentrations than 10^{-4} mol/liter [35, 38] and that it competes with Na^+ for its binding sites. Thus the inhibitory effect would also depend on the Na^+ concentration [38, 46]. The effect of amiloride on Na^+/Ca^{2+} exchange was then further investigated using higher amiloride concentrations and also low Na^+ concentration. At an outwardly directed Na^+ gradient with a vesicle inside Na^+ concentration of 200 mmol/liter, an amiloride-

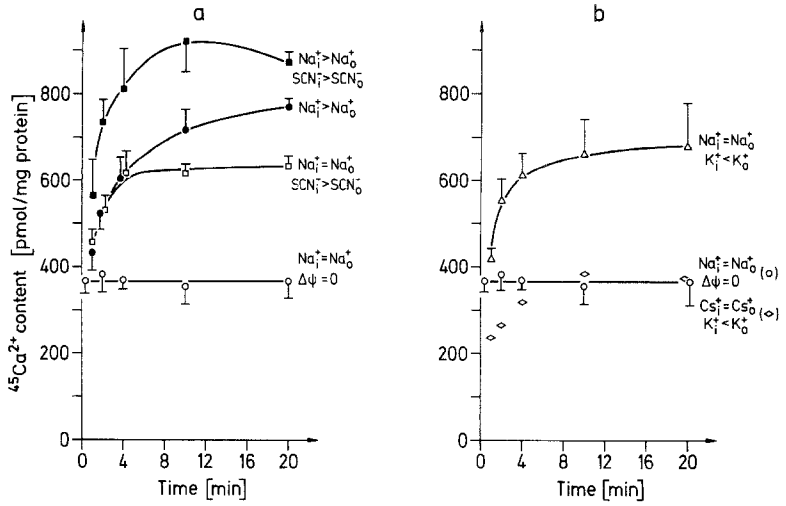

Fig. 9. Effect of a SCN^- (*a*) or a K^+ (*b*) diffusion potential on the Na^+/Ca^{2+} countertransport system. Control vesicles were preincubated for 60 min in 100 mmol/liter Na_2SO_4 and 10^{-5} mol/liter $^{45}CaCl_2$ and were diluted by 40-fold into a medium containing 100 mmol/liter Na_2SO_4 (○) or 100 mmol/liter $choline_2SO_4$ (●) and 10^{-5} mol/liter $^{45}CaCl_2$. 66 mmol/liter K^+ and 2×10^{-6} mol/liter valinomycin were present at both sides of the membrane in these measurements. To set an inside positive SCN diffusion potential (*a*), the vesicles were preincubated in 200 mmol/liter NaSCN and 10^{-5} mol/liter $^{45}CaCl_2$ and were diluted by 40-fold into a medium containing 100 mmol/liter Na_2SO_4 (□) or 100 mmol/liter $choline_2SO_4$ (■) and 100 mmol/liter mannitol to adjust the osmolarity and 10^{-5} mol/liter $^{45}CaCl_2$. To set an inside-positive K^+ diffusion potential (*b*), vesicles were preincubated in 50 mmol/liter Na_2SO_4 (△) or 50 mmol/liter Cs_2SO_4 ( ), 250 mmol/liter mannitol and 10^{-5} mol/liter $^{45}CaCl_2$ and were diluted into 50 mmol/liter Na_2SO_4 (△) or 50 mmol/liter Cs_2SO_4 (◇), 83 mmol/liter K_2SO_4, 10^{-5} mol/liter $^{45}CaCl_2$ and 2×10^{-6} mol/liter valinomycin

Table 2. Inhibition (in % of control) of Na^+/Ca^{2+} countertransport by amiloride at high and low Na^+ concentrations at the vesicle inside[a]

Amiloride concentration (mol/liter)		10^{-4}	3×10^{-4}	10^{-3}	3×10^{-3}	10^{-2}
Na^+ concentration	200 mmol/liter	0	0	0	15 ± 10	37 ± 10
	30 mmol/liter	0	8 ± 7	11 ± 4	44 ± 4	61 ± 8

[a] The data represent the inhibition of sodium gradient-driven $^{45}Ca^{2+}$ uptake by various amiloride concentrations in the presence of two different vesicle outwardly directed sodium gradients. Vesicles were preincubated for 60 min in the presence of 200 mmol/liter gluconate and 66 mmol/liter K gluconate, or in the presence of 30 mmol/liter Na gluconate and 170 mmol/liter choline gluconate, and the indicated amiloride concentration. Then vesicles were diluted 40-fold into a medium containing 200 mmol/liter choline gluconate, 66 mmol/liter K gluconate, 2×10^{-6} mol/liter valinomycin, 10^{-5} mol/liter $^{45}CaCl_2$ and the indicated amiloride concentration. The vesicle outside Na^+ concentration was kept at 5 mmol/liter. Samples were taken at 20 and 80 sec of incubation and $^{45}Ca^{2+}$ uptake was calculated for 1 min of incubation. Controls were performed with equal choline concentrations at both sides of the vesicle membrane for each amiloride concentration. The data give the inhibition in % of sodium-driven $^{45}Ca^{2+}$ uptake in the absence of amiloride. They are the mean ± SE from three preparations.

effected inhibition of Na^+/Ca^{2+} exchange was measurable at a concentration of 3×10^{-3} mol/liter and reached 37% inhibition at 10^{-2} mol/liter (Table 2). When the Na^+ concentration at the vesicle inside was lowered, the inhibitory potency of amiloride was increased about 10-fold and inhibition of Na^+/Ca^{2+} exchange was measurable at 3×10^{-4} mol/liter amiloride concentration. At 10^{-2} mol/liter amiloride the inhibitory effect reached 61% (Table 2). CFCCP (10^{-5} mol/liter) together with or without amiloride (3×10^{-4} mol/liter) had no influence on sodium-driven $^{45}Ca^{2+}$ uptake (Fig. 8).

Effect of Membrane on Na^+/Ca^{2+} Countertransport

Previous investigators provided evidence for a 3 : 1 coupling [5, 28, 30] of Na^+ and Ca^{2+} and also coupling in a 4 : 1 ratio was suggested [22, 26]. Since Ca^{2+} transport ratios of 3 : 1 or 4 : 1 would generate a membrane potential [8, 10, 32], it can be expected that a membrane potential (= $\Delta\varphi$) also influences the Na^+/Ca^{2+} countertransport system. Independently from the exact transport ratio, a transport ratio greater than 2 : 1 for the exchange of Na^+ versus Ca^{2+} would generate an inside-negative potential across the vesicle membrane. Thus the effect of an imposed inside-positive diffusion potential over the membrane on Na^+/Ca^{2+} countertransport seemed to be a useful tool to demonstrate a possible electrogenicity of the Na^+/Ca^{2+} countertransport system. Since passive Ca^{2+} influx would be stimulated only by an inside-negative membrane potential, the change of passive Ca^{2+} influx was neglected

for these experimental conditions. Diffusion potentials were generated with either K^+ in the presence of valinomycin or SCN^-. When SCN^- was used to create an inside-positive membrane potential, vesicles were loaded with 200 mmol/liter NaSCN and were diluted either into a medium of 100 mmol/liter Na_2SO_4 and 100 mmol/liter mannitol, or into 100 mmol/liter $choline_2SO_4$ and 100 mmol/liter mannitol. In the absence of a Na^+ gradient and with an outwardly directed SCN^- gradient, $^{45}Ca^{2+}$ uptake during 20 min was stimulated by 51% as compared to the control (Fig. 9*a*). In the presence of an outwardly directed SCN^- gradient and an additional outwardly directed Na^+ gradient, $^{45}Ca^{2+}$ uptake during 20 min was stimulated by 14% as compared to the control without a SCN^- gradient but with a Na^+ gradient (Fig. 9*a*). To set an inside- positive K^+ diffusion potential, vesicles were loaded with 50 mmol/liter Na_2SO_4 or Cs_2SO_4 and 250 mmol/liter mannitol and were diluted into a medium that contained 50 mmol/liter Na_2SO_4, or Cs_2SO_4 and 83.3 mmol/liter K_2SO_4 plus 2×10^{-6} mol/liter valinomycin thus creating an inside-positive diffusion potential. As can be seen from Fig. 9*b*, under these conditions $^{45}Ca^{2+}$ uptake during 20 min was stimulated by 62% as compared to the control without K^+ gradient. When sodium was replaced by caesium, $\Delta\varphi$-driven $^{45}Ca^{2+}$ uptake was below the control (Fig. 9*b*) indicating that $\Delta\varphi$-driven $^{45}Ca^{2+}$ uptake in the presence of sodium was mediated by the Na^+/Ca^{2+} countertransport system. $\Delta\varphi$-driven Ca^{2+} uptake mediated by Na^+/Ca^{2+} exchange increased linearly with the diffusion potentials generated by different SCN^- concentration differences across the vesicle membrane excluding unspecific effects of SCN^- on the plasma membrane (*data not shown*).

Stoichiometry of Na^+/Ca^{2+} Countertransport

In order to get further insight in the transport mechanism of the Na^+/Ca^{2+} countertransport system we calculated the Hill coefficient using the data presented in Fig. 7 for the Na^+ concentration dependence of sodium-driven Ca^{2+} uptake. A Hill plot of the results obtained is presented in Fig. 10. The Hill coefficient was calculated to be $n = 2.74 \pm 0.55$ from three experiments suggesting a Na^+/Ca^{2+} transport ratio of 3 : 1.

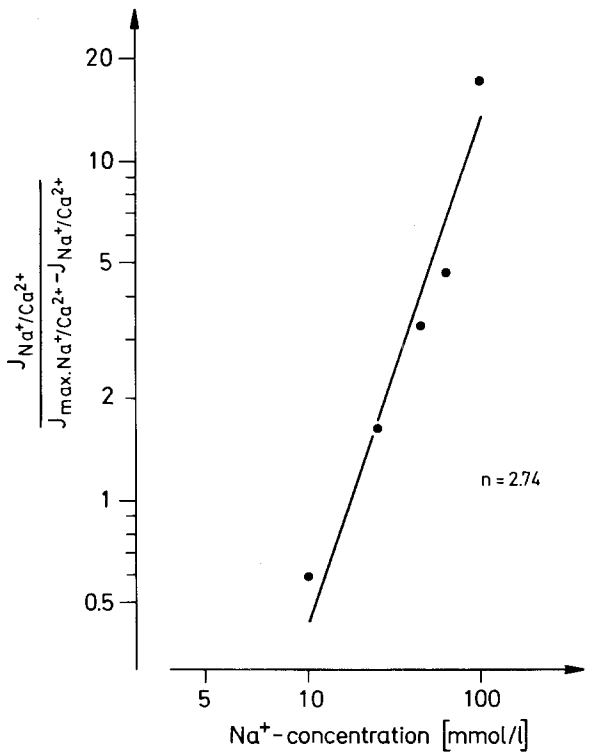

Fig. 10. Stoichiometry of Na^+/Ca^{2+} countertransport. Hill plot of the Na^+ concentration dependence of Na^+-driven Ca^{2+} uptake between 20 and 80 sec of incubation as shown in Fig. 7. The Hill coefficient n obtained from three experiments was 2.74 ± 0.55. Ordinate: $\log J_{Na^+Ca^{2+}}/J_{maxNa^+/Ca^{2+}} - J_{Na^+/Ca^{2+}}$. Abscissa: log vesicle inside/out Na^+ concentration difference

Discussion

Cytosolic free calcium concentration is regulated by calcium pumps in mitochondria, endoplasmic reticulum and plasma membrane, and also by a process of Na^+/Ca^{2+} exchange across the plasma membrane in different tissues [2, 6, 9–12, 18, 27, 31, 33, 36, 44, 45]. While short-term buffering of the cytosolic calcium concentration may be effected by sequestration of calcium into mitochondria and endoplasmic reticulum, these mechanisms must ultimately have a limited capacity, and long-term regulation of the cytosolic free calcium concentration must be due to calcium extrusion from the cell. Studies in various tissues [3, 5, 8, 10–12, 18, 21, 27] indicate that both, a Na^+/Ca^{2+} countertransport system, energized by the sodium gradient, and a $Ca^{2+}(Mg^{2+})$-ATPase are involved in the uphill extrusion of calcium, in order to maintain the cytosolic free calcium concentration at its physiological level of about 10^{-7} mol/liter [43].

For the exocrine pancreas indirect evidence for the presence of Na^+/Ca^{2+} countertransport system has been obtained from ^{45}Ca flux measurements in isolated cells [42]. In the presence of the (Na^+ + K^+)-ATPase inhibitor ouabain or in the absence of extracellular sodium, calcium extrusion from the cell was inhibited during hormone stimulation. These results suggested that calcium extrusion may be partially achieved by a Na^+/Ca^{2+} countertransport system located in the plasma membrane.

Advanced isolation procedures for plasma membrane vesicles derived from pancreatic acinar cells [3] enabled us to further investigate calcium extrusion mechanisms. We now report evidence for the existence of a Na^+/Ca^{2+} countertransport system in pancreatic acinar cells.

Preparation of Plasma Membranes

The isolation procedure for plasma membranes from pancreatic acinar cells as described previously [3] had to be modified, since the final protein yield was too low to investigate Na^+/Ca^{2+} countertransport. The $MgCl_2$ precipitation method used originally [3] consisting of three subsequent precipitation steps was shortened to two steps which raised the protein yield by about fourfold and the plasma membrane yield by twofold (Table 1) with a decrease of 50% in purity. The enrichment of $(Na^+ + K^+)$-ATPase activity was 23-fold as compared to the starting material (Table 1) indicating a purity comparable with other plasma membrane preparation methods [25]. Electron-microscopical investigation (Fig. 1) showed that about 50% 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 [47]. Decrease in the activity of the marker enzyme for the smooth endoplasmic reticulum, NADPH cytochrome *c* reductase, by threefold in the final plasma membrane fraction suggests that nearly all smooth membranes in the final fraction derived from the plasma membrane. It can be expected that shortening the isolation procedure improves the viability of the final membrane fraction. By the same reason the original method for cell preparation was modified by reducing the collagenase concentration by about 15% and the EDTA washing step from 15 to 5 min. These methodological changes seemed to maintain the impermeability of plasma membranes for sodium as judged by an increased sodium-driven $^{45}Ca^{2+}$ uptake as compared to uptake measurements without modifications (*data not shown*).

Localization of Na^+/Ca^{2+} Countertransport

Since the membrane fraction used in this study did not contain plasma membranes exclusively, the investigated Na^+/Ca^{2+} countertransport system should be localized. Previous investigations on Na^+/Ca^{2+} countertransport suggest that Na^+/Ca^{2+} countertransport systems are exclusively localized in the plasma membrane. We therefore correlated $^{45}Ca^{2+}$ uptake with the activity of the typical plasma membrane marker enzyme $(Na^+ + K^+)$-ATPase (Fig. 2). A purer plasma membrane fraction that was used previously [3] was compared with the membrane fraction enriched in plasma membranes as used in this study. As seen in Fig. 2 sodium-driven $^{45}Ca^{2+}$ uptake as measured after 1 min of incubation correlated with the $(Na^+ + K^+)$-ATPase activity by $r = 0.856$, indicating that Na^+/Ca^{2+} countertransport is localized in the plasma membrane. Furthermore correlation of Na^+/Ca^{2+} countertransport with the plasma membrane marker can be taken as indication for the presence of a Na^+/Ca^{2+} countertransport itself. If the data would represent binding of $^{45}Ca^{2+}$, correlation with the plasma membrane marker was unlikely.

Characterization of Na^+/Ca^{2+} Countertransport

The presence of a Na^+/Ca^{2+} countertransport system in plasma membranes of pancreatic acinar cells was investigated as Ca^{2+} uptake into and Ca^{2+} efflux from plasma membrane vesicles in the presence or absence of an outwardly or inwardly directed sodium gradient, respectively (Figs. 3 and 4). Sodium-driven Ca^{2+} uptake as measured after 1 min of incubation was 62 pmol/mg protein × min and similar as described for Na^+/Ca^{2+} countertransport in the small intestine and kidney cortex [9, 16].

Sodium-driven Ca^{2+} efflux was also investigated to demonstrate the symmetrical operation of the Na^+/Ca^{2+} countertransport system as described for other tissues [5, 7, 12, 16, 27, 31]. Sodium-induced Ca^{2+} efflux reached an absolute amount of 250 pmol/mg protein after 20 min (Fig. 4) which was in a similar range as sodium-driven Ca^{2+} uptake.

The ion specificity of Na^+/Ca^{2+} countertransport was determined by replacing sodium by lithium, rubidium, or caesium and replacing calcium by strontium. Lithium could replace sodium for Na^+/Ca^{2+} countertransport by about 75% in Ca^{2+} uptake measurements (Fig. 5) and about 60% in Ca^{2+} efflux studies (Fig. 4), whereas caesium or rubidium gradients could not drive Ca^{2+} uptake significantly (Fig. 5). This specificity of lithium for Na^+/Ca^{2+} countertransport agrees with the results from other tissues [2, 10, 12, 22]. Strontium has some affinity to this carrier since it was able to replace Ca^{2+} by about 35%.

Since the affinity of the Na^+/Ca^{2+} countertransport system for Ca^{2+} is important for the regulation of the cytosolic Ca^{2+} level, we have studied the dependence of sodium-driven Ca^{2+} transport into plasma membranes on the free Ca^{2+} concentration.

Ca^{2+} uptake into plasma membranes were measurable at concentrations higher than 10^{-7} mol/liter free Ca^{2+} and reached its maximum as plateau between 3×10^{-6} and 3×10^{-5} mol/liter free Ca^{2+} concentration. Half-maximal and maximal sodium-driven Ca^{2+} uptake was observed at 6.2×10^{-6} and 10^{-5} mol/liter free Ca^{2+} concentration, respectively (Fig. 6). These data seem reasonable regarding the regulation of the cytosolic free Ca^{2+} to about 10^{-7} mol/liter [5, 43] as a possible function of this Na^+/Ca^{2+} countertransport system. Furthermore Ca^{2+} concentrations for half-maximal and maximal sodium-driven Ca^{2+} uptake were similar to those obtained for MgATP-dependent Ca^{2+} transport in the same structure (Table 3, ref. 3). Similarly, submicromolar K_m values for sodium gradient-driven Ca^{2+} uptake were described for the kidney cortex [16] and the small intestine [9]. The K_m value for the Na^+/Ca^{2+} countertransport in these tissues was also close to that for ATP-dependent Ca^{2+} transport [9, 16]. In excitable tissues such as heart muscle, smooth muscle and nerve [5, 8, 10, 13, 22], as well as in the red blood cell and lymphocyte [27, 44], K_m values of Na^+/Ca^{2+} countertransport for Ca^{2+} were reported 10- to 100-fold higher. It should be noted, however, that Ca^{2+} uptake rates as measured between 20 and 80 sec of incubation must be considered with caution, since they could underestimate the true transport rate. Although rates from values taken after 20 sec were similar to those taken after 80 sec of incubation, initial rates were not measured because of limited yield of plasma membranes and specific activity of $^{45}Ca^{2+}$ available. Those who did measure initial rates [8, 30] obtained higher V_{max} of Na^+/Ca^{2+} exchange than the V_{max} of Ca^{2+}-ATPase. Thus, the difference in V_{max} of Na^+/Ca^{2+} exchange may result from methodology rather than from inherent differences between different systems.

Na^+/Ca^{2+} countertransport was also dependent on the sodium concentration inside the vesicles (Na_i^+). Sodium-driven Ca^{2+} uptake steeply increased at low outwardly directed sodium gradients to be half-maximal and maximal at a sodium gradient of 15 mmol/liter Na_i^+ at 5 mmol/liter Na_o^+ and 150 mmol/liter Na_i^+ at 5 mmol/liter Na_o^+, respectively (Fig. 7). With respect to the physiological intracellular and extracellular sodium concentrations, it seems reasonable that Na^+/Ca^{2+} countertransport was saturated at about 150 mmol/liter sodium in the presence of 5 mmol/liter at the other vesicle side. For the small intestine a sodium affinity in the same range has been reported [9]. A similar K_m value for sodium has also been recently described for a Na^+/H^+ exchange system in pancreatic acinar cells [17].

Inhibition of sodium-driven Ca^{2+} uptake by the Na^+/H^+ exchanger monensin (Fig. 8) provided another indication for coupling of Ca^{2+} transport to sodium via Na^+/Ca^{2+} countertransport and not via a combination of both Na^+/H^+ and Ca^{2+}/H^+ exchange. Furthermore, the effects of the diuretic amiloride and the protonophore CFCCP were tested to exclude coupling of the Na^+/H^+ exchange system described for pancreatic acinar cells [17] with a possible Ca^{2+}/H^+ exchange system. Amiloride which inhibits Na^+/H^+ exchange in pancreatic acinar cells in a concentration of 10^{-4} mol/liter [17] did not change sodium-driven Ca^{2+} uptake significantly in a concentration of 3×10^{-4} mol/liter (Fig. 8). The inhibitory effect of amiloride generally depends on the Na^+ concentration, since it competes with Na^+ for binding sites at the exchanger molecule [35, 46]. Higher concentrations of amiloride have been reported to inhibit Na^+/Ca^{2+} exchange in erythroleukemia cells [40], brain microsomes [35] and heart muscle [38]. When the Na^+ concentration inside the vesicles was lowered from 200 to 25 mmol/liter, the inhibitory potency of amiloride increased about 10-fold which was similar to that described in [46]. At maximal effective amiloride concentration of 10^{-2} mol/liter inhibition of the Na^+ gradient-driven initial Ca^{2+} uptake rate was 61% (Table 2). Since it has been proposed that the main site of action of amiloride is the cytoplasmic surface [38, 40], considerable effects of amiloride can be already expected at much lower concentrations because of low intracellular Na^+ concentration. The data suggest that the Na^+/Ca^{2+} countertransport system in pancreatic acinar cells behaves comparable to other Na^+/Ca^{2+} exchange systems [35, 38, 40] and could be a target for ana-

Table 3. Comparison of the plasma membrane-located $Ca^{2+}(Mg^{2+})$-ATPase and the Na^+/Ca^{2+} countertransport system in rat pancreatic acinar cells

$^{45}Ca^{2+}$ transport characteristics	Plasma membrane	
	Ca^{2+}-(Mg^{2+})-ATPase	Na^+/Ca^{2+} countertransport
Ca^{2+}: concentr. for		
max. uptake	10 μmol/liter	10 μmol/liter[b]
app.[a] K_m	0.88 μmol/liter	0.62 μmol/liter[b]
uptake	1.5 nmol/min × mg prot.	0.12 nmol/min × mg prot.[c]
Mg^{2+}: concentr. for		
max. uptake	3 mmol/liter	no Mg^{2+}
app. K_m	0.3 mmol/liter	dependence
energy supply:	ATP	Na^+ gradient via (Na^+ + K^+)-ATPase
concentr. for		
max. uptake	5 mmol/liter	c = 150 mmol/liter[b]
app. K_m	2 mmol/liter	c = 15 mmol/liter[b]
specificity	only ATP	$Na^+ > Li^+$

[a] app. = apparent.
[b] Values for $^{45}Ca^{2+}$ uptake during 1 min of incubation (*see* Results).
[c] Ca^{2+} uptake was calculated from experiments using the pure plasma membrane fraction (*see* Results and Fig. 3).

logs of amiloride with increased potency as described [38]. The protonophore CFCCP showed no effect of Na^+/Ca^{2+} countertransport [13] at a concentration of 10^{-5} mol/liter (Fig. 8) suggesting an operation of Na^+/Ca^{2+} countertransport independently from Na^+/H^+ exchange.

Stoichiometry of the Na^+/Ca^{2+} Countertransport

Transmembrane K^+ or SCN^- diffusion potentials ($\Delta\varphi$) stimulated Na^+-dependent Ca^{2+} uptake several-fold (*see* Results) (Figs. 9*a* and *b*), indicating that Na^+/Ca^{2+} exchange operates electrogenically in pancreatic plasma membranes similar to that in other tissues [8–10, 30, 32]. Since electrogenicity of Na^+-driven Ca^{2+} uptake indicates a transport ratio of Na^+ and Ca^{2+} greater than 2 : 1, we calculated the Hill coefficient to obtain a value for the Na^+/Ca^{2+} transport ratio (Fig. 10). This value of $n = 2.74$ is likely to be underestimated since relative high permeabilities of isolated membranes for Na^+ can be assumed. Also, since true initial rates of Ca^{2+} uptake have not been measured, driving forces during the observation period might not be constant. However, the value for n being close to 3 suggests electrogenicity and a transport ratio of at least 3 : 1 for the Na^+/Ca^{2+} exchange system.

Conclusion

By using a purified plasma membrane fraction from pancreatic acinar cells, Na^+/Ca^{2+} countertransport in this structure could be demonstrated. Na^+/Ca^{2+} exchange operates electrogenically and the transport ratio is proposed to be 3 : 1. The affinity of this system for Ca^{2+} was similar to the recently described ATP- and Mg-dependent Ca^{2+} transport in the same structure. This suggests that the extrusion of Ca^{2+} and thus the regulation of the cytosolic Ca^{2+} level in pancreatic acinar cells is performed by the parallel operation of Na^+/Ca^{2+} exchange system and a Ca^{2+} (Mg^{2+})-ATPase.

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