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Kinetic Properties of the ATP-Dependent Ca^{2+} Pump and the Na^+/Ca^{2+} Exchange System in Basolateral Membranes from Rat Kidney Cortex

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Summary. Basolateral plasma membranes from rat kidney cortex have been purified 40-fold by a combination of differential centrifugation, centrifugation in a discontinuous sucrose gradient followed by centrifugation in 8% percoll. The ratio of leaky membrane vesicles (L) versus right-side-out (RO) and inside-out (IO) resealed vesicles appeared to be L : RO : IO = 4 : 3 : 1. High-affinity Ca^{2+}-ATPase, ATP-dependent Ca^{2+} transport and Na^+/Ca^{2+} exchange have been studied with special emphasis on the relative transport capacities of the two Ca^{2+} transport systems. The kinetic parameters of Ca^{2+}-ATPase activity in digitonin-treated membranes are: K_m = 0.11 μM Ca^{2+} and V_{max} = 81 ± 4 nmol P_i/min · mg protein at 37°C. ATP-dependent Ca^{2+} transport amounts to 4.3 ± 0.2 and 7.4 ± 0.3 nmol Ca^{2+}/min · mg protein at 25 and 37°C, respectively, with an affinity for Ca^{2+} of 0.13 and 0.07 μM at 25 and 37°C. After correction for the percentage of IO-resealed vesicles involved in ATP-dependent Ca^{2+} transport, a stoichiometry of 0.7 mol Ca^{2+} transported per mol ATP is found for the Ca^{2+}-ATPase. In the presence of 75 mM Na^+ in the incubation medium ATP-dependent Ca^{2+} uptake is inhibited 22%. When Na^+ is present at 5 mM an extra Ca^{2+} accumulation is observed which amounts to 15% of the ATP-dependent Ca^{2+} transport rate. This extra Ca^{2+} accumulation induced by low Na^+ is fully inhibited by preincubation of the vesicles with 1 mM ouabain, which indicates that (Na^+-K^+)-ATPase generates a Na^+ gradient favorable for Ca^{2+} accumulation via the Na^+/Ca^{2+} exchanger. In the absence of ATP, a Na^+ gradient-dependent Ca^{2+} uptake is measured which rate amounts to 5% of the ATP-dependent Ca^{2+} transport capacity. The Na^+ gradient-dependent Ca^{2+} uptake is abolished by the ionophore monensin but not influenced by the presence of valinomycin. The affinity of the Na^+/Ca^{2+} exchange system for Ca^{2+} is between 0.1 and 0.2 μM Ca^{2+}, in the presence as well as in the absence of ATP. This affinity is surprisingly close to the affinity measured for the ATP-dependent Ca^{2+} pump. Based on these observations it is concluded that in isolated basolateral membranes from rat kidney cortex the Ca^{2+}-ATPase system exceeds the capacity of the Na^+/Ca^{2+} exchanger four- to fivefold and it is therefore unlikely that the latter system plays a primary role in the Ca^{2+} homeostasis of rat kidney cortex cells.

Key Words Ca^{2+}-ATPase · ATP-dependent Ca^{2+} transport · Na^+/Ca^{2+} exchange · rat kidney cortex · basolateral membranes

Introduction

The major fraction of the filtered calcium is reabsorbed in the proximal tubule and the bulk of this Ca^{2+} reabsorption is generally thought to be passive (Agus, Chiu & Goldberg, 1977; Ng, Peraino & Suki, 1982). *In situ* microperfusion experiments of proximal tubules demonstrate a high calcium permeability and therefore passive Ca^{2+} reabsorption is most likely paracellular (Murayama, Morel & Legrimellec, 1972; Bomsztyk & Wright, 1982). There is also evidence for a small active component of Ca^{2+} reabsorption in proximal tubules in the presence of a mannitol diuresis (Lassiter, Gottschalk & Mylle, 1963) and in the presence of an equilibrium solution in the lumen (Ullrich, Rumrich & Kloss, 1976). In the latter study more calcium was transported than could be accounted for by the electrochemical gradient. Moreover, this active Ca^{2+} reabsorption was dependent on peritubular Na^+ and inhibited by ouabain (Ullrich et al., 1976). Active Ca^{2+} reabsorption must be transcellular and Ca^{2+} pumps in the basolateral membrane have to be involved to overcome the steep energy barriers at the peritubular cell side. Gmaj, Murer and Kinne (1979) demonstrated in isolated basolateral membranes from rat kidney cortex ATP-dependent Ca^{2+} transport as well as Na^{2+}/Ca^{2+} exchange activity. They concluded that Na^+/Ca^{2+} exchange is responsible for transcellular Ca^{2+} transport, whereas Ca^{2+}-ATPase is involved in fine regulation of intracellular calcium. However, this view is not supported by Ca^{2+} flux studies on slices and isolated cells from rat kidney cortex (Borle, 1981; 1982). These studies demonstrated that in kidney cells low extracellular Na^+ depresses Ca^{2+} influx as well as Ca^{2+} efflux, but no evidence could be found for involvement of a Na^+/Ca^{2+} exchange system in Ca^{2+} efflux.

Since the original demonstration of ATP-dependent Ca^{2+} transport in basolateral membranes, high-affinity calmodulin-dependent Ca^{2+}-ATPase activity has been described (De Smedt et al., 1981; Gmaj, Murer & Carafoli, 1982) and a phosphorylated intermediate of this ATPase has been identified on SDS-acrylamide gels (De Smedt et al., 1983). No further studies on the properties of the Na^+/Ca^{2+} exchange system have been reported. For this reason we investigated Ca^{2+}-transport mechanisms in a highly purified basolateral membrane preparation from rat kidney cortex with special emphasis on the transport capacities of Ca^{2+}-ATPase and Na^+/Ca^{2+} exchange. From this study we conclude that Na^+/Ca^{2+} exchange in rat kidney cortex cells is of minor importance since the ATP-dependent Ca^{2+} pump exceeds the Na^+/Ca^{2+} exchange capacity four- to five-fold. Surprisingly, the affinity for Ca^{2+} of both systems appears to be identical.

Materials and Methods

Membrane Preparation

Three male Wistar rats (180 to 220 g) are decapitated, the kidneys removed and decapsulated. Cortical slices are homogenized in 30 ml homogenization buffer (250 mM sucrose, 25 mM NaCl, 5 mM Hepes titrated to pH 7.4 with Tris, 0.5 mM dithiothreitol (DTT) and 0.2 mM phenylmethylsulfonylfluoride (PMSF) with a loose-fitting dounce apparatus (15 strokes). The homogenate is filtered through cheesecloth and then centrifuged at $50 \times g_{av}$ for 8 min to remove glomeruli, whole cells and cell debris. The supernatant is brought at 38% (wt/wt) sucrose by adding 60% (wt/wt) sucrose buffered with 5 mM Hepes/Tris (pH 7.4), poured into centrifuged tubes, overlayed with 250 mM sucrose in 5 mM Hepes/Tris and centrifuged at $200{,}000 \times g_{av}$ for 60 min. The bands appearing at the interface of 8 and 38% sucrose, containing predominantly plasma membranes, are collected and suspended in 250 mM sucrose in 5 mM Hepes/Tris and brought at 8% percoll in a total volume of 20 ml. After 30 min centrifugation at $50{,}000 \times g_{av}$ the bands appearing in the upper part of the percoll gradients are collected and diluted with the uptake medium without the Ca^{2+} buffers (20 mM Hepes/Tris, pH 7.4, 5 mM $MgCl_2$ and 150 mM KCl or NaCl). The membrane material is pelleted at $95{,}000 \times g_{av}$ for 30 min in a fixed angle rotor. The fluffy layer on top of the glassy percoll pellet, containing basolateral membranes, is resuspended again in the same medium by using a syringe with a fine needle.

Enzyme Assays

Protein is determined with a commercial Coomassie blue kit (BioRad). The following marker enzymes are assayed according to previously published procedures: $(Na^+$-$K^+)$-ATPase for basolateral membranes (Mircheff & Wright, 1976); maltase (Dahlqvist, 1964) and alkaline phosphatase (Mircheff & Wright, 1976) for brush border membranes; succinic acid dehydrogenase for mitochondria (Pennington, 1961) and NADPH-dependent cytochrome-*c* reductase for smooth endoplasmic reticulum (Sottocasa et al., 1967). Ca^{2+}-ATPase is assayed in the range of 0.05 to 1 μM free Ca^{2+} in a medium containing (mM): 100 NaCl or KCl, 50 Tris-maleate buffer (pH 7.4), 1 theophylline, 5 $MgCl_2$, 3 Tris-ATP, 0.5 EGTA, 0.5 NTA and calculated amounts of $CaCl_2$ to bring the free Ca^{2+} concentration at the desired level (*see* Appendix).

The reaction was started by adding 0.5 ml of the above medium to aliquots of 10 to 25 μl basolateral membrane suspensions. After incubation for 30 min at 37°C, the reaction is stopped with 0.5 ml 5% trichloroacetic acid and phosphate is analyzed as described by Bonting (1970).

For membrane sidedness studies the vesicles are suspended in the uptake medium with 150 mM NaCl. When necessary the vesicles are further diluted with the same medium and an amount of detergent is added. The vesicles are then assayed for $(Na^+$-$K^+)$-ATPase activity. To unmask inside-out oriented resealed vesicles, $(Na^+$-$K^+)$-ATPase activity is determined in the presence of ouabain (1 mg/ml) and the difference in ATP hydrolysis in the presence and absence of 10 mM KCl is determined. Vesicles assayed in KCl containing medium are preincubated with 10 mM KCl for 10 min on ice.

ATP-Dependent Ca^{2+} Uptake Studies

In general, the basolateral membrane vesicles are suspended in the uptake medium with 150 mM KCl. Further additions or replacements will be referred to under these particular experiments. At zero time two volumes of an uptake medium are added to one volume of vesicle suspension containing 1 to 1.5 mg/ml protein. The final concentrations are 150 mM KCl, 20 mM Hepes/Tris, pH 7.4, no ATP or 1 to 6 mM Tris-ATP, 0.5 mM EGTA, 0.5 mM NTA, an amount of calculated $CaCl_2$ to bring the free Ca^{2-} concentration to the desired level (0.025 to 1 μM) and a calculated amount of $MgCl_2$ to keep the free Mg^{2+} concentrations at about 1.7 mM (*see* Appendix). Finally, the medium contained 3 μCi/ml ^{45}Ca. Four minutes before the experiment, the vesicles and the uptake medium are brought to the desired temperature in a water bath. At certain time intervals 50-μl aliquots are taken and quenched into 1 ml stopping solution containing 1 mM $LaCl_3$, 150 mM KCl and 20 mM Hepes/Tris, pH 7.4. Membranes are collected on 0.45 μm Sartorius filters (type SM11306), precooled with 2 ml ice-cold stopping solution. The filter with sample is washed twice with 2 ml stopping solution. The radioactivity on the filter is counted in Bray's solution and compared to the activity of 5-μl samples of the incubation mixtures.

To study the influence of sodium on the ATP-dependent Ca^{2+} uptake, the vesicles in 150 mM KCl solutions are mixed with an equimolar NaCl solution in order to get the desired sodium concentration. When ouabain is used it is added already during the isolation after the percoll centrifugation step. In this way ouabain is present at least one hour before the experiment to make sure that it is present inside the vesicles. When other additions have to be made, they are present in the vesicle suspension at least 10 min before the experiment, unless indicated otherwise.

Na^+ Gradient-Driven Ca^{2+} Uptake

Basolateral membrane vesicles are pelleted and resuspended in the uptake medium with 150 mM NaCl instead of KCl. The reaction mixture contained either 150 mM KCl or NaCl. The further composition was such that after a 15-fold dilution of the vesicles in the medium, the mixture contains 20 mM Hepes/Tris, 75 μCi ^{45}Ca/ml, 0.5 mM EGTA, 0.5 mM NTA, $CaCl_2$ and $MgCl_2$ in calcu-

lated amounts so that the free concentrations are 0.1 to 5 μM Ca^{2+} and 1.7 mM Mg^{2+}. Mixing is accomplished in a test tube where a droplet of membrane vesicles is pipetted against the wall above the uptake medium. By activating the vortex for mixing an automatic pipettor is triggered which quenches the uptake with stopping solution after a preset time. Collection of the membranes and counting of radioactivity are done as described above. The radioactivity on the filter is compared to the activity of a 2.0-μl sample of the stopped mixture.

Results

Basolateral Membrane Isolation and Orientation

The recovery and purification of several marker enzymes during isolation of basolateral membranes from rat kidney cortex is given in Table 1. The (Na^+-K^+)-ATPase activity is enriched eightfold after discontinuous sucrose gradient centrifugation. This fraction is still heavily contaminated with brush border membranes and fragments from smooth endoplasmic reticulum. Additional centrifugation in a self-generating percoll gradient yields a highly purified basolateral membrane fraction, although the recovery is only 14% of the activity found in the homogenate. The combination of sucrose and percoll gradients gives the highest purification of (Na^+-K^+)-ATPase activity reported so far for kidney basolateral membranes (Heidrich et al., 1972; Gmaj et al., 1979; Inui et al., 1981; Gmaj et al., 1982). Starting from 3 rats the yield of basolateral membranes is about 1.4 mg protein.

The degree of resealing and the orientation of the basolateral membrane vesicles have been studied by subjecting the membranes to several treatments such as osmotic shocks, lyophilyzing, freeze-thawing and incubation with detergents. Assuming that resealed vesicles are impermeable for either ATP and ouabain, then (Na^+-K^+)-ATPase activity of untreated vesicles reflects the leaky vesicle fraction (Fig. 1). After an osmotic shock the vesicles appear to reseal while lyophylizing or freeze-thawing decreases the (Na^+-K^+)-ATPase activity. Detergent treatment gives the highest stimulation of (Na^+-K^+)-ATPase activity. The results with SDS, Triton X-100 and digitonin are most promising while Tween-80, deoxycholic and cholic acid give less reproducible results. In Table 2 the effects of SDS, Triton X-100 and digitonin on (Na^+-K^+)-ATPase latency are shown. Digitonin is the most convenient detergent since its effect on (Na^+-K^+)-ATPase activity is independent of the protein concentration in the sample. Digitonin treatment inhibited Mg-ATPase activity by 40% and stimulated (Na^+-K^+)-ATPase twofold (Table 2). SDS and Triton X-100 have an optimal concentration range and higher concentrations inhibit (Na^+-K^+)-ATPase activity.

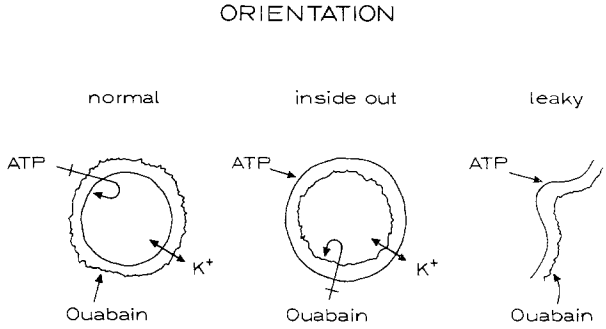

Fig. 1. Schematic representation of the assay of the percentage of inside-out-oriented vesicles. For explanation *see* text

Table 1. Relative recoveries and enrichment factors of marker enzymes in rat kidney cortex basolateral membranes

	Homogenate		Band at 8–38% sucrose interface		8% Percoll band final pellet		
	SA	% recovery	SA	% recovery	SA	% recovery	*n*
Protein	442 ± 8	100	38.9 ± 3.1	8.8	1.4 ± 0.02	0.31	(10)
(Na^+-K^+)-ATPase (37°C)	1.43 ± 0.13	100	12.2 ± 0.6	75 ± 6	57 ± 6	14.1	(8)
enrichment		1×		8.3×		44×	
Alkaline phosph. (20°C)	3.3 ± 0.2	100	6.9 ± 0.3	17 ± 2	9.2 ± 1.3	0.9 ± 0.2	(10)
enrichment		1×		2.1×		2.8×	
Maltase (37°C)	—	100	—	27 ± 3[a]	—	0.6 ± 0.3	(4)
enrichment				3.3×		2.2×	
Succinic dehydrogenase	—	100	—	9.2 ± 1	—	0.3 ± 0.1	(7)
(20°C) enrichment		1×		1.2 ± 0.1×		1×	
NADPH-dependent cyt-*c*-reductase	—	100	—	18.6 ± 2	—	1.2 ± 0.2	(4)
(20°C) enrichment		1×		2.3 ± 0.2×		3.5×	

[a] Maltase activity overestimated due to high sucrose content of this fraction. Mean values are given with SEM. Specific Activity (SA) of (Na^+-K^+)-ATPase and alkaline phosphatase is expressed as μmol P_i/hr · mg protein.

The conclusion of these studies are that 50% of the vesicle population is resealed for ATP and ouabain. When the homogenate is treated with 0.04% digitonin, the (Na^+-K^+)-ATPase increases from 1.43 to 3.58 μmol P_i/hr · mg (n = 4). This result suggests that 60% of the vesicle population in the homogenate is resealed. Therefore, the enrichment factor for (Na^+-K^+)-ATPase in the final BLM preparation is 32 instead of 44 when activities are compared in digitonin-treated samples. However, in literature usually values of untreated membrane fractions are compared as given in Table 1.

The percentage of resealed vesicles with an inside-out orientation (IO) can be obtained by measuring the stimulation of ATP hydrolysis induced by K^+ ions in a medium containing Na^+ and ouabain. During short incubations, K^+ ions will equilibrate across the vesicle membrane quickly while ouabain does not (*see* Fig. 1). The percentage stimulation of ATP hydrolysis by K^+ appeared independent of the incubation time up to 20 min and then decreased. Apparently, after 20 min ouabain equilibrates across the membrane and inhibits (Na^+-K^+)-ATPase in IO vesicles. Valinomycin did not further increase the K^+ stimulation of ATP hydrolysis which indicates that K^+ ions easily permeate. In Table 3 the results of this approach are summarized. K^+ ions stimulation of ATP hydrolysis amounts to 30% of the (Na^+-K^+)-ATPase activity in untreated membranes and to 15% of digitonin-treated membranes. Therefore 15% of the pump sites are inaccessible to ouabain, hence 15% of the population is inside-out oriented. When basolateral vesicles are first treated with digitonin there is 3% stimulation of ATP hydrolysis by K^+ ions in the presence of ouabain, indicating that the membrane barrier for ouabain is destroyed. This rest activity of K^+ stimulation of ATP hydrolysis after digitonin treatment may be caused by solubilization of (Na^+-K^+)-ATPase or loss of ouabain sensitivity. However, correction for this small effect leaves us with 12% IO vesicles. In conclusion, the ratio of leaky (L) versus right-side-out (RO) versus inside-out in this membrane preparation is roughly: L : RO : IO = 4 : 3 : 1.

Table 2. Effects of detergents on (Na^+-K^+)-ATPase activity in basolateral membranes of rat kidney cortex

Concentration range tested	Stimulation of (Na^+-K^+)-ATPase at the optimal concentration
Digitonin (0.005 to 0.1% wt/vol)	2.01 × (±0.14)[a] (n = 4)[b]
Sodium dodecyl sulfate (0.3 to 1.3 mM)	1.98 × (±0.23) (n = 4)[b]
Triton X-100 (0.4 to 1.3 mg/mg protein)	2.26 × (±0.10) (n = 4)[b]

	Mg^{2+}-ATPase	(Na^+-K^+)-ATPase
Control	105 ± 7 (n = 32)	58 ± 6 (n = 13)
Digitonin (0.04% wt/vol)	64 ± 8 (n = 13)	114 ± 8 (n = 13)
Stimulation		1.96×

[a] Values at 0.04% wt/vol. Specific activities of Mg^{2+}-ATPase and (Na^+-K^+)-ATPase in μmol P_i/hr · mg.
[b] Digitonin, SDS and Triton X-100 effects on the same 4 batches of membranes.

Kinetics of ATP-Dependent Ca^{2+} Uptake and Ca^{2+}-ATPase

In Fig. 2 the effect of ATP and the Ca^{2+} ionophore A23187 on Ca^{2+}-uptake by rat kidney cortex basolateral membranes is shown at 25°C and 1 μM free Ca^{2+}. At 8 min a 10-fold stimulation by ATP is observed above the plateau reached in the absence of ATP. The plateau reached in the presence of ATP can be further increased by using higher ATP and lower protein concentrations in the uptake assay, since ATP depletion is the major cause of slowing down of the uptake rate in Fig. 2. In the presence of 6 mM ATP the initial rate of uptake is constant up to 10 min (*see* Fig. 8). Addition of A23187 after 8 min of Ca^{2+} uptake causes a rapid release of 70% of the accumulated Ca^{2+}. The remaining 30% is probably bound to the inside. Control experiments in the presence of 10 μg/ml oligomycin or 10 μg/ml valinomycin excluded a possible mitochondrial contribution to the ATP-dependent Ca^{2+} uptake or an effect of membrane potential on this uptake.

In Figs. 3 and 4 the kinetic properties of ATP-dependent Ca^{2+} uptake are shown at 25 and 37°C, respectively. Initial rates are presented which have been determined from Ca^{2+} uptake values at 0.5 and 1.0 min in the presence of 3 mM ATP. The maximal rates of uptake occur around 1 μM free Ca^{2+} and are

Table 3. Stimulation of ATPase activity by 10 mM K^+ in the presence of ouabain and Na^+

	Untreated membranes stimulation of ATPase activity	Digitonin-treated membranes stimulation of ATPase activity
10 mM K^+	30 ± 4%[a] (n = 8)	3 ± 1%[b] (n = 4)
% inside-out vesicles of total population	15 ± 1%	12 ± 1%[c]

[a] K^+ stimulation of ATPase activity expressed as % of (Na^+-K^+)-ATPase activity measured in untreated basolateral membranes.
[b] K^+ stimulation of ATPase activity expressed as % of the (Na^+-K^+)-ATPase activity measured in digitonin-treated membranes.
[c] % inside-out vesicles after correction for K^+ effect on digitonin-treated vesicles.

4.3 ± 0.2 and 7.4 ± 0.3 nmol Ca^{2+}/min · mg protein at 25 and 37°C. Realizing that only IO resealed vesicles contribute to ATP-dependent Ca^{2+} uptake, then the estimated V_{max} at 37°C should be multiplied by a factor of 8. This brings the maximal transport capacity of the ATP-dependent Ca^{2+} pump to 59 nmol Ca^{2+}/min · mg protein.

The increase in V_{max} with temperature yields a Q_{10} of 1.54 ± 0.08 or an apparent activation energy of 7.9 ± 1.0 kcal/mol. This Q_{10} value is comparable to the value of 1.49 reported by Kasson and Levin (1981) for pancreatic islet Ca^{2+}-ATPase. The affinity of the Ca^{2+} pump for Ca^{2+} ions extrapolated from the Eadie-Hoffstee plots is 0.13 μM at 25 and 0.07 μM at 37°C. These apparent K_m values are comparable to the free Ca^{2+} concentration in the cytosol of proximal tubular epithelium (Lee, Taylor & Windhager, 1980; Borle & Snowdowne, 1982).

The enzymatic expression of the ATP-dependent Ca^{2+} pump has been studied with basolateral membranes treated with 0.04% digitonin. In these preparations Ca^{2+}-ATPase activity should be optimal since all vesicles have lost their barrier towards ATP. The assay of high-affinity Ca^{2+}-ATPase remains troublesome since addition of 1 μM Ca^{2+} only stimulates the ATP hydrolysis between 5 and 10% above the background hydrolysis due to Mg^{2+}-ATPase activity. Figure 5 shows the relative rates of ATP hydrolysis as a function of free Ca^{2+} concentrations between 0.025 and 1 μM at 37°C. These results are mean values of eight experiments. Note that the standard errors are considerably larger than those in Figs. 3 and 4. This is entirely due to the relatively small increase in ATP hydrolysis measured against a high blackground level. The kinetic parameters which can be extrapolated from the

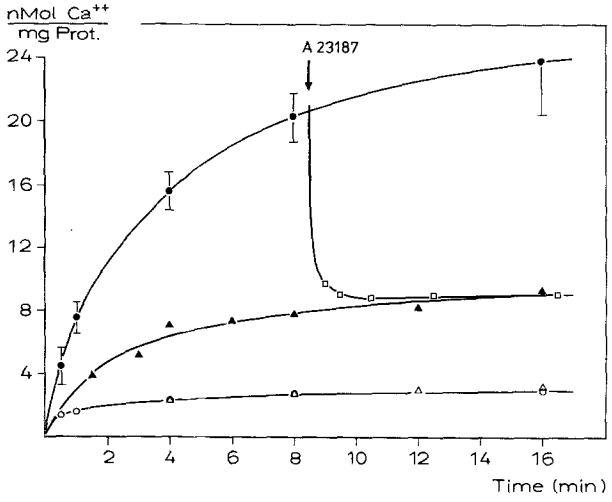

Fig. 2. Effect of ATP and A23187 on Ca^{2+} uptake by basolateral membranes of rat kidney cortex. ●—● uptake in the presence of 1 mM ATP; O—O uptake in the absence of ATP. Free Ca^{2+} concentrations is 1 μM and free Mg^{2+} is 1.7 mM in both situations. □—□ release of Ca^{2+} by addition of 10 μg/ml A23187. ▲—▲ uptake in the presence of ATP and A23187. △—△ uptake in the absence of ATP and presence of A23187. Mean values of 4 experiments are given

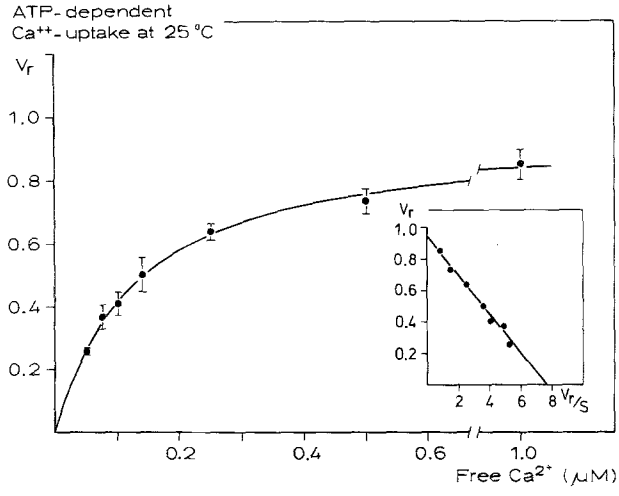

Fig. 3. Kinetics of ATP-dependent Ca^{2+} uptake in basolateral membranes of rat kidney cortex at 25°C. Relative rates which are mean values of nine experiments are given. Initial rates of ATP-dependent Ca^{2+} uptake have been corrected for ATP independent uptake. The inset shows an Eadie-Hoffstee plot of the data. K_m for Ca^{2+} is 0.13 μM and V_{max} = 4.3 ± 0.2 nmol Ca^{2+}/min · mg protein

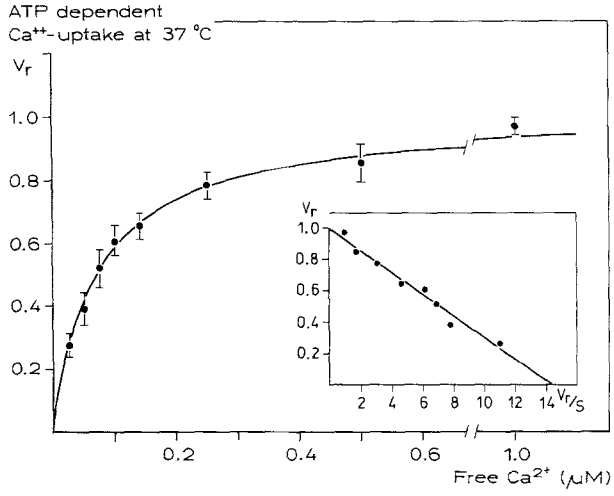

Fig. 4. Kinetics of ATP-dependent Ca^{2+} uptake in basolateral membranes of rat kidney cortex at 37°C. Relative rates which are mean values of six experiments are given. Initial rates of ATP-dependent Ca^{2+} uptake have been corrected for ATP-independent Ca^{2+} uptake. The inset shows an Eadie-Hoffstee plot of the data. K_m for Ca^{2+} is 0.07 μM and V_{max} = 7.4 ± 0.3 nmol Ca^{2+}/min · mg protein

Eadie-Hoffstee plot (inset in Fig. 5) are a V_{max} value of 81 ± 4 nmol P_i/min · mg protein and a K_m value of 0.11 μM. The affinity for Ca^{2+} of the Ca^{2+}-ATPase is very similar to the affinity of the ATP-dependent Ca^{2+} uptake and it is therefore unlikely that digitonin treatment influences the affinity. Whether digitonin affected the V_{max} of Ca^{2+}-ATPase was checked in the following way. Starting the isolation procedure with frozen cortex slices yielded a basolateral membrane fraction in which (Na^+-K^+)-ATPase could not be stimulated by detergent treatment. Apparently the freeze-thaw cycle before homogenization yields leaky vesicles. In four such preparations the Ca^{2+}-ATPase activity at 1 μM free Ca^{2+} amounted to the same level as the activity measured in digitonin-treated membranes.

When the V_{max} value of Ca^{2+}-ATPase is compared with the one for ATP-dependent Ca^{2+} uptake after correction for the IO vesicle population, it can be concluded that the stoichiometry of the ATP-driven Ca^{2+} pump is 0.73 mol Ca^{2+} per mol ATP. This value is close to 1.0, the stoichiometry found for reconstituted Ca^{2+}-ATPase purified from erythrocyte membranes (Niggli et al., 1981). Our value for stoichiometry is likely to be an underestimate since the degree of resealing was determined for ATP and ouabain and it is likely that a smaller percentage is resealed for Ca^{2+}. However, our estimate of 0.73 indicates that ATP-dependent Ca^{2+} transport and Ca^{2+}-ATPase activity are expressions of the same enzyme system.

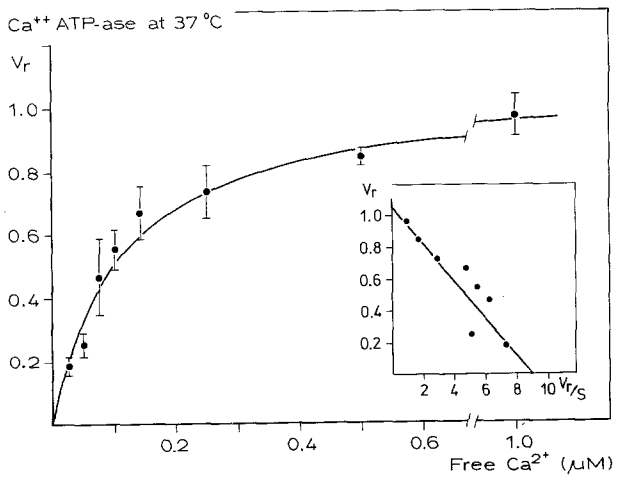

Fig. 5. Kinetics of Ca^{2+}-induced ATP hydrolysis in basolateral membranes of rat kidney cortex. Relative rates, which are mean values of eight experiments are given. The inset shows an Eadie-Hoffstee plot from which the following parameters are extrapolated: V_{max} = 81 ± 4 nmol P_i/min · mg protein and K_m = 0.11 μM

Presence and Properties of Na^+/Ca^{2+} Exchange

Figures 6*A* and *B* give a schematic representation of the two possibilities for Na^+/Ca^{2+} exchange to interfere with ATP-driven Ca^{2+} uptake. When Na^+ is present in the uptake medium in relatively high concentrations, it will release Ca^{2+} that has been accumulated by Ca^{2+}-ATPase (Fig. 6*A*). This approach was used by Gmaj et al. (1979) and their results were the first indication that Na^+/Ca^{2+} exchange is present in rat kidney cortex basolateral membranes. Another possibility is that the (Na^+-K^+)-ATPase generates a Na^+ gradient which is favorable for Ca^{2+} accumulation by the Na^+/Ca^{2+} exchange system. This second possibility is most likely to occur with low Na^+ concentrations in the uptake medium (Fig. 6*B*). Both possibilities have been tested and the results are summarized in Fig. 7. The initial rate of ATP-dependent Ca^{2+} uptake at 150 mM KCl and 0 mM NaCl is not influenced by ouabain. When 5 mM Na^+ is present in the incubation medium there is a 15% stimulation in Ca^{2+} uptake rate which is inhibited by ouabain. The ouabain inhibition is only observed after prolonged preincubation of the vesicles with ouabain (1 hr at 0°C). Apparently, the Na^+ pump generates a Na^+ gradient, $[Na]_i > [Na]_o$, which leads to further Ca^{2+} accumulation by the Na^+/Ca^{2+} exchanger. With 10 mM Na^+ present there is still a statistically significant Ca^{2+} accumulation above the value observed in the presence of ouabain. At 75 mM Na^+ there is no difference between the ouabain and the ouabain-free values. In this situation, 75 mM Na^+ reduces the ATP-dependent Ca^{2+} uptake by 22%, apparently by inducing a Ca^{2+} leak via the Na^+/Ca^{2+} exchanger. The percentage inhibition by high external Na^+ is independent of the time of incubation, provided sufficient ATP is present to prolong the time of a linear rate of Ca^{2+}

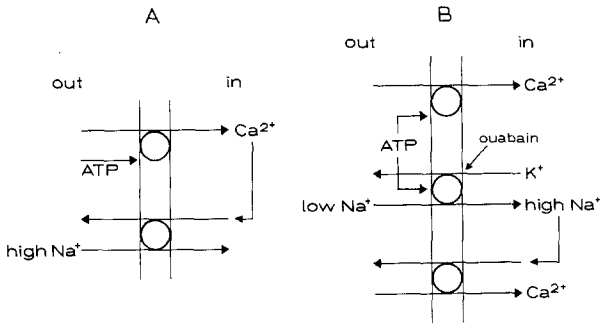

Fig. 6. Schematic representation of possible interference of Na^+ ions with ATP-dependent Ca^{2+} uptake. For explanation *see* text

uptake. In Fig. 8 a typical experiment is shown of prolonged incubation with high external Na^+ and sufficient ATP supply. The percentage inhibition of ATP-dependent Ca^{2+} uptake by high external Na^+ is 29% at 1 min, 18% at 4 min and 28% at 10 min. These values are comparable to the mean value obtained after 1 min incubation in Fig. 7. Therefore, the data in Fig. 7 strongly suggest that the Na^+/Ca^{2+} exchange capacity is maximally 22% of the V_{max} value of the ATP-dependent Ca^{2+} pump.

The affinity of the Na^+/Ca^{2+} exchanger for Ca^{2+} was tested by varying the free Ca^{2+} concentration between 0.05 and 1.0 μM Ca^{2+} and measuring ATP-dependent Ca^{2+} uptake in the presence of 5 mM Na^+ with or without ouabain. The result is shown in Fig. 9. Over the range of Ca^{2+} concentration tested, the presence of 5 mM Na^+ in the absence of ouabain increases the ATP-dependent Ca^{2+} uptake above the control level measured in membranes preincubated with 1 mM ouabain. This result strongly suggests that the affinity of the Na^+/Ca^{2+} exchanger is comparable to the one for the ATP-dependent system, which is around 0.1 μM Ca^{2+}.

We also tried to demonstrate more directly the presence of Na^+/Ca^{2+} exchange by measuring a Na^+ gradient-, $[Na]_i > [Na]_o$, dependent Ca^{2+} uptake in the absence of ATP. For these experiments, vesicles are loaded with 150 mM NaCl and diluted 15-fold into an equimolar KCl or NaCl solution con-

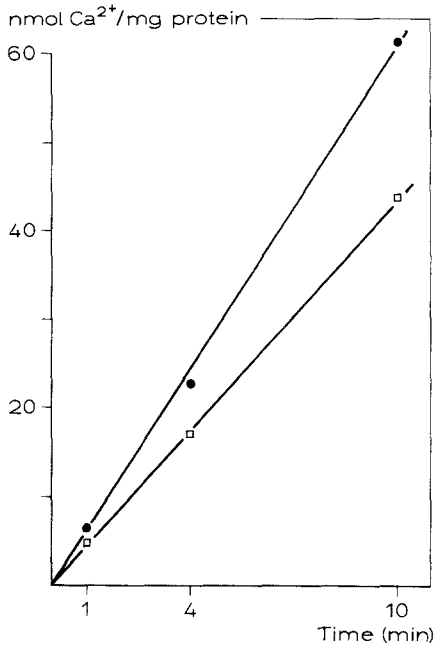

Fig. 8. ATP-dependent Ca^{2+} uptake in the absence and presence of high external Na^+ ions. Ca^{2+} uptake is measured in a medium containing 150 mM KCl (●—●) or 75 mM NaCl and 75 mM KCl (□—□). The free Ca^{2+} and Mg^{2+} concentrations are 1 μM and 1.7 mM, respectively, and the ATP concentration is 6 mM. Mean values are given of one experiment in triplicate. Ca^{2+} uptake in the presence of ATP is corrected for ATP-independent uptake

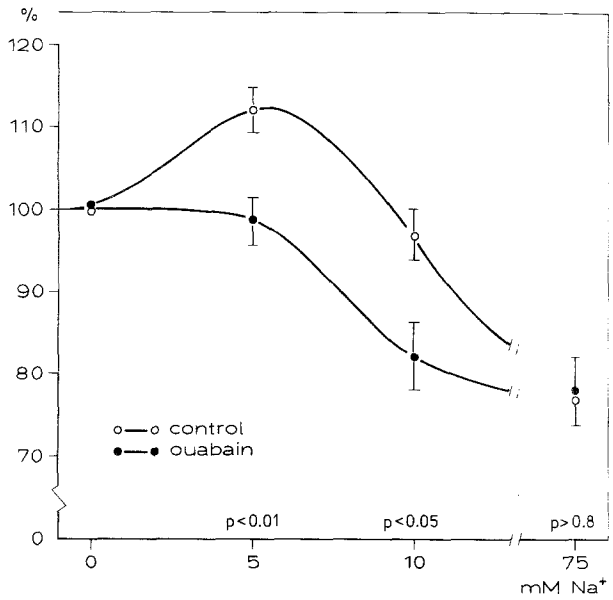

Fig. 7. Effects of Na^+ on ATP-dependent Ca^{2+} uptake in basolateral membranes from rat kidney cortex. Initial rates of Ca^{2+} uptake have been determined by 1-min incubations at 37°C in triplicate. Mean values are given of experiments on six membrane preparations. The control value at zero NaCl and 150 mM KCl is 7.2 ± 0.2 nmol Ca^{2+}/min · mg protein, which value has been corrected for ATP-independent Ca^{2+} uptake. ●—● experiments with membranes preincubated with 1 mM ouabain for 1 hr at 0°C. NaCl is substituted for KCl, hence the osmolarity and ionic strength of the media are constant

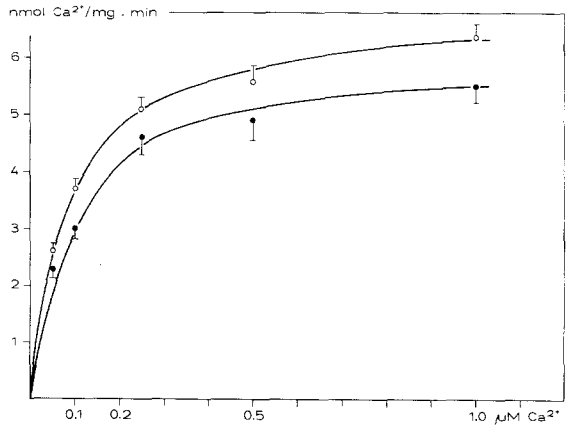

Fig. 9. Kinetics of the Na^+/Ca^{2+} exchange system in the presence of ATP. Initial rates of Ca^{2+} uptake have been determined by 1-min incubations at 37°C in triplicate. Mean values are given of four experiments, corrected for ATP-independent binding. ●—● membranes preincubated with 1 mM ouabain for 1 hr at 0°C. ○—○ membranes without ouabain. Uptake medium contained 5 mM NaCl, 145 mM NaCl, 145 mM KCl, 3 mM ATP and 1.7 mM free Mg^{2+}

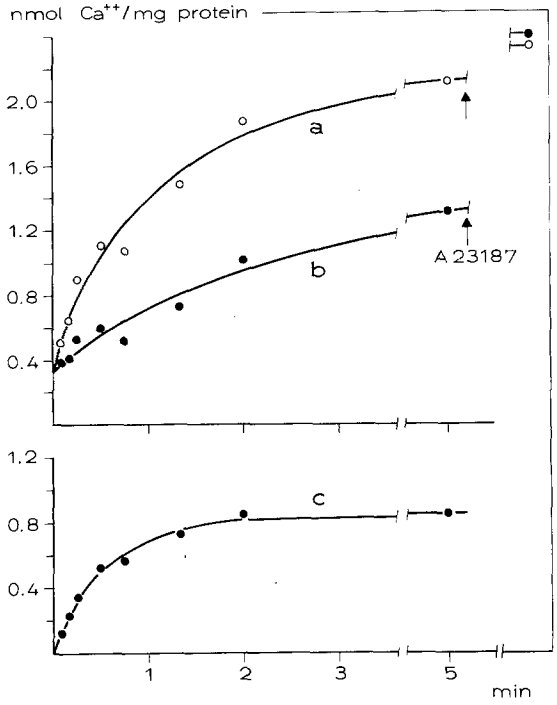

Fig. 10. Effect of a Na^+ gradient on Ca^{2+} uptake in basolateral membranes from rat kidney cortex in the absence of ATP. Membrane vesicles containing 150 mM NaCl are diluted 15-fold into equimolar KCl (curve *a*, open circles) or NaCl (curve *b*, closed circles) at 37°C. The difference, $a - b = c$, is plotted below. The free Ca^{2+} concentration is 5 μM. Mean values are given from four experiments in triplicate. Standard errors are 6% or less for all points, in curve *a* and *b*. After 5-min incubation, A23187 is added and an equilibrium value is reached within 30 sec for both curves

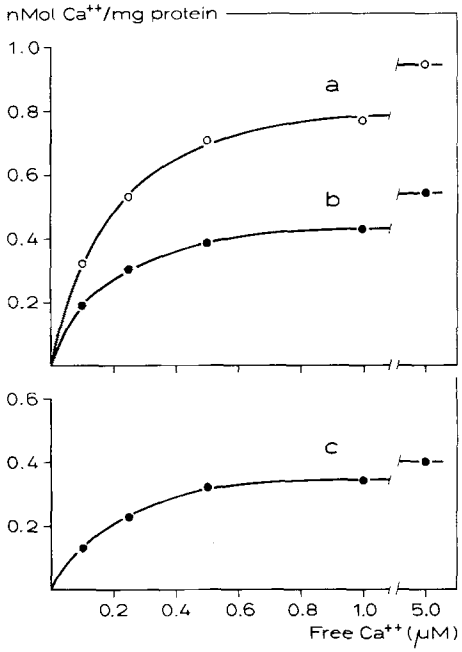

Fig. 11. Kinetic analysis of Na^+ gradient-dependent Ca^{2+} uptake in basolateral membranes of rat kidney cortex. Membrane vesicles containing 150 mM NaCl are diluted 15-fold into equimolar KCl (curve *a*) or NaCl (curve *b*) containing various free Ca^{2+} concentrations at 37°C. The difference ($a - b = c$) is plotted below. The incubations are quenched 15 sec after dilution. Mean values are given of five experiments in triplicate. Standard errors are 8% or less for all points in curves *a* and *b*

taining 5 μM free Ca^{2+}. The time course of Ca^{2+} uptake in the presence of and in the absence of a Na^+ gradient is shown in Fig. 10. The difference in uptake rates between the two conditions is statistically significant during the whole period ($P < 0.05$ at $t = 5$ sec). When the extra Ca^{2+} uptake in the presence of a Na^+ gradient is due to a Na^+/Ca^{2+} exchange system, then the difference should decrease or disappear when the membranes contain a Na^+ ionophore. In three experiments with 10 μM monensin present, the Na^+ gradient-dependent uptake was reduced to 27% of the control value at 15 sec, while after 2 min monensin had completely inhibited the Na^+ gradient-dependent Ca^{2+} uptake. In three other experiments in the presence of valinomycin we did not find a stimulatory effect of an inside-positive membrane potential. In two experiments valinomycin stimulated 5 and 17%, while in the third experiment valinomycin inhibited Na^+ gradient-dependent uptake 13%.

In Fig. 10 it is also shown that when the Ca^{2+} ionophore is added after 5-min incubation periods, equilibrium values are reached within 30 sec which are not significantly different for curve *a* and *b*. This result indicates that there is no significant Na^+ inhibition of Ca^{2+} binding to the inside of the vesicles.

In Fig. 10 the Ca^{2+} uptake was measured at 5 μM free Ca^{2+} and therefore we also studied the Na^+ gradient-driven Ca^{2+} uptake at various free Ca^{2+} concentrations. The results of 15-sec incubation points, which reflects reasonably the initial rate values, are shown in Fig. 11. The affinity for Ca^{2+} is about 0.2 μM, which is indeed as low as already indicated by the results of Fig. 9.

Since the Na^+/Ca^{2+} exchanger has been shown to be a symmetrical carrier at least in heart sarcolemma (Philipson & Nishimoto, 1982), and our data in Fig. 7 support this, all resealed vesicles (RO and IO vesicles) will contribute to Na^+ gradient-dependent Ca^{2+} uptake. Therefore, the maximal activity of Na^+/Ca^{2+} exchange determined as shown in Fig. 11 amounts to $0.4 \times 2 \times 4 = 3.2$ nmol Ca^{2+}/min · mg protein. This value is only 5% of the ATP-dependent pump activity.

Discussion

The main conclusions of our study with rat kidney cortex plasma membranes are that the ATP-driven Ca^{2+} pump has a much larger capacity than the Na^+/Ca^{2+} exchanger but the affinities for Ca^{2+} of both systems do not differ significantly. Since it is conceivable that during the membrane isolation procedure the Na^+/Ca^{2+} exchanger has become inactivated or that an important cofactor has been lost, we have to be cautious in extrapolating our *in vitro* findings to the *in vivo* system, the proximal tubular cell. In addition, we have to discuss the factors which could be responsible for over- or underestimation of the transport capacities of both Ca^{2+} pumps assessed in basolateral membrane vesicles.

It is known that intracellular organelles such as the endoplasmic reticulum contain ATP-dependent Ca^{2+} transport but no Na^+/Ca^{2+} exchange activity (Moore et al., 1974; Bruns, McDonald & Jarett, 1976), and therefore contamination with these fragments can lead to overestimation of the ATP-dependent Ca^{2+}-transport capacity of plasma membranes. Although $(Na^+\text{-}K^+)$-ATPase activity in our basolateral membrane preparation is purified 13-fold with respect to NADPH-dependent cyt-*c* reductase (Table 1), there is still significant contamination with smooth endoplasmic reticulum fragments (SER). Our kinetic studies on ATP-dependent Ca^{2+} uptake (Figs. 3, 4, 5) reveal only one high-affinity site for Ca^{2+}. In view of the lower affinity for Ca^{2+} of the microsomal Ca^{2+} pump (Moore et al., 1974, 1975; Friedman & Carafoli, 1982), a more complex kinetic behavior should be anticipated when SER fragments contribute significantly to ATP-dependent Ca^{2+} uptake. Moreover, the presence of 0.015% digitonin during the Ca^{2+} uptake experiments abolished ATP-dependent Ca^{2+} uptake completely (2 controls, *data not given* in Results section), while it had no effect on Ca^{2+}-ATPase activity (*see* Results, Fig. 5). Since digitonin action depends on a specific interaction with cholesterol-rich plasma membranes (Amar-Costesec et al., 1974), it is unlikely that SER fragments accumulate Ca^{2+}. A similar conclusion was reached by Gmaj et al. (1979) based on the observation that ATP-dependent Ca^{2+} uptake in several fractions after free-flow electrophoresis correlated best with the distribution of $(Na^+\text{-}K^+)$-ATPase and by van Os and Ghijsen (1983) with a basolateral membrane preparation of rat small intestine.

When endogenous calmodulin is lost during isolation of basolateral vesicles, this may lead to underestimation of Ca^{2+}-ATPase activity. However, this possibility is remote in view of the affinity of Ca^{2+} found for Ca^{2+}-ATPase and for ATP-dependent Ca^{2+} uptake. With a calmodulin-depleted Ca^{2+}-ATPase preparation of rat kidney cortex Gmaj et al. (1982) reported a K_m value of 1.2 μM Ca^{2+}. After repletion with calmodulin this value became 0.2 μM, close to 0.1 μM the value we found in freshly prepared membranes. In control experiments we have added 0.5 μM bovine brain calmodulin to freshly prepared membranes and found no effect on Ca^{2+}-ATPase activity nor on ATP-dependent Ca^{2+} uptake. When basolateral membranes were washed with 5 mM EGTA, ATP-dependent Ca^{2+} uptake was reduced to 51% of the untreated control value (n = 4). Adding 0.5 μM calmodulin to EGTA-treated membranes brought the Ca^{2+} uptake back to 81% of the control value. These results make it unlikely that the isolation procedure interferes with the calmodulin-Ca^{2+}-ATPase interaction. Moreover, we find similar Ca^{2+}-ATPase activities as those reported by Gmaj et al. (1982) in native membranes of rat kidney cortex.

With respect to the transport capacity of Na^+/Ca^{2+} exchange the following is important. Measuring the inhibition of ATP-dependent Ca^{2+} uptake by high external Na^+ forms an indirect method for assessing Na^+/Ca^{2+} exchange activity and it is most probable that this indirect method provides a good estimate of the V_{max} value, since under these conditions the exchanger is transporting Ca^{2+} as well as Na^+ downhill and its rate is therefore not limited by the driving force of the Na^+ gradient. After 1-min incubations we find a reduction of 22% in ATP-dependent Ca^{2+} uptake by high external Na^+ and this percentage reduction did not increase after prolonged incubations. Gmaj et al. (1979) reported a much larger effect of Na^+ on ATP-dependent Ca^{2+} uptake and the effect increased after prolonged incubations. The main differences with our results are a much smaller initial rate of ATP-dependent Ca^{2+} uptake (~1 nmol/min · mg protein) and the rate of Ca^{2+} transport decreased already after 1 min of incubation. This discrepancy can be explained when vesicles that have been subjected to free-flow electrophoresis are less tightly sealed for Ca^{2+} than those isolated by centrifugation only. Support for this hypothesis is provided by earlier work of Kinne, Schmitz and Kinne-Saffran (1971) in which they measured by detergent treatment that only one-third of basolateral membranes were still resealed after free-flow electrophoresis. Another point is that after prolonged incubations the ATP supply may be exhausted, a condition which inhibits ATP-dependent Ca^{2+} uptake but not Ca^{2+} release by Na^+/Ca^{2+} exchange. We have shown that even after 10 min of incubation with sufficient ATP,

the rate of ATP-driven Ca^{2+} uptake is still linear and that the Na^+ effect does not increase with time. Under these conditions Na^+/Ca^{2+} exchange should work at its maximal rate but in the opposite direction of the ATP-dependent Ca^{2+} pump, also operating at a maximal rate. Moreover, we studied Na^+ interference with Ca^{2+} uptake rates at 35°C instead of 25°C (Gmaj et al., 1979). Therefore, our values reflect the physiological situation and exclude the possible error introduced when the two Ca^{2+} transport systems would have largely different apparent activation energies.

With Na^+ present at low concentrations in the incubation medium we were able to demonstrate an extra Ca^{2+} accumulation on top of the ATP-dependent Ca^{2+} uptake which amounted to 15% of the transport rate of the latter system. The extra Ca^{2+} accumulation is ouabain-sensitive implying a role of $(Na^+\text{-}K^+)$-ATPase in realizing a Na^+ gradient, $[Na]_i > [Na]_o$. Although 15% accumulation does not equal exactly the 22% release by high external Na^+, it is clear from this result that Na^+/Ca^{2+} exchange is a symmetrical carrier as shown before with heart sarcolemma (Philipson & Nishimoto, 1982), and that it is situated on the same membrane as the $(Na^+\text{-}K^+)$ pump. The small difference between Ca^{2+} gain and loss depending on the direction of the Na^+ gradient may be related with an inhomogeneity in IO resealed vesicles. It is possible that fewer IO vesicles can establish a sufficient Na^+ gradient than IO vesicles involved in ATP-dependent Ca^{2+} uptake. Another possibility is that high intravesicular Na^+ displaces Ca^{2+} from intravesicular binding sites and in this way reduce Ca^{2+} accumulation. In basolateral membranes of rat duodenal cells this aspecific effect of Na^+ was as large as the effect of the Na^+/Ca^{2+} exchanger (Ghijsen et al., 1983) on ATP-dependent Ca^{2+} uptake. In summary, the transport capacity of Na^+/Ca^{2+} exchange assessed by measuring the effect of high external Na^+ represents an upper limit, while the one assessed by low external Na^+ is more likely a minimal value.

In the absence of ATP, an outwardly directed Na^+ gradient stimulated Ca^{2+} uptake but this transport rate was only 5% of the ATP-dependent Ca^{2+} transport rate. The reason for this small rate is not clear. It may either be due to a rapidly dissipating Na^+ gradient or to a direct effect of ATP on the Na^+/Ca^{2+} exchange system. ATP has been found to stimulate Na^+/Ca^{2+} exchange in axonal membranes (Baker & McNaughton, 1976) and in heart sarcolemmal vesicles (Reinlib, Caroni & Carafoli, 1981). We were unable to show a stimulatory effect of valinomycin. A stimulatory effect of an electrical potential can be anticipated since Na^+/Ca^{2+} exchange is electrogenic with 3 Na^+ ions exchanged for each Ca^{2+} ion (Caroni, Reinlib & Carafoli, 1980; Philipson & Nishimoto, 1980; Reeves & Sutko, 1980). The absence of an effect of valinomycin may be explained by a high intrinsic membrane permeability for K^+ ions. We dilute Na^+ containing vesicles into K^+ solutions which could induce a K^+ diffusion potential when the membranes are more permeable for K^+ than for Na^+. Absence or presence of valinomycin is then not an all-or-none response on the membrane potential but merely an improvement of K^+ selectivity.

The affinity for Ca^{2+} of the Na^+/Ca^{2+} exchanger, in the presence as well as in the absence of ATP, is one order of magnitude higher than the one reported for Na^+/Ca^{2+} exchange in heart sarcolemma and smooth muscle plasma membrane (Caroni et al., 1980; Grover et al., 1983). Our results classify Na^+/Ca^{2+} exchange in rat kidney cortex as a high-affinity low-capacity system in contrast to Na^+/Ca^{2+} exchange in heart sarcolemma which is viewed as a low-affinity high-capacity system (Carafoli, 1981). A comparable high-affinity for Ca^{2+} of Na^+/Ca^{2+} exchange in small intestinal basolateral membranes has been reported (Ghijsen, de Jong & van Os, 1983). These findings are significant, since both intestinal and kidney epithelial cells are nonexcitable. During excitation of heart and smooth muscle the free cytosolic Ca^{2+} concentration is undoubtedly higher than in nonexcitable cells, hence the affinity for Ca^{2+} of Na^+/Ca^{2+} exchange in excitable cells may be less.

A last point which has to be discussed is that the percentage IO-resealed vesicles is relatively small with respect to the percentage RO-resealed vesicles. This finding may be due to the relatively mild homogenization procedure. Recently, Forbush (1982) described a $(Na^+\text{-}K^+)$-ATPase-rich fraction from dog kidney outer medulla which had a complete right-side-out orientation. He used a Teflon® glass homogenizer with a very loose serrated pestle. In our homogenization procedure a dounce apparatus with a loose pestle has to be used to leave glomeruli intact which can then be removed by filtering. We noticed that the presence of 25 mM NaCl or KCl in the homogenization medium improved the rate of ATP-dependent Ca^{2+} uptake in purified basolateral membranes. Apparently, the presence of low salt concentrations either improve resealing or induce more inside-out resealed vesicles.

In conclusion, the results of our study suggest that the Ca^{2+}-ATPase system in rat kidney cortex cells plays a primary role in the Ca^{2+} homeostasis of these cells. Our results support the data of Borle (1982), whose study on Ca^{2+} fluxes in rat kidney cortex slices reveal no evidence for a role of Na^+/Ca^{2+} exchange. Moreover, the results obtained pre-

viously with basolateral membranes of rat duodenum (Ghijsen et al., 1982; 1983) are qualitatively and quantitatively similar to the results described here.

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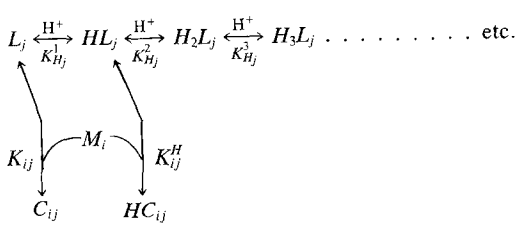
Appendix

Calculation procedure for the total amount of metal ion that must be added to a system with several ligands and metal ions to reach the desired free metal ion concentrations is now discussed.

A system with several ligands (L_j) and metal ions (M_i) at a certain pH can be described by the following scheme:



in which C_{ij} and HC_{ij} are the unprotonated and the protonated complexes between a metal i and a ligand j. H_qL_j represents ligand j with q protons bound. Composing a system in which the total concentration of each ligand is known (L_j^o), one can calculate exactly the total concentration of each metal ion (M_i^o) necessary to reach the required free metal ion concentration at a certain pH.

The fraction of a metal in the free ion form is:

$$\mu_i = \frac{M_i}{M_i^o} = \frac{M_i}{M_i + \sum_{j=1}^{l} \{C_{ij} + HC_{ij}\}}. \tag{A.1}$$

The summation is over all the ligands j in a complexed form with metal i. Knowing the stability constants

$$K_{ij} = \frac{C_{ij}}{M_i \cdot L_j} \tag{A.2}$$

$$K_{ij}^H = \frac{HC_{ij}}{M_i \cdot HL_j} \tag{A.3}$$

$$K_{H_j}^1 = \frac{HL_j}{[H^+] \cdot L_j} \tag{A.4}$$

and dividing numerator and denominator by M_i yields

$$\mu_i = \left[1 + \sum_{j=1}^{l} \{K_{ij} + \{H^+\} \cdot K_{ij}^H \cdot K_{Hj}^1\} L_j\right]^{-1}. \tag{A.5}$$

We only need to know L_j for each ligand. In the same way the fraction uncomplexed and unprotonated ligand is:

$$\lambda_j = \frac{L_j}{L_j^o} = \frac{L_j}{L_j + \sum_{i=1}^{m} \{C_{ij} + HC_{ij}\} + \sum_{q=1}^{h} H_qL_j}. \tag{A.6}$$

The first summation is over all the metals i in a complexed form with ligand j. The second summation is over all protonated but uncomplexed forms of ligand j.

Knowing the general form of the protonation constants for the q^{th} protonation

$$K_{H_j}^q = \frac{H_qL_j}{[H^+] \cdot H_{(q-1)}L_j} \qquad \text{with } q = 1 \text{ to } h \tag{A.7}$$

it follows that

Table A1. Logarithm of the binding constants of H^+, Mg^{2+}, Ca^{2+} to ATP, EGTA and NTA and their protonated forms at 37 and 25°C

log K	Binding to					
	ATP	HATP	EGTA	HEGTA	NTA	HNTA
H^+ 37°C	6.60	4.08	9.36	8.73	9.61	2.65
25°C	6.53	4.03	9.54	8.87	9.75	2.63
Ca^{2+} 37°C	3.80	2.13	10.67	5.33	6.36	—
25°C	3.70	2.13	10.89	5.33	6.40	
Mg^{2+} 37°C	4.26	2.27	5.44	3.36	5.36	—
25°C	4.22	2.24	5.28	3.36	5.36	

$$\lambda_j = \left[1 + \sum_{q=1}^{h} [H^+]^q \prod_{k=1}^{q} K_{Hj}^k + \sum_{i=1}^{m} M_i\{K_{ij} + K_{ij}^H K_{Hj}^l [H^+]\}\right]^{-1}. \quad (A.8)$$

Knowing the constants, choosing the pH and the total of each ligand, the total concentration for each metal can be calculated for any desired free metal ion concentration.

Recognizing $M_i\{K_{ij} + K_{ij}^H K_{Hj}^l [H^+]\}$ and $\{K_{ij} + K_{ij}^H K_{Hj}^l [H^+]\}L_j$ as vector matrix multiplications, the calculations can even be programmed on a pocket calculator with a solid-state matrix-vector multiplication program. This was done for a three metal-three ligand system, taking into account only the first two protonation steps since the influence of further protonations at physiological pH can be neglected. The logarithms of the binding constants are summarized in Table A1 and were taken from: Schwarzenbach et al., 1955; Martell and Schwarzenbach, 1956; Hull et al., 1964; Sillen and Martell, 1964; Boyd et al., 1965; Taqui Khan and Martel, 1966; Scharff, 1979.