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EVIDENCE AGAINST PARALLEL OPERATION OF SODIUM/CALCIUM ANTIPORT AND ATP-DRIVEN CALCIUM TRANSPORT IN PLASMA MEMBRANE VESICLES FROM KIDNEY TUBULE CELLS

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The present study aimed to clarify the existence of a Na^+/Ca^{2+} antiport device in kidney tubular epithelial cells discussed in the literature to represent the predominant mechanistic device for Ca^{2+} reabsorption in the kidney. (1) Inside-out oriented plasma membrane vesicles from tubular epithelial cells of guinea-pig kidney showed an ATP-driven Ca^{2+} transport machinery similar to that known to reside in the plasma membrane of numerous cell types. It was not affected by digitalis compounds which otherwise are well-documented inhibitors of Ca^{2+} reabsorption. (2) The vesicle preparation contained high, digitalis-sensitive ($Na^+ + K^+$)-ATPase activities indicating its origin from the basolateral portion of plasma membrane. (3) The operation of a Na^+/Ca^{2+} antiport device was excluded by the findings that steep Ca^{2+} gradients formed by ATP-dependent Ca^{2+} accumulation in the vesicles were not discharged by extravesicular Na^+, and did not drive $^{45}Ca^{2+}$ uptake into the vesicles via a Ca^{2+}-$^{45}Ca^{2+}$ exchange. (4) The ATP-dependent Ca^{2+} uptake into the vesicles became increasingly depressed with time by extravesicular Na^+. This was not due to an impairment of the Ca^{2+} pump itself, but caused by Na^+/Ca^{2+} competition for binding sites on the intravesicular membrane surface shown to be important for high Ca^{2+} accumulation in the vesicles. (5) Earlier observations on Na^+-induced release of Ca^{2+} from vesicles pre-equilibrated with Ca^{2+}, seemingly favoring the existence of a Na^+/Ca^{2+} antiporter in the basolateral plasma membrane, were likewise explained by the occurrence of Na^+/Ca^{2+} competition for binding sites. The weight of our findings disfavors the transcellular pathway of Ca^{2+} reabsorption through tubule epithelium essentially depending on the operation of a Na^+/Ca^{2+} antiport device.

Introduction

Na^+ and Ca^{2+} clearance as well as Na^+ and Ca^{2+} reabsorption from kidney proximal tubule are known to correlate closely with each other (reviewed in Refs. 1–5).

From microperfusion studies in the proximal tubule [1] and from studies on Ca^{2+} uptake and release in basolateral plasma membrane vesicles from kidney tubular cells, Gmaj et al. [6] concluded that a Na^+/Ca^{2+} antiport represents the predominant mechanism for Ca^{2+} reabsorption in the tubule whereas the ATP-driven Ca^{2+} pump [6,7] might be involved in the fine regulation of the intracellular Ca^{2+} concentration. However, the experimental protocol of the vesicle studies [6] is open to question as will be shown in the present

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Abbreviations: $(Na^+ + K^+)$-ATPase, EC 3.6.1.3, membrane adenosine-5-triphosphate phosphohydrolase requiring Na^+, K^+, and Mg^{2+} for full activity; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid.

paper. Observations in isolated perfused proximal renal tubules on the role of cytosolic Ca^{2+} levels in the regulation of transepithelial Na^+ transport were interpreted by Taylor and Windhager [8] in terms of the involvement of Na^+/Ca^{2+} antiport although they admitted the possibility that the reduction of the Na^+ transport rate at elevated Ca^{2+} concentrations could also be caused by an inhibitory Ca^{2+} effect on $(Na^+ + K^+)$-ATPase activity. On the basis of studies in kidney slices, Matsushima and Gemba [9] as well as Borle [10] stated that in the tubular plasma membrane there is no Na^+/Ca^{2+} antiport as described in several excitable tissues (reviewed in Ref. 11).

Our present studies in basolateral plasma membrane vesicles from kidney tubular epithelial cells aimed to clarify the existence of the parallel operation of ATP-driven Ca^{2+} transport and Na^+/Ca^{2+} antiport as concurring in the plasma membrane of excitable tissues [12–14].

Materials and Methods

Materials. $^{45}CaCl_2$ was purchased from the Radiochemical Centre, Amersham. Enzymes and nucleotides were obtained from Boehringer, Mannheim. The sources of antibiotics were Serva, Heidelberg (oligomycin) and Eli Lilly, Indianapolis (A23187). Ficoll 400 was from Pharmacia, Uppsala; Cibacron Blue F3G-A from Ciba-Geigy, Basle; prednisolone-3,20-bisguanylhydrazone dihydrochloride from Bayer, Leverkusen; ouabain, digitoxigenin, ammonium metavanadate as well as other chemicals were from Merck, Darmstadt. Sucrose was recrystallized from methanol. The sodium salt of ATP was converted into the imidazole salt by ion exchange.

Preparation of vesicles from guinea-pig kidney. The vesicles were prepared as described by Walter [15]. Briefly (cf. Table I), the membranes from the homogenate (fraction A) containing 1.65 M sucrose, were allowed to move upward from the bottom of a centrifuge tube for 2.5 h at 100000 × *g* into a linear gradient (0.95–1.45 M sucrose). The membrane fraction marked by the yellowish band (fraction B), which appeared in the upper half of the gradient, was either purified further by means of centrifugation in a linear Ficoll gradient (4–10%) in 0.8 M sucrose, yielding a white band (fraction C) on the top of the gradient, or was dialysed over night at 5°C against 150 mM KCl, 3 mM $MgCl_2$, 40 mM imidazole adjusted to pH 7.6 with HCl (dialysis medium), yielding fraction D. Among the various layers of sucrose or Ficoll gradient, the fraction B or C, respectively, showed the highest specific activity of $(Na^+ + K^+)$-ATPase.

Determination of protein and ATPase activities. Protein was determined by the procedure of Bensadoun and Weinstein [16] using bovine serum albumin as the standard. For estimation of $(Na^+ + K^+)$-ATPase activity the assay medium contained 2 mM ATP, 4 mM $MgCl_2$, 80 mM NaCl, 5 mM KCl, 80 mM imidazole-HCl (pH 7.6), 0.1 mM EDTA, 0.3 mM phospho*enol*pyruvate, 0.2 mM NADH, and 9 IU pyruvate kinase as well as lactate dehydrogenase each in a volume of 2 ml. After addition of aliquots of the membrane fractions, the decrease of absorbance at 334 nm was recorded [17]. The activity suppressed by 0.1 mM ouabain or 0.1 mM digitoxigenin was taken as

TABLE I

ATPase ACTIVITIES IN DIFFERENT FRACTIONS OF PREPARATIONS FROM GUINEA-PIG KIDNEY

The values are the means ± standard error of at least ten preparations.

Fractions	Protein (mg per 10 g kidney)	Mg^{2+}-ATPase ($\mu mol \cdot mg^{-1} \cdot h^{-1}$)	$(Na^+ + K^+)$-ATPase ($\mu mol \cdot mg^{-1} \cdot h^{-1}$)	Yield (%)
A Homogenate	2050 ± 450	6.9 ± 3.2	2.7 ± 0.6	100
B Yellowish band of sucrose gradient	60 ± 17	20.2 ± 4.1	25.4 ± 8.3	27 ± 6
C Top band of Ficoll gradient in 0.8 M sucrose	21 ± 10	5.4 ± 1.6	33.4 ± 10.3	12 ± 4
D KCl dialysate from the yellowish band	58 ± 20	6.2 ± 1.8	23.2 ± 9.1	25 ± 4

$(Na^+ + K^+)$-ATPase activity, and the remaining activity was designated as Mg^{2+}-ATPase activity. The $(Ca^{2+} + Mg^{2+})$-ATPase activity was taken as the Ca^{2+}-induced increment of activity determined by means of the optical assay in a medium containing 1 mM ATP, 3 mM $MgCl_2$, 100 mM KCl, 40 mM imidazole-HCl (pH 7.6) and either 0.1 mM $CaCl_2$ or 0.1 mM EGTA. All measurements of ATPase activities were done at 25°C.

Ca^{2+} uptake and release. The $^{45}Ca^{2+}$ content of the vesicles was assayed by a filtration technique using a Millipore apparatus and glass fibre filters, GF/C (Whatman, U.K.), 13 mm diameter. Fraction C suspended in a solution containing 0.8 M sucrose, about 4% Ficoll, 1 mM EDTA adjusted to pH 7.2 with Tris, or fraction D suspended in the dialysis medium (see above) was used. Samples of these fractions were incubated at 25°C in media specified in the legends to the figures and tables. The incubation was terminated by 10-fold dilution with an ice-cold stop solution containing 3 mM $MgCl_2$, 0.1 mM EGTA, 40 mM imidazole-HCl (pH 7.6) and, corresponding to the primary suspension medium of the vesicles, either 1 M sucrose or 150 mM KCl, respectively. The diluted aliquots containing 0.1–0.4 mg protein in a volume of 2 ml were filtered immediately by suction. The filters were washed twice each with 1.5 ml of the ice-cold stop solution. These procedures reduced considerably the background radioactivity, but not the amount of ATP-dependent $^{45}Ca^{2+}$ uptake. The whole procedure took no more than one minute. The filters were transferred into vials for liquid-scintillation counting. In the absence of ATP, the Ca^{2+} uptake was the sum of Ca^{2+} binding to the non-vesiculated and vesiculated membranes and the amount of Ca^{2+} equilibrated with the intravesicular space. The net Ca^{2+} uptake remaining after diluting and washing the vesicles was not accelerated by the presence of the antibiotic A23187, a Ca^{2+} ionophore (Fig. 1). The ATP-dependent increment of Ca^{2+} uptake was abolished by A23187 application (Fig. 1) and thus reflected intravesicular Ca^{2+} accumulation with formation of an outwardly directed Ca^{2+} gradient. When the intravesicular space was assumed to be 1 μl/mg vesicle protein (cf. Ref. 6), a 20-fold enhancement of the Ca^{2+} concentration in the vesicles could be calculated. For studying Ca^{2+} release, the vesicle suspension was not diluted by the stop solution before filtration, but was washed as above. The retention of protein on the filter depended especially on the age of the vesicles, and amounted to $40 \pm 12\%$ (40 determinations). The efficiency of ATP-dependent Ca^{2+} uptake into the vesicles uniformly decreased to one-half within a week when storing the vesicles in the dialysis medium at 5°C. After freezing, this activity of the vesicles was nearly completely destroyed.

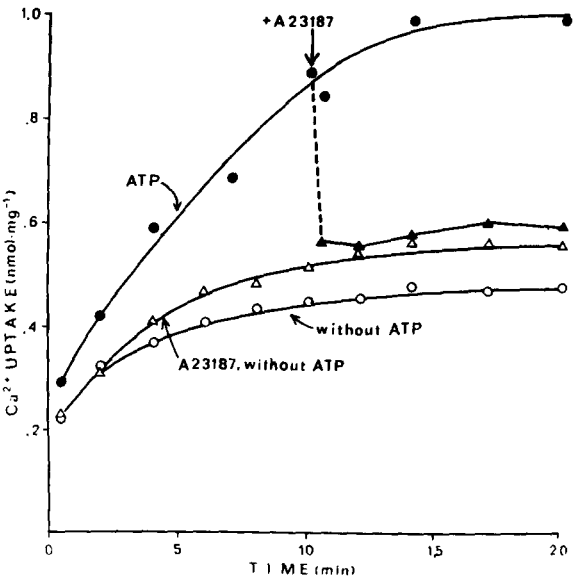

Fig. 1. ATP-independent and ATP-dependent uptake of Ca^{2+} into plasma membrane vesicles: effect of the ionophore A23187. The incubation medium contained 20 μM $^{45}CaCl_2$, 3 mM $MgCl_2$, 150 mM KCl, 40 mM imidazole-HCl buffer (pH 7.6), 1.97 mg/ml protein of vesicle fraction D without (○) or with 1 mMATP (●) and additionally (arrow) 8 μM A23187 (▲). △, Ca^{2+} uptake without ATP and with the ionophore from the start.

Mathematical treatment of the data. Generally, total Ca^{2+} uptake was not linear with time. Theoretically, at least the ATP-dependent Ca^{2+} uptake could be described by the following equation

$$\text{uptake}_t = (\text{uptake}_0 - \text{uptake}_\infty)\, e^{-kt} + \text{uptake}_\infty \qquad (1)$$

in which uptake means the amount of Ca^{2+} associated with the vesicles, and the subscripts refer to zero time (0), to equilibrium (∞) or to any time in between (t) [18]. From Eqn. 1 one can derive the

equation

$$\ln \frac{d(\text{uptake}_t)}{dt} = \ln k(\text{uptake}_\infty - \text{uptake}_0) - kt \quad (2)$$

in which the product $k(\text{uptake}_\infty - \text{uptake}_0)$ is the initial velocity (v_0) of Ca^{2+} uptake. v_0 was determined by plotting the logarithmic values of Ca^{2+} uptake per time interval (Δuptake/Δt) as a rough approximation to the required differential quotient versus time. The intercept with the ordinate delivered ln v_0 (cf. Fig. 2). The computation of v_0 was carried out by linear regression. The initial velocity of Ca^{2+} uptake was used to calculate the parameters as K_m for ATP and Ca^{2+}, and K_i for ADP. For all calculations of parameters, the measured data were transformed into a suitable form for linear regression using either the Lineweaver-Burk equation

$$\frac{1}{v} = \frac{1}{V} + \frac{K}{V \cdot [S]} \quad (3)$$

or the logarithmic form of the Hill equation

$$\log \frac{\gamma}{100 - \gamma} = h \cdot \log[I] - \log K' \quad (4)$$

in which γ means percent inhibition of Ca^{2+} uptake by an effector I. The latter equation was used only for the calculation of the concentration of I producing 50% inhibition of Ca^{2+} uptake defined as the intercept with the abscissa ($h \cdot \log[I] - \log K' = 0$).

All reported experiments were repeated at least twice with similar results. Only representative data are shown in the figures and tables.

Results

Characterization of sidedness of the vesicles and their tightness for Ca^{2+}

Electron microscopic studies showed that the membrane preparation consisted predominantly of vesicles with a diameter of 200–400 nm (not demonstrated). According to the results of freeze-etching analysis [19], allowing only a conservative valuation of the sidedness of the vesicles, the great majority was right-side oriented. Only 10 of 72 vesicles were estimated to have inside-out orientation. This is in accordance with the low ($Na^+ + K^+$)-ATPase activity of the vesicle preparation measured under isotonic conditions (19% of maximum, cf. Table II). Walter [15] and Kinsella et al. [20] found in similar membrane preparations under identical conditions 15% and 13%, respectively.

Since the vesicles showed an ATP-dependent Ca^{2+} uptake into the intravesicular space, the small percentage of inside-out oriented vesicles observed must have been poorly permeable for Ca^{2+}. The tightness of the vesicles for Ca^{2+} was estimated by measuring the release of Ca^{2+} from the vesicles loaded with Ca^{2+} in the presence of ATP (cf. Fig. 5). On the average, the half-time for the release of the Ca^{2+} from fraction D vesicles was as long as 6.4 ± 1.0 min (eleven determinations).

Properties of the ATP-dependent Ca^{2+} uptake system

Active Ca^{2+} uptake obeyed an exponential function (Fig. 2 and Table III). From the kinetics, followed under various conditions, there was no hint of more than one pumping system. The ATP-dependent Ca^{2+} uptake was clearly a Ca^{2+} accu-

TABLE II

ESTIMATE OF PERCENTAGE OF INSIDE-OUT VESICLES PRESENT UNDER ISOTONIC CONDITIONS

On the understanding that ATP did not penetrate into the vesicles whereas Na^+ and K^+ sufficiently rapidly did, the percentage of ($Na^+ + K^+$)-ATPase activity found in isotonic sucrose could be assumed to be related to both inside-out vesicles and membrane fragments. The reference activity set to be 100% was estimated under non-isotonic conditions. It could not be enhanced in the presence of various detergents (0.01% Triton X-100, 0.05% deoxycholate, 1% Tween 80) contrary to the activity found under isotonic conditions. For the measurements, fraction C was used exclusively. The values are the means ± standard error of ten preparations.

Assay	Reference activity without sucrose (μmol·mg^{-1}·h^{-1})	Activity in 1 M sucrose (% of reference activity)
($Na^+ + K^+$)-ATPase [a]	33.4 ±10.3	19± 6
Mg^{2+}-ATPase	5.4 ± 1.6	53±11
($Ca^{2+} + Mg^{2+}$)-ATPase	0.29± 0.23	62±28

[a] Decrement of activity in the presence of 0.1 mM ouabain which produced the full inhibitory effect on this enzyme since the same decrement was obtained in the presence of 0.1 mM digitoxigenin or after omission of Na^+ from the medium.

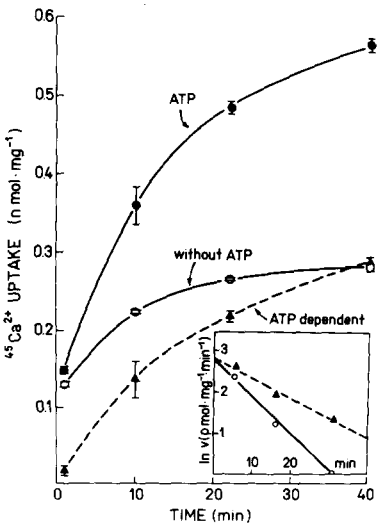

Fig. 2. Uptake of Ca^{2+} into plasma membrane vesicles: effect of ATP. The incubation medium contained 20 μM $^{45}CaCl_2$, 0.2 mM EDTA, 2 mM $MgCl_2$, 1 M sucrose, 40 mM imidazole-HCl buffer (pH 7.6), 1.15 mg/ml protein of vesicle fraction C without (○) or with 1 mM ATP (●), ▲, difference values. Vertical bars denote S.E. of the mean for three separate determinations. Inset: The same data shown in a semilogarithmic plot with the intercept representing ln v_0 and the slope $-k$. In this experiment, v_0 for the ATP-dependent and ATP-independent Ca^{2-} uptake were incidentally similar and amounted to 17.0 and 16.6 pmol·mg^{-1}·min^{-1}, respectively. The half-times ((1/k)ln 2), however, were different and amounted to 14.3 and 7.5 min, respectively.

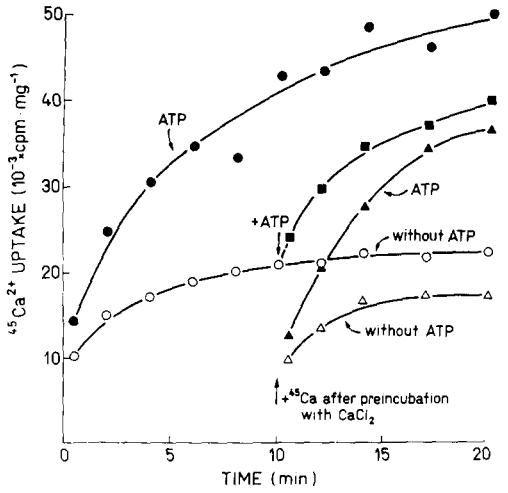

Fig. 3. Uptake of Ca^{2+} into plasma membrane vesicles: independence of pre-loading with Ca^{2+}. The incubation medium contained 20 μM $^{45}CaCl_2$, 3 mM $MgCl_2$, 150 mM KCl, 40 mM imidazole-HCl buffer (pH 7.6), 10 mM phospho*enol*-pyruvate, 5 U/ml pyruvate kinase and 1.97 mg/ml protein of vesicle fraction D. $^{45}Ca^{2+}$ uptake was started at zero-time either with $CaCl_2$ and $^{45}CaCl_2$ in the absence (○) or presence of 1 mM ATP (●); with 1 mM ATP after 10 min preincubation with $CaCl_2$ and $^{45}CaCl_2$ (■); with $^{45}CaCl_2$ after 10 min preincubation with 20 μM unlabelled $CaCl_2$ in the absence (△) or presence of 1 mM ATP (▲). The specific radioactivity of the mixture of unlabelled and labelled Ca^{2+} was 962 cpm/pmol $CaCl_2$.

mulation in the intravesicular space as the effect of the ionophore A23187 showed (Figs. 1 and 5). The rapidly released Ca^{2+} stemmed from both free intravesicular Ca^{2+} and intravesicularly loosely bound Ca^{2+}, as will be shown later. The residual vesicular Ca^{2+} left in the presence of the ionophore was as low as that taken up in the absence of ATP and identified as tighly bound Ca^{2+}.

The decrease of Ca^{2+} uptake rate with time was due to an increase of the Ca^{2+} efflux in consequence of intravesicular Ca^{2+} accumulation. This was concluded from the experiment shown in Fig. 3. The kinetics of ATP-dependent $^{45}Ca^{2+}$ uptake was similar in vesicles not loaded with Ca^{2+}, equilibrated with $^{45}Ca^{2+}$ from the medium or preloaded with unlabelled Ca^{2+} in the presence of ATP.

Table III shows some parameters characterizing the ATP-dependent Ca^{2+} uptake into the vesicles. Half-maximum velocity of Ca^{2+} uptake was reached at 0.04 mM ATP. Other nucleotides (GTP, ITP, and CTP, 1 mM each) were completely ineffective. The Ca^{2+} uptake was inhibited by ADP in a competitive manner toward ATP. Without adding creatine phosphokinase, creatine phosphate (5 mM) was able to promote the Ca^{2+} uptake in the presence of ATP concentrations near the K_m value or in the presence of ADP instead of ATP (not demonstrated). The effect was similar to that of an added ATP regenerating system as used in other experiments (cf. Fig. 3). The results of variations of the ATP and the Mg^{2+} concentrations resulting in much uncomplexed ATP or Mg^{2+}, respectively, showed that Ca^{2+} uptake required by all means free Mg^{2+}. The K_m value was found to be 0.02 mM uncomplexed Mg^{2+} at saturating ATP concentrations. The requirement of Mg^{2+} for ATP-dependent Ca^{2+} transport across the plasma membrane in various tissue preparations is well documented (e.g. Refs. 7, 23), but the concentration of

TABLE III

SOME KINETIC PARAMETERS OF ATP-DEPENDENT CALCIUM UPTAKE INTO PLASMA MEMBRANE VESICLES

Parameter	Numerical value	Fraction used
Initial velocity [a]	22 ± 4 (11) pmol·mg^{-1}·min^{-1}	C
	106 ±32 (15) pmol·mg^{-1}·min^{-1}	D
Half-time [a,b]	9.6± 2.4 (7) min	C
	4.0± 1.5 (14) min	D
K_m(ATP) [c]	0.04 mM	C
K_i(ADP) [d]	0.1 mM	C
K_m(Mg^{2+}) [e]	0.02 mM	D
K_m(Ca^{2+})	0.4 μM [f]	C
	0.3 μM [g]	D

[a] The values are the means ± S.E.; in parenthesis, the number of experiments.

[b] Time, after which the initial velocity of Ca^{2+} uptake decreased to one-half.

[c] [ATP] was varied from 0.03 to 1 mM at 2 mM total Mg^{2+} and 1.8 μM free Ca^{2+} in the presence of an ATP regenerating system with pyruvate kinase and phospho*enol*pyruvate.

[d] [ATP] was varied from 0.03 to 1 mM at 0.1 and 0.3 mM ADP. After having established the competitive nature of the inhibition by means of the Lineweaver-Burk-plot, K_i was derived from the Dixon-plot.

[e] Total [Mg^{2+}] was varied from 0.1 to 3 mM at 40 μM total Ca^{2+} and 1 mM ATP. Free [Mg^{2+}] was calculated with the association constants for ATP with H^+, Mg^{2+}, and Ca^{2+}, respectively, as given by Sanui and Pace [21].

[f] Total [Ca^{2+}] was varied from 5 to 40 μM at 3 mM Mg^{2+} and 0.5 mM EDTA.

[g] Total [EDTA] was varied from 0 to 0.5 mM at 3 mM Mg^{2+} and 20 μM Ca^{2+}.

[f,g] Free [Ca^{2+}] was calculated with the association constants for EDTA with H^+, Mg^{2+}, and Ca^{2+}, respectively, as given by Portzehl et al. [22].

needed free Mg^{2+} was not reported. Half-maximum velocity of ATP-dependent Ca^{2+} uptake was reached at about 0.3 μM free Ca^{2+}. The addition of calmodulin (0.75 μg/ml) from pig brain had no influence on Ca^{2+} uptake at 0.9 μM or 20 μM free Ca^{2+} (not demonstrated).

In Table IV, the effects of some ATPase inhibitors are shown. Oligomycin, a potent inhibitor of mitochondrial ATP-dependent Ca^{2+} transport, had little effect on ATP-dependent Ca^{2+} uptake into the vesicles. The inhibition by NaN_3 was due to Na^+ (see next paragraph). Neither the polar cardiac glycoside ouabain nor the apolar aglycone digitoxigenin diminished the Ca^{2+} uptake in concentrations which completely inhibited the (Na^+ + K^+)-ATPase activity of these preparations. The glycoside-insensitivity of ATP-driven Ca^{2+} accumulation in various plasma membrane vesicle preparations was generally observed [6,23,24]. However, prednisolone-3,20-bisguanylhydrazone, a well-known inhibitor of (Na^+ + K^+)-ATPase, but also of Ca^{2+} transport across the plasma membrane of neural cells [25], was effective. Additionally, the ATP-dependent Ca^{2+} uptake was sensitive to inhibition by vanadate and the dye Cibacron Blue F3G-A. A competition of Cibacron blue (10 μM) to ATP (0.04–3 mM) as a possible mechanism of inhibition was not observed.

TABLE IV

EFFECT OF VARIOUS AGENTS ON ATP-DEPENDENT CALCIUM UPTAKE INTO PLASMA MEMBRANE VESICLES

The Ca^{2+} uptake under control conditions as given in Fig. 1 was taken as 100%.

Additions	Ca^{2+} uptake after 10 min (%)
Oligomycin (1 μg/ml)	87
10 mM NaN_3	62
10 mM NaCl	69
0.1 mM ouabain	108
0.1 mM digitoxigenin	97
0.027 mM prednisolone-3,20-bisguanylhydrazone	50
0.015 mM Cibacron blue F3G-A	50
0.007 mM vanadate	50

Evidence against parallel operation of Na^+/Ca^{2+} antiport and ATP-driven Ca^{2+} transport

Speed and directionality of Na^+/Ca^{2+} antiport require a cation gradient over the vesicle membrane. As we thought that the tightness of the vesicles could be insufficient for Na^+ accumulation through preequilibration with high Na^+ concentration or through operation of (Na^+ + K^+)-ATPase, we decided to employ for these studies Ca^{2+} gradients that were formed through ATP-driven Ca^{2+} accumulation in the vesicles. The presence of a steep outwardly directed Ca^{2+} gradient was proven by the almost immediate release of Ca^{2+} from the vesicles after application of the Ca^{2+} ionophore A23187 (Figs. 1 and 5). If the

Na^+/Ca^{2+} antiporter would coexist with ATP-driven Ca^{2+} transport in plasma membrane vesicles from kidney epithelial cells as in those from cells of excitable tissues [12,13,24,26], then extravesicular Na^+ should provoke a Ca^{2+} efflux from the vesicles almost as rapid as the addition of the Ca^{2+} ionophore did (Figs. 1 and 5). However, as shown in Fig. 4, the velocity of Ca^{2+} release from the vesicles after interruption of Ca^{2+} uptake by dilution and complexation of Ca^{2+} with EGTA was in the presence of Na^+ not only not faster but, if changed, even slower than in its absence. In the experiment demonstrated in Fig. 4 the half-times were 10 min or 7 min, respectively. Further, as shown in Fig. 5, the addition of Na^+ together with additionally a Mg^{2+} chelator to the suspension of vesicles, that were preloaded with Ca^{2+} by means of ATP, did not significantly influence Ca^{2+} efflux. Finally, there was no hint of the Ca^{2+}/Ca^{2+} antiport which is said to be effected by the Na^+/Ca^{2+} antiporter [14,27]. Several aliquots of the vesicle preparation were or were not preloaded with unlabeled Ca^{2+} by means of ATP. $^{45}Ca^{2+}$ uptake was then measured partially in the presence of hexokinase and glucose to reduce the ATP

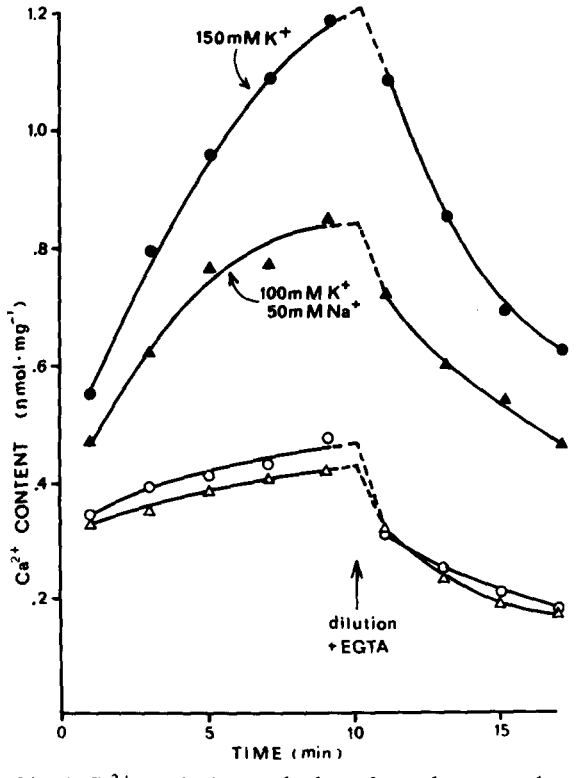

Fig. 4. Ca^{2+} uptake into and release from plasma membrane vesicles: effect of Na^+. The uptake of Ca^{2+} was measured in a medium containing 20 μM $CaCl_2$, 3 mM $MgCl_2$, 40 mM imidazole-HCl buffer (pH 7.6), 5 mM phospho*enol*pyruvate, 5 U/ml pyruvate kinase, 0.59 mg/ml protein of vesicle fraction D, 150 mM KCl (○, ●) or 100 mM KCl and 50 mM NaCl (△, ▲) in the absence (○, △) or the presence of 1 mM ATP (●, ▲). The release was started, after 10 min of incubation, by 10-fold dilution with a solution containing 3 mM $MgCl_2$, 40 mM imidazole-HCl buffer (pH 7.6), 0.1 mM EGTA and 150 mM KCl (○, ●) or 100 mM KCl and 50 mM NaCl (△, ▲).

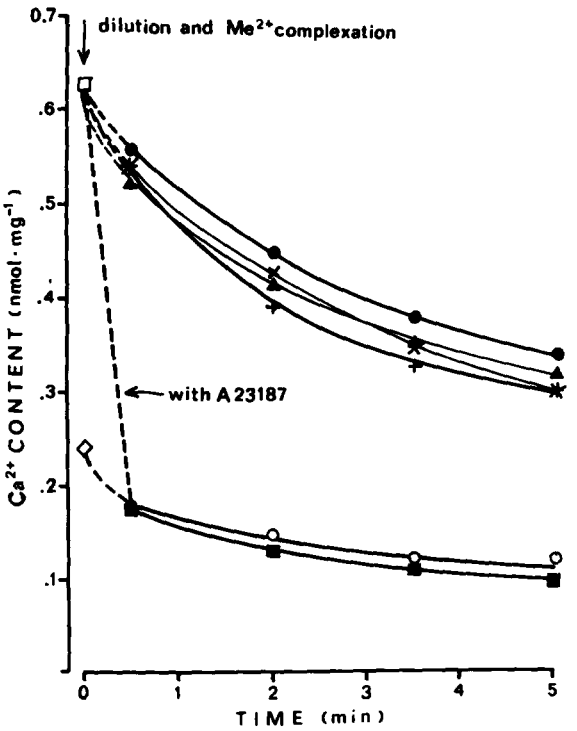

Fig. 5. Ca^{2+} release from vesicles preloaded with Ca^{2+} in the absence or presence of ATP after chelation of extravesicular Ca^{2+}, or Ca^{2+} and Mg^{2+}, in the presence or the absence of Na^+. After 10 min incubation of the vesicles in a medium containing 20 μM $^{45}CaCl_2$, 3 mM $MgCl_2$, 150 mM KCl, 40 mM imidazole-HCl buffer (pH 7.6), 10 mM phospho*enol*-pyruvate, 5 U/ml pyruvate kinase, 0.81 mg/ml protein of vesicle fraction D in the absence (◇) or the presence of 1 mM ATP (□), the release of Ca^{2+} from the vesicles was started at zero-time by 10-fold dilution with a solution containing 40 mM imidazole-HCl buffer (pH 7.6) and additionally: ○, ●, 150 mM KCl, 0.1 mM EGTA; ▲, 150 mM KCl, 0.5 mM EDTA; +, 100 mM KCl, 50 mM NaCl, 0.1 mM EGTA; ×, 100 mM KCl, 50 mM NaCl, 0.5 mM EDTA; ■, 150 mM KCl, 0.1 mM EGTA, 2 μM A23187.

concentration and thus ATP-driven inward transport of Ca^{2+}. The outwardly directed Ca^{2+} gradient, produced beforehand by the ATP supported pump, did not enhance $^{45}Ca^{2+}$ uptake as should occur if a Ca^{2+}/Ca^{2+} antiporter were present. On the contrary, in the experiment shown in Fig. 6, the Ca^{2+} gradient even slightly reduced the $^{45}Ca^{2+}$ uptake rate.

Effects of Na^+ on ATP-driven Ca^{2+} uptake into the vesicles

The initial velocity of Ca^{2+} uptake was not different with or without Na^+ in the medium. In the experiment shown in Fig. 4, v_0 amounted to 0.17 and 0.18 nmol · mg^{-1} · min^{-1} in the absence and in the presence of 50 mM NaCl, respectively. Clearly, Na^+ did not impair the Ca^{2+} pump itself as stated earlier by Gmaj et al. [6]. However, the

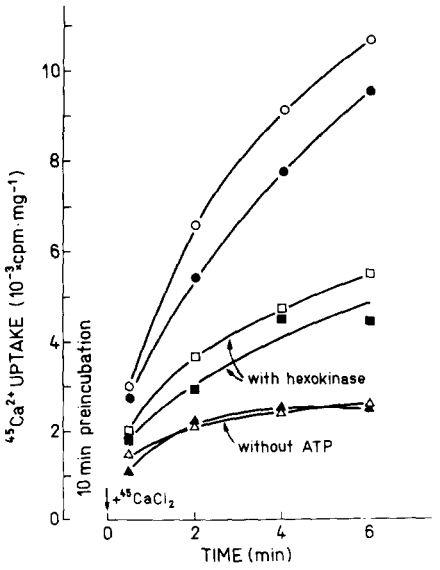

Fig. 6. Non-existence of Ca^{2+}/Ca^{2+} antiport between intra- and extravesicular space of vesicles loaded with Ca^{2+} in the absence or presence of ATP. The vesicles were preincubated for 10 min in a medium containing 3 mM $MgCl_2$, 150 mM KCl, 40 mM imidazole-HCl buffer (pH 7.6), 5 mM phospho*enol*-pyruvate, 2.5 U/ml pyruvate kinase, 0.91 mg/ml protein of vesicle fraction D in the absence (open symbols) or the presence of 20 μM unlabelled $CaCl_2$ (filled symbols) without (△, ▲) or with 1 mM ATP (○, ●, □, ■). $^{45}Ca^{2+}$ uptake was started at zero-time by adding 20 μM $CaCl_2$ and $^{45}CaCl_2$ (open symbols) or $^{45}CaCl_2$ (filled symbols). In two batches (□, ■), extra addition of 12 mM glucose and 2 U/ml hexokinase at zero-time. The specific radioactivity of the mixture of unlabelled and labelled Ca^{2+} was 14.7 cpm/pmol $CaCl_2$ each.

amount of Ca^{2+} taken up by the vesicles became diminished in a time-dependent manner in the presence of Na^+ partially replacing K^+. In the experiment shown in Fig. 4, the time in which v_0 became reduced to one-half in the presence or absence of Na^+ amounted to 1.8 min and 3.2 min, respectively. The Na^+ concentration required to halve the amount of Ca^{2+} taken up within 10 min was 27 mM on the average. This value varied considerably for different vesicle preparations (cf. Table IV). Neither the variation of the Mg^{2+} concentration from 0.3 mM to 10 mM (total) nor the variation of the concentration of uncomplexed Ca^{2+} in the medium from 2.6 μM to 40 μM altered the percent inhibitory effect of 50 mM Na^+ on the amount of Ca^{2+} taken up within 10 min. Under conditions allowing activity of ($Na^+ + K^+$)-ATPase, 100 μM ouabain and 100 μM digitoxigenin, respectively, both sufficient to suppress this activity, did not affect Ca^{2+} uptake into the vesicles (cf. Table IV). This excludes the possibility that the inhibitory Na^+ effect on ATP-driven Ca^{2+} uptake was caused by an adverse, vesicle lumen-positive membrane potential resulting from the electrogenicity of concomitant Na^+/K^+ antiport.

Discussion

Origin of the vesicle preparation showing ATP-dependent Ca^{2+} uptake activity

The vesicle preparation studied presumably originated essentially from the proximal tubule since the vesicle population exhibiting the highest Ca^{2+} uptake activity was obtained from the kidney cortex section (not demonstrated), the known localization of proximal tubules [28,29], and because vesicle preparations that were satisfactory for transport studies have been reported to date only for membranes of proximal tubular cells [29].

The kinetic characteristics of the ATP-dependent Ca^{2+} transport system found by us (especially the K_m-values for Ca^{2+} and ATP; the specificity for ATP; the requirement for Mg^{2+}; the sensitivity to prednisolone-bis-guanylhydrazone and VO_4^{3-}; the insensitivity to oligomycin, azide and ouabain; the full reversal of Ca^{2+} uptake by the Ca^{2+} ionophore A23187) are similar to the properties of the ATP-dependent Ca^{2+} transport systems described to reside in plasma membranes of erythrocytes

[30], lymphocytes [23], cardiac muscle cells [12,31], brain synaptosomes [13], and tubular cells of rat kidney [6,7]. These similarities favor the conclusion that the vesicle preparation from guinea-pig kidney characterized by us as to the ATP-dependent Ca^{2+} transport consisted of vesiculated plasma membranes.

The origin of our vesicle preparation from the basolateral membrane of tubular epithelial cells is defined by the distribution of $(Na^+ + K^+)$-ATPase as membrane marker. In the various fractions obtained after density gradient centrifugation, the highest activities of the $(Na^+ + K^+)$-ATPase and of the ATP-dependent Ca^{2+} uptake system were found in the same, i.e., the top layer (cf. Fig. 7). The concurrence of both activities in the basolateral membrane was also found by Gmaj et al. [6] after membrane fractionation by free-flow electrophoresis. The $(Ca^{2+} + Mg^{2+})$-ATPase involved in ATP-driven Ca^{2+} transport was likewise localized in the basolateral membrane [32]. The $(Na^+ + K^+)$-ATPase activity was in our preparation as high as or even higher than described in other basolateral membrane preparations highly purified by different procedures [20,33]. Accordingly, the ATP-dependent Ca^{2+} transport system could safely be localized in the basolateral membrane of tubule cells.

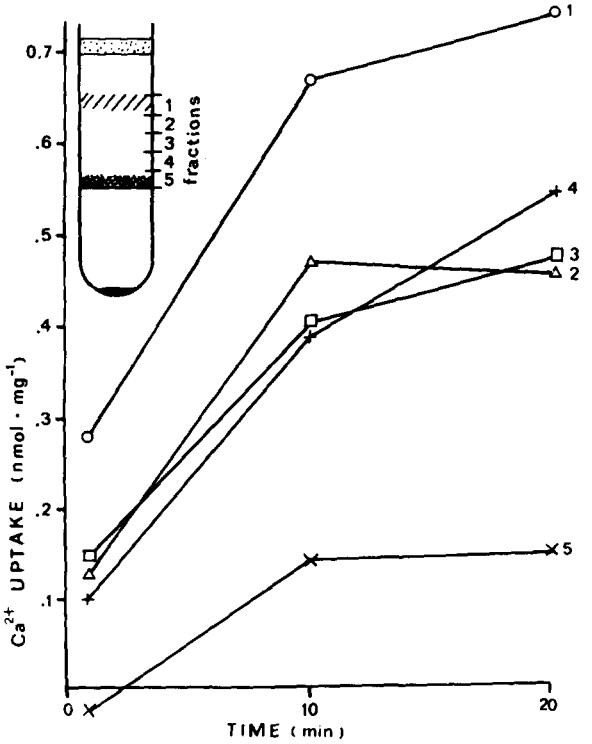

Fig. 7. ATP-dependent Ca^{2+} uptake: efficiency of different fractions from the sucrose gradient. The different fractions (1–5) were collected after the first density gradient centrifugation (see methods). Fraction 1 contained the top visible band (yellowish), which is identical with fraction B (cf. Table I). Fraction 5 (brownish) lays on the interphase to the clear red lower phase. The incubation medium contained 40 μM $^{45}CaCl_2$, 3 mM $MgCl_2$, 0.5 mM EDTA, 1 M sucrose, 40 mM imidazole-HCl buffer (pH 7.6), 1 mM ATP and protein from fraction 1: ○ (1.80 mg/ml); fraction 2: △ (1.38 mg/ml); fraction 3: □ (1.90 mg/ml); fraction 4: +(2.75 mg/ml) or fraction 5: × (4.25 mg/ml), respectively. The Ca^{2+} uptake in the absence of ATP was subtracted.

Functional aspects of ATP-dependent Ca^{2+} uptake into the vesicles

The ATP-driven transport system in the plasma membrane of kidney tubule cells can be assumed to exhibit the same vectorial operation mode as in the other, above-mentioned cell types which are known to extrude Ca^{2+} from the cytoplasm into the extracellular space in utilizing the Gibbs energy of ATP hydrolysis. Thus, one can assume that the catalytic center of an involved Ca^{2+}-ATPase is exposed to the cytoplasmic membrane surface. Thus, only the 14% of inside-out oriented vesicles exposing the catalytic center to the medium responded to added ATP. This may help to explain the seemingly low transport efficiency of our vesicle preparation.

The initial rates of Ca^{2+} uptake given in Table III are tantamount to *V*-values because they were estimated with saturating concentrations of Ca^{2+}, ATP, and Mg^{2+}. Actually, the Ca^{2+} transport rates and capacities of our vesicle preparation from guinea-pig kidney were lower than those in similar preparations from rat kidney [6]. If possible species differences in enzyme density per unit membrane area were not involved, the seemingly lower efficiency of our preparation could be caused by a higher percentage of right-side-out oriented vesicles or non-vesiculated sheets of basolateral membrane both inactive in ATP-dependent Ca^{2+} uptake.

The Ca^{2+} affinity of the ATP-driven Ca^{2+} pump in our vesicle preparation was as high as the Ca^{2+} affinity of $(Ca^{2+} + Mg^{2+})$-ATPase in isolated kid-

ney cortex plasma membranes determined after addition of calmodulin [32] (the K_m values were 0.3 μM and 0.24 μM, respectively). Hence, our vesicular ATP-dependent Ca^{2+} uptake system appeared to have retained a saturating calmodulin concentration. Actually, addition of calmodulin elicited no change of Ca^{2+} uptake rate.

At ATP concentrations lower than 100 μM, the rate and degree of Ca^{2+} accumulation in the vesicles decayed rapidly with time in the absence, but not in the presence of an ATP regenerating system. Thus, the decay was the outcome of a decreasing chemical potential of ATP due to the decrease of the [ATP]:[ADP] ratio. Therefore, generally 1 mM ATP was used, and in case of an eventual substantial ATP consumption, throughout an ATP regenerating system was added keeping the [ATP]:[ADP] ratio high. Even under optimized conditions, an early deceleration of Ca^{2+} uptake rate was observed. If our transport system operated like the Ca^{2+} pump of erythrocyte membrane as an electroneutral Ca^{2+}/H^+ antiporter, the deceleration of transport rate could have been due to the formation of an outwardly directed Ca^{2+} gradient opposite to the Ca^{2+} uptake direction, and to the exhaustion of protons in the intravesicular space [34]. However, such interpretation can be rejected in our case, since the kinetics was similar when the Ca^{2+} uptake was studied in vesicles either without or with Ca^{2+} preloading (Fig. 3). Apparently, the leakiness of the vesicles for Ca^{2+} and H^+ was sufficient to compensate for increasing Ca^{2+} gain and H^+ loss.

The activity of the $(Ca^{2+} + Mg^{2+})$-ATPase in our vesicle preparation was difficult to measure accurately because it amounted to 5% of the Mg^{2+}-ATPase and 0.9% of the $(Na^+ + K^+)$-ATPase activity only (cf. Table II). The $(Ca^{2+} + Mg^{2+})$-ATPase activity was nevertheless 45-fold in excess to the activity calculated to be required for Ca^{2+} transport under same conditions if splitting of one molecule ATP was assumed to transport one calcium ion (cf. Ref. 34). Similar observations were described for plasma membrane preparations of various origin [7,23,32]; several possible reasons for this anomalous $(Ca^{2+} + Mg^{2+})$-ATPase activity are discussed in Ref. 7.

Earlier observations seemingly favoring the existence of a Na^+/Ca^{2+} antiporter in plasma membrane vesicles of kidney proximal tubule cells

Gmaj et al. [6] inferred the existence of Na^+/Ca^{2+} antiporter in basolateral plasma membrane vesicles from two findings. First, they observed that the Ca^{2+} content of vesicles preequilibrated with Ca^{2+} became by addition of Na^+ somewhat more reduced than by addition of K^+. However, they did not show, by means of the characteristic releasing effect of the Ca^{2+} ionophore, that the preequilibration had led to Ca^{2+} accumulation in the vesicles. On the contrary, they stated that the Ca^{2+} uptake under equilibration conditions represented to the greatest extent binding to membrane sites. Their observations that Na^+ effected only a very slow and incomplete reduction of vesicle-Ca^{2+}, and that this Na^+ effect scarcely exceeded that of K^+, are incompatible with the Na^+ effects on the Na^+/Ca^{2+} antiport system, well documented in excitable tissues, that are characterized by rapid and complete Ca^{2+} release from vesicular plasma membrane preparations, and by irreplaceability of Na^+ by K^+. In conclusion, the Na^+ effect reported by Gmaj et al. [6] appears to be essentially caused by Na^+/Ca^{2+} competition for binding sites. In line with this interpretation, Na^+ and Ca^{2+} are known to compete for binding sites at the extracellular membrane surface [10,35] (i.e., at the intravesicular surface in our inside-out vesicles), and Na^+ is described to show higher affinity to such sites than K^+ [35].

Second, Gmaj et al. [6] observed and we confirmed (Fig. 4) that in the presence of Na^+ the rate of ATP-dependent Ca^{2+} uptake into the vesicles, when beginning to result in Ca^{2+} accumulation, became increasingly depressed with time. They concluded that the reduced Ca^{2+} uptake was caused by a Na^+-induced increase of the Ca^{2+} permeability of the membrane resulting in enhanced backleak of Ca^{2+}. Although the authors did not give a more detailed mechanistic account, the involvement of a long-lasting, inwardly directed Na^+ gradient over the membrane can be excluded. The then required low Na^+ permeability of the vesicle membrane would render possible the formation of an outwardly directed Na^+ gradient due to the operation of $(Na^+ + K^+)$-ATPase present under

the conditions chosen by Gmaj et al. (Fig. 6) and by us (Fig. 4). Such Na^+ gradient should have increased the Ca^{2+} uptake into the vesicles. Since this never occurred, we may conclude with some confidence that the vesicle membrane had a Na^+ leakiness which did not allow the build-up and maintenance of a Na^+ gradient in either direction. In conclusion, we alternatively propose as mechanism for the inhibitory effect of Na^+ on ATP-driven Ca^{2+} accumulation in the vesicles that Na^+ competitively filled the low-affinity Ca^{2+} binding sites otherwise favoring Ca^{2+} accumulation in the vesicles. If so, the preexisting membrane leaks allowed increasing backfluxes of Ca^{2+} the magnitude of which was a function of free Ca^{2+} concentration in the vesicles.

Concluding remarks

Taken together, the experimental findings presented in Results and the objections raised here against the interpretation of earlier observations [6], appear to disfavor strongly the existence of the Na^+/Ca^{2+} antiporter in the basolateral plasma membrane of kidney tubule cells. However, the conclusiveness of our experimental evidence could be felt debatable on three grounds.

First, the Na^+/Ca^{2+} antiporter could have been inactivated during the long isolation procedure of the vesicles. Although instability of the antiporter has never been reported, we also studied the vesicle preparations from which the sucrose was not removed (fractions B and C). Sucrose is known to stabilize macromolecules in solution and to prevent the loss of enzymic activities [36]. However, a comparison of the pertinent data in Figs. 2 and 4, and Table III shows that the ATP-dependent Ca^{2+} uptake rate was in the presence of sucrose much slower than in the dialyzed KCl vesicle preparation (fraction D). Since sucrose exerts a deleterious effect on vesicle membrane permeability to Ca^{2+} [10], the sucrose-containing vesicle preparations did not promise to improve the traceability of the Na^+/Ca^{2+} antiporter.

Second, the examined vesicle preparations could not have been originated from the right part of the nephron or were not formed from the right membrane area where the Na^+/Ca^{2+} antiporter is eventually localized. This possibility, however, seems unlikely. When demonstrated beyond doubt, the antiporter was found to co-exist with the ATP-dependent Ca^{2+} transport system [12,13,24, 26]. Since the latter transport system was present in our vesicle preparations, we could not have missed the Na^+/Ca^{2+} antiporter, if it existed in kidney tubule epithelium.

Third, the activity of the antiporter in the tubular epithelium could be so low that it escaped detection. However, in excitable tissues where it had been demonstrated to function, the limiting rate of the Na^+/Ca^{2+} antiporter was at least 30-times higher than that of the ATP-dependent Ca^{2+} transport system [31,37]. A similarly high-activity ratio should be present in kidney tubular epithelial membrane, if the Na^+/Ca^{2+} antiporter played the dominant role in transepithelial Ca^{2+} transport as assumed by several authors [1,6,8].

Finally, when viewing our negative findings in isolated plasma membrane vesicles from kidney tubule epithelium in context with the likewise negative findings in intact epithelial cell layers [9,10], we may conclude with some confidence that a Na^+/Ca^{2+} antiporter does not operate in the indicated kidney preparations that allow to analyze the transmembrane or the transcellular Ca^{2+} movements, respectively, but not the transjunctional paracellular Ca^{2+} movements.

The Na^+/Ca^{2+} antiporter proposed to provide the major device for the transcellular pathway of Ca^{2+} reabsorption in the kidney does not appear to exist in the basolateral plasma membrane of kidney tubule cells. The weight of this conclusion favors the transjunctional paracellular mechanism of Ca^{2+} reabsorption in the kidney as derived from a series of convincing experimental findings (cf. Refs. 38–40).

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