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Effects of divalent cations and pH on amiloride-sensitive Na^+ fluxes into luminal membrane vesicles from pars recta of rabbit proximal tubule

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The effect of Ca^{2+}, Cd^{2+}, Ba^{2+}, Mg^{2+} and pH on the renal epithelial Na^+-channel was investigated by measuring the amiloride-sensitive $^{22}Na^+$ fluxes into luminal membrane vesicles from pars recta of rabbit proximal tubule. It was found that intravesicular Ca^{2+} as well as extravesicular Ca^{2+} substantially lowered the channel-mediated flux. Amiloride sensitive Na^+ uptake was nearly completely blocked by 10 μM Ca^{2+} at pH 7.4. The inhibitory effect of Ca^{2+} was dependent on pH. Thus, 10 μM Ca^{2+} produced 90% inhibition of $^{22}Na^+$ uptake at pH 7.4, and only 40% inhibition at pH 7.0. The tracer fluxes measured in the absence of Ca^{2+} were pH independent over the range from 7.0 to 7.4. All the cations Ca^{2+}, Cd^{2+}, Ba^{2+} except Mg^{2+} inhibited the $^{22}Na^+$ influx drastically when added extravesicularly in millimolar concentrations. The cations Cd^{2+}, Ba^{2+} and Mg^{2+} in the same concentrations intravesicularly inhibited the $^{22}Na^+$ influx only slightly. A millimolar concentration of Ca^{2+} intravesicularly blocked the amiloride-sensitive $^{22}Na^+$ flux completely. The data indicate that Ca^{2+} inhibits Na^+ influx specifically by binding to sites composed of one or several deprotonated groups on the channel proteins.

Introduction

Gögelein and Greger demonstrated by using patch-clamp technique the existence of selective Na^+ channels in pars recta of rabbit proximal tubule, and suggested that part of the apical Na^+ uptake in this segment of the nephron occurred via these fluctuating Na^+ channels [1]. Recently, we described by using luminal membrane vesicles some properties of Na^+ selective channels in pars recta of rabbit proximal tubule. Initial experiments showed that the fluxes through these channels were dependent on Ca^{2+} [2]. In a previous paper we have provided evidence for the existence of a novel Ba^{2+}-sensitive K^+-channel in membrane vesicles isolated from pars convoluta of rabbit proximal tubule. The K^+ flux through these channels was found to be inhibited by intravesicular Ca^{2+}. The inhibitory effect of Ca^{2+} was strongly dependent on pH. Thus 100 nM Ca^{2+} produced maximal inhibition of $^{86}Rb^+$ influx at pH > 7.4 but had no effect at pH < 7.0 [3]. In the present communication we have studied the effect of pH and Ca^{2+} and of other appropriate divalent cations on the activity of the amiloride-sensitive Na^+-channel in luminal membrane vesicles from pars recta. We observed that Ca^{2+} (10 μM) blocked the amiloride-sensitive radioactive $^{22}Na^+$-fluxes. Furthermore, we found that the regulatory effect of Ca^{2+} was strongly dependent on pH, while variations in intravesicular pH in the absence of Ca^{2+} ion influenced neither the amiloride-sensitive nor the amiloride-insensitive uptake of radioactive $^{22}Na^+$ via the Na^+-channel.

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Materials and Methods

Preparation of luminal membrane vesicles

Luminal membrane vesicles were isolated from pars recta ('outer stripe of outer medulla') of the proximal tubule of rabbit kidney according to Ref. 4 and the method is only briefly described here. First, outer cortical tissue was removed by taking slices < 0.3 mm thick from the surface of the kidney. This contained

pars convoluta. Next, strips of the outer medulla tissues approx. 1 mm thick were dissected and these represent predominantly pars recta. Unless otherwise stated the tissue was homogenized and vesicles were prepared by differential centrifugation and by Mg^{2+} precipitation analogous with Ca^{2+} precipitation as described in Ref. 4. Unless otherwise stated the final pellet was washed for 20 min by a solution consisting of 3.75 mM EDTA, 10 μM of the Ca^{2+}-ionophore A23187, 298 mM sucrose and 27 mM Hepes-Tris buffer (pH 7.4) and resuspended in the same medium minus ionophore. The purity of the membrane vesicle preparations was examined by electron microscopy [5,6] and by measuring specific activities of various enzyme markers as in Ref. 7. The activities of various enzyme markers in the luminal membrane vesicle fractions were enriched as compared with the corresponding homogenates by the following factors (n = 15): 12.4 ± 1.8-fold (alkaline phosphatase), 31.5 ± 2.7-fold (leucine aminopeptidase), and 9.4 ± 2.4-fold (maltase). Average enrichments in specific activity of the basolateral marker, Na^+-K^+-stimulated ATPase, and that of the mitochondrial marker, succinate dehydrogenase, were in all cases < 0.4 and < 0.04, respectively. The amount of protein was determined as described by Lowry et al. [8] and as modified by Peterson [9] and with bovine serum albumin as a standard. All solutions used in this study were sterilized before use.

Assay of Na^+ flux

Influx of Na^+ was measured in NaCl (55 mM), KCl (55 mM) or Na_2SO_4 (27.5 mM) loaded vesicles analogous to flux measurement procedures reported previously [2,3]. All experiments were performed at 20°C. Dowex AG50W-X8 columns were prepared in 1 ml tuberculin syringes. The resin was washed with 1 M Tris to bring it on $Tris^+$ ionic form, followed by 5–6 volumes of 298 mM sucrose, 27 mM Hepes-Tris (pH 7.4) (except in experiments in which the effect of pH on the Na^+-channel activity was examined), containing 1 mg/ml bovine serum albumin. In the standard loading procedure, vesicles, 500 μl (approx. 10 mg/ml), were suspended in a buffer of 185 mM sucrose, 3.75 mM EGTA, 1 mM ouabain, 55 mM KCl, 27 mM Hepes-Tris (pH 7.4) for 1.5 h on ice. Alternatively, the loading was conducted by homogenizing the vesicles (45 strokes with teflon piston at 1500 rpm) in a buffer of 55 mM NaCl or 55 mM KCl, 185 mM sucrose, 3.75 mM EGTA, 27 mM Hepes-Tris, and 0.1 mM to 4.75 mM divalent cations to give the desired concentrations of free divalent cations [10]. The vesicles were collected by centrifugation (25 000 × g for 30 min at 2°C) and resuspended in the same buffer, to a protein concentration of approx. 15 mg/ml. Portions of 300 μl were applied on separate columns. The eluates were pooled and diluted to 1.0 ml with 298 mM sucrose, 27 mM Hepes-Tris (pH 7.4) buffer, and 75 μl were withdrawn for protein determination. In reference experiments amiloride was added in final concentrations of 350 μM. The experiment was initiated by adding $^{22}Na^+$ (122 kBq; 0.5 nmol) to the incubation medium. Samples of 145 μl were taken out at various time intervals (1, 3, 5, 7, 10, and 15 min) and uptake was stopped by passing the samples through Dowex columns by centrifugation. The separate eluates were diluted to 500 μl and used for radioactivity determinations. In experiments where $^{22}Na^+$ uptakes were expressed in percentage of control the values generally were obtained after a 10 min incubation. However, similar percentages were obtained when values at 1, 3, 5 or 7 min uptakes were calculated.

In the measurements of Ca^{2+}-dependent transport, the Ca^{2+}-depleted vesicles were incubated for 1 min at 20°C with 298 mM sucrose, 27 mM Hepes-Tris (pH 7.0–7.4), containing 3.75 mM EGTA and 1.5–4.75 mM $CaCl_2$ to give the desired concentration of free Ca^{2+} before addition of tracer $^{22}Na^+$. The ionophore A23187 incorporated in the membrane in the preparation procedure permits equilibration of the Ca^{2+} concentration over the membrane. Experiments using $^{45}Ca^{2+}$ tracer showed that 1 min incubation of vesicles pretreated with ionophore was sufficient to obtain a maximum Ca^{2+} concentration inside the vesicles. Although 2 H^+ move out the vesicles each time one Ca^{2+} moves in (electroneutral exchange) the fast equilibration time combined with a reasonable high buffer capacity (27 mM Hepes-Tris) was considered to be enough for maintaining pH in- and outside the vesicles at the desired value. The free Ca^{2+} in the Ca^{2+}/EGTA buffer system was calculated according to Pershadsingh and McDonald [11]. Divalent cation/EGTA buffer solutions were made so they contained defined free concentrations of Cd^{2+}, Ba^{2+} and Mg^{2+}. The free concentrations of Cd^{2+}, Ba^{2+} and Mg^{2+} were calculated using the tables and equations summarized by Bartfai [10].

Results and Discussion

The luminal membrane vesicles

Previously, we have described a procedure for isolation and separation of purified luminal membrane and basolateral membrane vesicles from rabbit renal cortex by using Mg^{2+} precipitation, differential centrifugation and a self-orienting Percoll gradient centrifugation [4,7]. The purity of the membrane vesicle preparations was examined by electron microscopy and by measuring specific activities of various enzyme markers as in [5–7]. The electron microscopical examinations of negatively stained membrane vesicle preparation revealed that the vesicles were covered with a coat of small particles on their outer surface, indicating right-side-out

orientation and intact vesicles [5–7]. Although the luminal membrane vesicles obtained from this segment of proximal tubule are highly purified as judged by electron microscopic examination, yet minor contamination of membrane vesicles from subcellular structure other than the luminal membrane cannot be excluded. However, a number of studies based on tubular transport of organic solutes utilizing vesicles formed by the brush border membranes of renal tubule support that majority of the vesicles isolated by using the above described procedure are oriented right side out. Thus, we have shown a number of different Na^+-coupled cotransport systems: at least two different systems for hexoses [12], multiple transport systems for monocarboxylic acids [13–16] and several systems for neutral α-amino acids [17–20]. In addition, a novel H^+-coupled transport system for L-proline [21–22] and L- and D-alanine [20,23,24] and at least three different Na^+-dependent systems and a unique H^+-gradient dependent transport system for β-alanine has recently been reported [25].

The Na^+ flux measurements

The principle of the flux assay is as follows. The vesicles are prepared to contain a relatively high concentration of NaCl. This is conducted by keeping the vesicles in a buffer solution containing NaCl for 1.5 h on ice. Shortly before starting the influx assay the external Na^+ is replaced by the relative impermeant ion $Tris^+$ by forcing the vesicles through the Dowex cation exchanger. The outwardly directed Na^+ gradient will set up an electrical diffusion potential, the magnitude of which will be determined by the relative permeabilities of Na^+, $Tris^+$ and Cl^-. In those vesicles containing the Na^+ channels the permeability of Na^+ is much greater than the $Tris^+$ and Cl^- permeabilities and accordingly an outwardly directed Na^+ diffusion potential, negative inside the vesicles, will be formed. $^{22}Na^+$ in very low concentration, below 0.5 μM, is added to the exterior solution and will accumulate selectively into that fraction of vesicle population containing the channels. The flux is usually measured in absence and presence of blocker of the channel. Isotope uptake is detected by taking aliquots of vesicle suspension at time intervals and separating vesicles from medium on Dowex columns. During the course of time the electrical driving force will dissipate as a result of progressive collapse of the cation gradients. The accumulation will therefore be transient, the magnitude and period of initial accumulation and the rate of the subsequent efflux depending on the relative permeabilities of all the ions present i the suspension.

The present study focuses on some properties of the Na^+ channels previously demonstrated to be present in luminal membrane vesicles from pars recta [2]. Thus, it is important to ascertain that the assay detect the

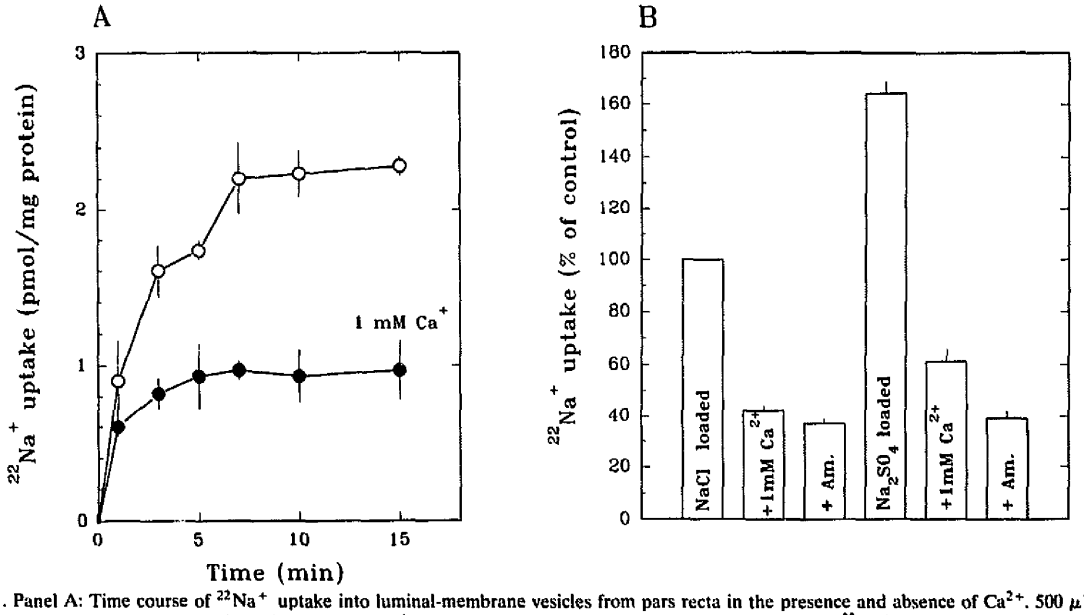

Fig. 1. Panel A: Time course of $^{22}Na^+$ uptake into luminal-membrane vesicles from pars recta in the presence and absence of Ca^{2+}. 500 μl of vesicles (protein concn. 8 mg/ml), Na^+_{in} = 55 mM, Na^+_{out} = 0 mM, was incubated at room temperature with $^{22}Na^+$ (122 kBq, 0.5 μM) in the absence (○), and in the presence of 1 μM Ca^{2+} (●). Panel B: $^{22}Na^+$ uptake into vesicles loaded with 27.5 mM Na_2SO_4 or 55 mM NaCl in the absence and presence of 1 mM free Ca^{2+} or 350 μM amiloride. NaCl (55 mM)-loaded vesicles were taken as 100%. Uptakes were measured for 10 min. Data are presented as means ± S.E. of three membrane preparations.

channel activity and not a possible neutral exchange pathways as Na^+/H^+ or Ca^{2+}/Na^+ exchange. Therefore, two different experimental protocols were applied to establish the electrical diffusion potential across the vesicle membranes. In the first one the vesicles were loaded with the procedure described above. In the second, vesicles were loaded with 55 mM KCl, followed by adding 10 μM of the K^+-ionophore valinomycin to the incubation medium just before adding $^{22}Na^+$. The latter protocol by loading with KCl and using K^+ ionophore excludes the possibility that the transient isotope accumulation occurs by electroneutral ion exchange via a carrier mediated countertransport kinetic mechanism [26]. It should be noticed that we previously have shown, that the Na^+/H^+ antiporter is predominantly operative in luminal membranes from pars convoluta and of minor significance in those from pars recta [27]. The Na^+/Ca^{2+} exchange systems are detected in the basolateral membranes, but not in the luminal cell membranes [28–30]. Thus, the assay used here should be appropriate for the measurements of the channel-mediated $^{22}Na^+$ fluxes into luminal membranes vesicles from pars recta.

Effects of Ca^{2+} and pH on Na^+ uptake

The time course of $^{22}Na^+$ uptake into NaCl-loaded vesicles in the absence (upper curve) and presence (lower curve) of 1 mM Ca^{2+} is shown in Fig. 1A. The vesicles were pretreated with the ionophore A23187 to equalize the Ca^{2+} concentration in- and outside the vesicles. In order to be able to measure the Ca^{2+} effect on the kinetics of the channel-mediated $^{22}Na^+$ uptake, a concentration of Ca^{2+} which lowered the tracer flux by approx. 60%, was chosen in this experiment. It was found that the presence of Ca^{2+} drastically reduced the initial rate and the maximal level of the $^{22}Na^+$ accumulation. Fig. 1B compares the effect of Ca^{2+} on the $^{22}Na^+$ uptake into Na_2SO_4- and NaCl-loaded vesicles. It is immediately apparent from the figure that Ca^{2+} inhibits the $^{22}Na^+$ uptake to the same relative extent under both experimental conditions. The result suggests that Ca^{2+} directly interacts with the Na^+-channel and that Ca^{2+} does not lower the $^{22}Na^+$ uptake by changing the membrane potential. From our previous studies on the mechanism of electrogenic transport of various organic solutes by luminal membrane vesicles isolated from pars recta, it has been established that the SO_4^{2-} anion is less permeant than the Cl^- anion. The cation diffusion potential is therefore short-circuited by the anion which is most permeant i.e. by Cl^- [14,17]. Furthermore, Fig. 1B shows that amiloride-insensitive $^{22}Na^+$ uptakes do not appear to be dependent upon the internal anions, indicating that amiloride-insensitive uptakes are not driven by an electrical diffusion potential. It should be noticed that a relatively high concentration of amiloride (350 μM) was needed for complete blockage of the Na^+ flux. Gögelein and Greger [1] made the same observation when using patch-clamp technique to study Na^+ channels in luminal membrane of rabbit straight proximal tubule segments, i.e. from the same segment of the tubule as in the present study. They needed a concentration of 1 mM amiloride to cause a complete block of the channel. Also Na^+ channels present in pig kidney membranes isolated from outer medulla required a relatively high dose of amiloride for complete blockage [31]. Kleyman et al. photoaffinity labeled a Na^+ channel in apical plasma membrane of bovine cortex [32]. The channel was identified by measuring $^{22}Na^+$ tracer flux into 55 mM NaCl-loaded vesicles prepared from bovine kidney cortical membranes. The $^{22}Na^+$ uptake in their preparations was still 70% of control in the presence of 10 μM amiloride [32]. Moran et al. [33] detected conductive sodium pathway with low affinity to amiloride in LLC-PK_1 cells and other epithelia. They studied amiloride-sensitive Na^+ flux into membrane vesicles containing 90 mM potassium gluconate. A negative inside membrane potential was imposed by adding valinomycin. Membrane vesicles isolated from LLC-PK_1, MDCK and OK cells gave in presence of 200 μM amiloride an amiloride-sensitive Na^+ uptake of 67%, 71% and 27%, respectively, calculated as percentage of total uptake. Apical brush border membrane vesicles isolated from rat kidney cortex exhibited an amiloride (200 μM) sensitive uptake of 56% of control. In our experiments we observed that the presence of 350 μM amiloride resulted in an amiloride sensitive $^{22}Na^+$ uptake into membrane vesicles isolated from pars recta of rabbit kidney proximal tubule of 63% of control. Generally, low doses (< 1 μM) of amiloride is considered to be a specific inhibitor of epithelial Na^+ channel. Thus, experiments carried out here and by others reveal the existence of amiloride-sensitive Na^+ conductive pathways, which are different from formerly known epithelial pathways. It is unclear what is the cellular role of this renal epitheial Na^+ transporter and how large is its contribution to Na^+ transport under physiological conditions. Nevertheless, the fact that it is present in many epithelia could suggest a fundamental role for this pathway [1,31–33]

Table I shows the effect of 10 μM free Ca^{2+} on the amiloride-sensitive $^{22}Na^+$ uptake. To include a defined amount of free Ca^{2+} in the vesicles two different experimental procedures were employed. In the first, renal tissue was homogenized and the membrane isolated in a medium that contained 10 μM free Ca^{2+} (Ca^{2+}/EGTA buffer). In the second, the vesicles were prepared in a Ca^{2+}-free medium containing a minimal amount of EGTA, and then incubated in a Ca^{2+}/EGTA mixture in the presence of Ca^{2+} ionophore A23187. It is seen that Ca^{2+} only completely blocks the amiloride-sensitive $^{22}Na^+$ uptake when present in- and

TABLE I

Effects of Ca^{2+} on $^{22}Na^+$ uptake in luminal-membrane vesicles from pars recta

Luminal membrane vesicles were prepared from outer medulla tissue by homogenization and differential centrifugation and by Mg^{2+} precipitation in media buffered to pH 7.4 containing either 3.75 mM EGTA (A) or Ca^{2+}/EGTA buffer giving a free Ca^{2+} concentration of 10 μM (B, C and D). The vesicles were incubated in media containing either EGTA (A, B and C) or 10 μM Ca^{2+} (D). Preparation B received 10 μM A23187 and A, C and D equal volumes of diluent (dimethylsulfoxide). The vesicle suspensions were assayed at 20°C at pH 7.4 for amiloride-sensitive $^{22}Na^+$ uptakes.

Homogenizing solution	Incubation solution	Amiloride-sensitive $^{22}Na^+$ uptake (%)
A. EGTA	EGTA	100 (control)
B. 10 μM Ca^{2+}	A23187+EGTA	72±3
C. 10 μM Ca^{2+}	EGTA	25±6
D. 10 μM Ca^{2+}	10 μM Ca^{2+}	3±2

outside the vesicles (D in Table I). This inhibition was not fully reversible. Incubating membrane vesicles, prepared to contain 10 μM free Ca^{2+}, in a Ca^{2+}-free EGTA medium plus A23187 restored only 72% of the control (maximal) level of $^{22}Na^+$ uptake (B in Table I). However, the fact that inhibition of $^{22}Na^+$ transport can be both induced and almost reversed by incubating membrane vesicles with Ca^{2+} and with Ca^{2+} and ionophore, respectively, suggests a direct Ca^{2+} and Na^+-channel interaction. By similar experimental protocol, Garty and co-workers have observed direct inhibition of toad urinary bladder epithelial Na^+-channels by Ca^{2+} and by other divalent ions [34–36].

Fig. 2 summarizes the results of the effect of increasing Ca^{2+} concentrations on the Na^+ uptake. The lower curve shows experiments with vesicles prepared in Ca^{2+}-free medium and then incubated in various Ca^{2+}/EGTA mixtures at pH 7.4 in the presence of Ca^{2+}-ionophore A23187. It is seen that the presence of nanomolar concentrations of Ca^{2+} drastically reduced the $^{22}Na^+$ uptake mediated by Na^+-channels in membrane vesicles from pars recta. The amiloride-inhibitable tracer flux is almost completely blocked by 10 μM Ca^{2+}. The upper curve shows the effect of Ca^{2+} on vesicles prepared in Ca^{2+}-free medium and incubated in a Ca^{2+}/EGTA buffer without pretreatment with Ca^{2+}-ionophore. In this case Ca^{2+} may exert its effect predominantly at the outer part of the Na^+ channel. At 10 μM Ca^{2+} the uptake was 50% of control. It means that Ca^{2+} ions also influence the Na^+ channel extravesicularly. This observation is in accordance to the findings outlined in Table I. Solely when Ca^{2+} was present in- and outside the vesicles the Na^+ flux could be completely blocked.

It has been shown that the inhibitory effect of Ca^{2+} on the amiloride-sensitive $^{22}Na^+$ flux in vesicles from toad urinary bladder was strongly dependent on pH [36]. In our laboratory we have demonstrated that the Ba^{2+}-sensitive $^{86}Rb^+$ flux into luminal membrane vesicles from pars convoluta was inhibited by intravesicular Ca^{2+} and furthermore varied by pH [3]. Therefore, we have examined the effect of various pH values on the Ca^{2+} inhibitory impact on $^{22}Na^+$ flux into membrane vesicles from pars recta. Fig. 3 shows the Ca^{2+} dose-response relationships measured in membrane vesicles buffered to various pH values. The pH effect was studied from 7.0 to 7.4, i.e. in the vicinity of the physiological intracellular pH. In these experiments the vesicles were preloaded and equilibrated to the given pH so that the intravesicular pH was the same as medium pH (i.e. $pH_{in} = pH_{out}$) and the uptake of $^{22}Na^+$ was examined at various free Ca^{2+} concentrations. The maximum amiloride-sensitive Na^+ tracer fluxes into membrane vesicles in the absence of Ca^{2+} were similar within this pH range (data not shown). It is apparent from Fig. 3 that the inhibition of channel-mediated $^{22}Na^+$ uptake by Ca^{2+} ions is dependent on pH. Lowering the pH shifted the inhibition curves to higher Ca^{2+} values. Thus 100 nM Ca^{2+} inhibited the Na^+ uptake about 70% at pH 7.4 but 1 mM Ca^{2+} is

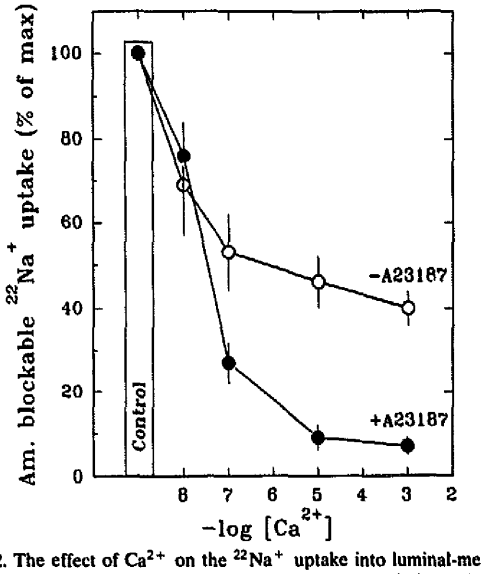

Fig. 2. The effect of Ca^{2+} on the $^{22}Na^+$ uptake into luminal-membrane vesicles. In one set of experiments (lower curve) the vesicles were depleted for Ca^{2+} by treatment with 3.75 mM EGTA and 10 μM Ca^{2+}-ionophore A23187 for 20 min at 20°C before loading the vesicles with 55 mM NaCl. The vesicles were incubated for 1 min at 20°C with 298 mM sucrose, 27 mM Hepes-Tris (pH 7.4), containing 3.75 mM EGTA and 1.5–4.75 mM $CaCl_2$ to give the desired concentration of free Ca^{2+} before adding tracer $^{22}Na^+$. In another set of experiments (upper curve, ○) vesicles prepared in a Ca^{2+}-free medium containing 3.75 mM EGTA were incubated in the above Ca^{2+}/EGTA buffer in absence of Ca^{2+}-ionophore A23187. The uptakes were measured for 10 min. Results are means ± S.E. of three independent preparations.

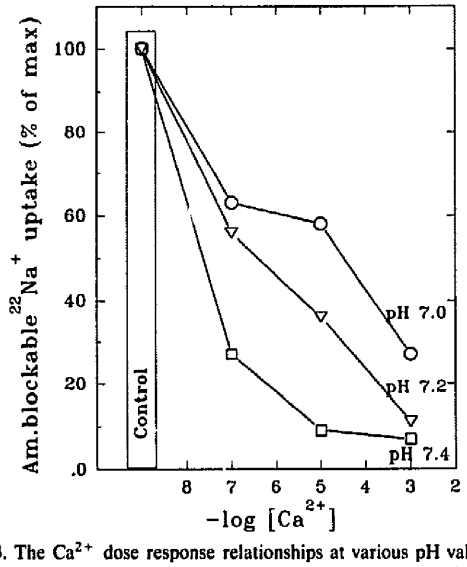

Fig. 3. The Ca^{2+} dose response relationships at various pH values. The vesicles were washed as described in Materials and Methods in a solutions containing 10 μM Ca^{2+}-ionophore A23187 for 20 min at 20°C before loading with 55 mM NaCl in 27 mM Hepes-Tris buffer (pH range 7.0–7.4), 185 mM sucrose, 1 mM ouabain and 3.75 mM EGTA and $CaCl_2$ to give the desired concentration of free Ca^{2+}. The free Ca^{2+} in the Ca^{2+}/EGTA buffer systems at the various pH values was calculated according to Pershadsingh and McDonald [11]. The control values were obtained from vesicles incubated without $CaCl_2$ at the same pH value.

needed to give the same inhibition at pH 7.0. One way to explain the curves could be to assume a Ca^{2+}-H^+ competition at a common binding site.

This assumption was subsequently tested in another series of experiments, shown in Fig. 4, in which the inhibition induced by a single concentration of Ca^{2+} at various pH values was evaluated. The vesicles were prepared to contain 100 nM free Ca^{2+} at intravesicular pH values ranging from 7.0 to 7.4. The amiloride-sensitive $^{22}Na^+$ flux by maintaining a constant extravesicular pH (pH 8.0) was examined. The figure shows that including 100 nM free Ca^{2+} in the vesicles did not change the $^{22}Na^+$ uptake notably within this pH range. Thus 100 nM Ca^{2+} reduced the amiloride-sensitive uptake of $^{22}Na^+$ from 60% at pH 7.0 to 47% at 7.4. The result of these experiments supported the view that there is only a slight modulation in the sensitivity of the channel to Ca^{2+} with internal pH. In contrast, previous findings showed that 100 nM intravesicular Ca^{2+} produced maximal inhibition of $^{86}Rb^+$ uptake into luminal membrane vesicles from pars convoluta of rabbit proximal tubule at pH 7.4 but had only slight effect at pH 7.0 [3].

Effects of other divalent cations on Na^+ uptake

To examine the specificity of the inhibition by Ca^{2+} ions, we studied the potential effects of other divalent ions Cd^{2+}, Ba^{2+} and Mg^{2+}. In the preparation procedure the vesicles initially were washed by 3.75 mM EDTA to remove traces of Mg^{2+} and Ca^{2+} ions. Next divalent cation/EGTA buffer solutions were made and free concentrations of Cd^{2+}, Ba^{2+} and Mg^{2+} were calculated using the tables and equations summarized by Bartfai [10]. Fig. 5A depicts the inhibition effect of these ions when included in the incubation medium together with tracer $^{22}Na^+$. Accordingly, this figure delineates the extravesicular effect of these ions. Addition of 1 mM Mg^{2+} did not affect the uptake significantly. Ca^{2+}, 1 mM, lowered the uptake to 61% of control. The amiloride-insensitive fluxes, i.e. 1 mM divalent cation + 350 μM amiloride, were in all cases at the same level, approximately 35% uptake, as in the presence of amiloride alone. Previously, we observed a large inhibition of Rb^+ flux into the luminal pars recta vesicles when Cd^{2+} was added in concentrations above 1 mM whereas addition of 10 mM $BaCl_2$ only affected the Rb^+ flux slightly [37]. The inhibitory effect of Ba^{2+} on the Na^+ channel mentioned here is higher than would have been expected for epithelial Na^+ channels, Some nonspecific interactions may account for this effect. However, Garty et al. reported that millimolar concentrations of Ba^{2+}, but not of Mg^{2+}, inhibited the amiloride-blockable $^{22}Na^+$ flux through epithelial Na^+ channels of toad bladder vesicles. They detected a maximal inhibition of 70% of the total amiloride-sensi-

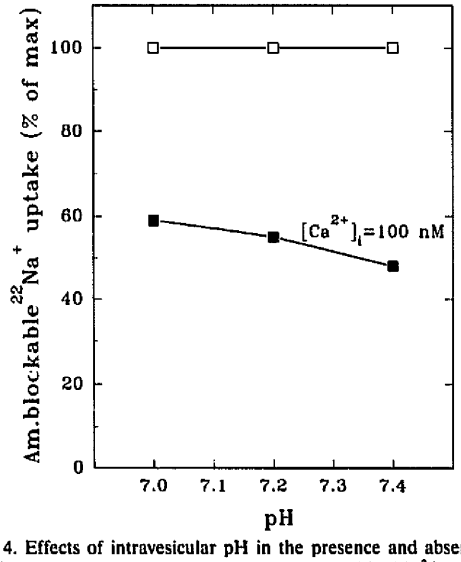

Fig. 4. Effects of intravesicular pH in the presence and absence of Ca^{2+}. Luminal membrane vesicles were prepared by Mg^{2+} precipitation in media buffered to pH 7.0, pH 7.2 or 7.4 containing either 3.75 mM EGTA or Ca^{2+}/EGTA buffer giving free Ca^{2+} concentration of 100 nM. The vesicles were incubated in media at pH 8.0 containing EGTA and with (■) and without (□) 10 μM A23187. Uptakes were measured in the absence and presence of 350 μM amiloride for 10 min.

tive Na^+ uptake, and noted that $BaCl_2$ concentrations at higher than 1 mM could give potential nonspecific effects [36].

The specificity of the intravesicular Ca^{2+} inhibition was studied by homogenizing the vesicles in the same buffer as above containing the divalent ions Ca^{2+}, Cd^{2+}, Ba^{2+} and Mg^{2+}. The reliability of this procedure was checked by comparing the inhibitory effect of 1 mM Ca^{2+} incorporated by the homogenization step with that in which Ca^{2+} was present initially during the preparation procedure. Either procedures resulted in comparable inhibition of Na^+ flux. Fig. 5B clearly shows that the divalent cations Cd^{2+}, Ba^{2+} and Mg^{2+} only have a modest inhibitory effect on Na^+ flux when present intravesicularly. Concentrations of 1 mM only reduced the uptake to approx. 80% of control. In contrast 1 mM free Ca^{2+} was as effective as 0.35 mM amiloride reducing the uptake to approximately 40% of control. Two different experimental protocols were employed to establish the electrical diffusion potential across the vesicle membranes. In the first, vesicles were loaded by homogenizing with 55 mM NaCl and divalent cation/EGTA buffer giving the desired free concentration of cation. In the second, vesicles were loaded by homogenizing with 55 mM KCl, the same divalent cation/EGTA buffer followed by adding 10 μM of the K^+-ionophore valinomycin to the incubation medium 1 min before adding Na^+ tracer. The latter protocol by loading with KCl and using K^+ ionophore has the advantage to exclude the possibility that the transient isotope accumulation could occur by electroneutral ion exchange via a carrier mediated countertransport kinetic mechanism [26]. As seen from Fig. 5B either protocols revealed the same $^{22}Na^+$ uptake pattern. The KCl-loaded vesicles are marked by filled bars in Fig. 5B. The results strongly support the view that Ca^{2+} ions in contrast to the other divalent cations exert their effect by specific interactions at the intravesicular part of the Na^+ channel.

In conclusion the data presented in this communication are compatible with the model that Ca^{2+} blocks a amiloride-inhibitable potential sensitive Na^+-channel located in pars recta of rabbit proximal tubule by binding specifically to a site composed of one or several deprotonated groups. The protonation of any of these groups prevents Ca^{2+} from binding to this site, but does not by itself block transport. Furthermore, the data indicate that Ca^{2+} in contrast to other divalent cations inhibits the Na^+ influx specifically inside the vesicles.

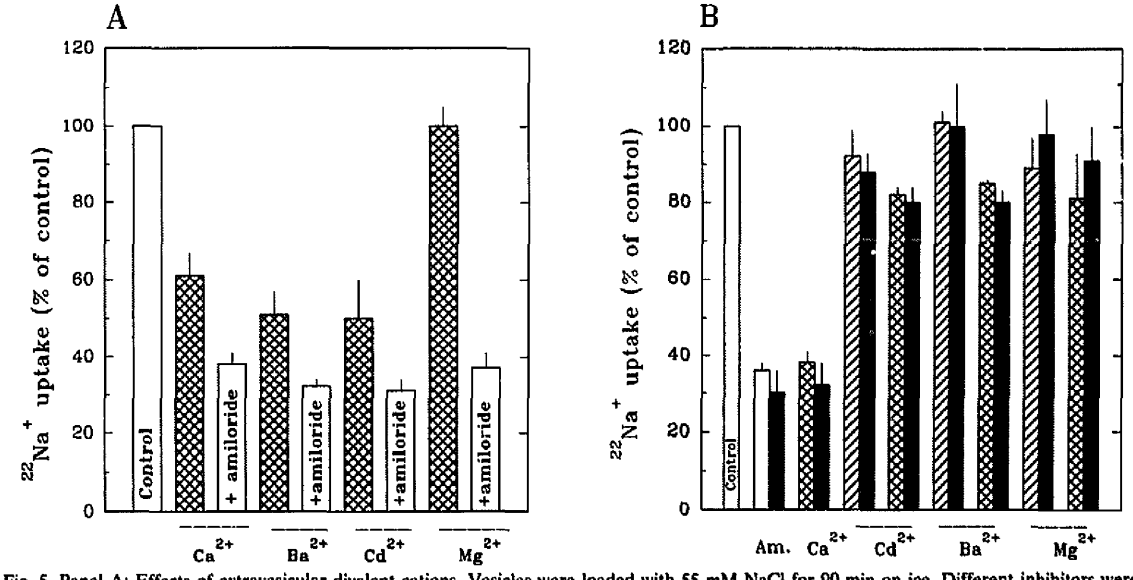

Fig. 5. Panel A: Effects of extravesicular divalent cations. Vesicles were loaded with 55 mM NaCl for 90 min on ice. Different inhibitors were added to the incubation medium and the $^{22}Na^+$ uptake was measured for 10 min. The free concentrations of Ca^{2+}, Cd^{2+}, Ba^{2+} and Mg^{2+} were calculated using the tables and equations summarized by Bartfai [10]. 5 μM (hatched to right); 1 mM (cross hatched). Control value was obtained by loading vesicles with 55 mM NaCl and without divalent cations in the medium. Panel B: Effect of intravesicular divalent cations. Two different protocols were employed to establish the electrical diffusion potential across the vesicle membranes. In the first, vesicles were loaded with 55 mM NaCl and divalent cation/EGTA buffer giving the desired free concentration of cation. In the second, vesicles were loaded with 55 mM KCl, the same divalent cation/EGTA buffer followed by adding 10 μM of the K^+-ionophore valinomycin to the incubation medium just before adding Na^+ tracer (solid bars). In either protocols the vesicles were loaded by homogenizing with 55 mM NaCl or 55 mM KCl, 185 mM sucrose, 27 mM Hepes-Tris and 3.75 mM divalent cation/EGTA giving the desired free concentration of the cations. 5 μM (hatched to right); 1 mM (cross hatched). Results are means ± S.E. of three membrane preparations.

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