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Demonstration of Na^+-selective channels in the luminal-membrane vesicles isolated from pars recta of rabbit proximal tubule

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Characteristics of $^{22}Na^+$ fluxes through Na^+ channels in luminal-membrane vesicles isolated from either pars recta or pars convoluta of rabbit proximal tubule were studied. In NaCl-loaded vesicles from pars recta, transient accumulation of $^{22}Na^+$ is observed, which is inhibited by amiloride. The isotope accumulation is driven by an electrical diffusion potential as shown in experiments using either these membrane vesicles loaded with different anions, or an outwardly directed K^+ gradient with a K^+ ionophore valinomycin. The vesicles containing the channel show a cation selectivity with the order $Li^+ > Na^+ > K^+$. The amiloride-sensitive $^{22}Na^+$ flux is dependent on intravesicular Ca^{2+}. In NaCl-loaded vesicles from pars convoluta, no overshoot for $^{22}Na^+$ uptake is observed. Furthermore, addition of amiloride to the incubation medium did not influence the uptake of $^{22}Na^+$ in these vesicle preparations. It is concluded that Na^+ channels are only present in pars recta of rabbit proximal tubule.

Na^+ channel; Potential sensitivity; Membrane vesicle; (Pars recta)

1. INTRODUCTION

Amiloride-sensitive Na^+ channels are generally found in epithelia which respond to the hormone aldosterone or antidiuretic hormone. However, recent studies employing the patch-clamp technique, suggested the involvement of Na^+-selective channels in transepithelial salt transport in the straight portion of rabbit proximal tubule [1]. The object of the present investigation has been to identify Na^+ channels in the luminal-membrane vesicles isolated from two distinct regions of rabbit proximal tubule, namely pars convoluta and pars recta, as an essential first step towards purification of the protein(s) involved and characterization of their role in the regulation of Na^+ reabsorption. The $^{22}Na^+$ uptake is measured against a large opposing chemical gradient of Na^+. For a detailed description of the principles involved in measuring isotope fluxes through ion channels by this method see [2].

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In short, for that fraction of vesicles containing Na^+ channels, the selective Na^+ permeability together with an outwardly directed Na^+ gradient are expected to produce an electrical diffusion potential, exterior positive, which should cause $^{22}Na^+$ to accumulate. When the chemical gradient of Na^+ is dissipated, isotope should then flow out of the vesicles. With this procedure we have been able to detect amiloride-sensitive Na^+ channels in the luminal-membrane vesicles from pars recta of rabbit proximal tubule.

2. MATERIALS AND METHODS

2.1. *Preparation of luminal-membrane vesicles*

Luminal-membrane vesicles were isolated from pars convoluta ('outer cortex') and from pars recta ('outer medulla') of the proximal tubule of rabbit kidney according to [3] and the method is only briefly described here. Our cortical tissue was obtained by taking slices ≤ 0.3 mm thick from the surface of the kidney containing pars convoluta. Strips of the outer medulla tissue approx. 1 mm thick (representing predominantly pars recta) were dissected from the outer strips of the outer medulla. We always prepared luminal-membrane vesicles from outer cortical and outer medulla tissue from the same kidneys and the two preparations were performed in parallel. The renal tissue

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was homogenized and vesicles were prepared by differential centrifugation and Mg^{2+} precipitation analogous to the Ca^{2+} precipitation described in [3]. The final pellet was washed by a solution containing EDTA (3.75 mM), sucrose (298 mM) and 27 mM Hepes/Tris buffer (pH 7.4) and resuspended in the same medium minus EDTA. In a series of experiments 10 μM Ca^{2+} ionophore (A23187) and 3.75 mM EGTA were added in the washing medium, in order to remove traces of intravesicular Ca^{2+}. The purity of the membrane vesicle preparations was examined by electron microscopy [4] and by measuring specific activities of various enzyme markers as in [3]. The amount of protein was determined as in [5] as modified in [6] with serum albumin as a standard. All solutions used in this study were sterilized before use.

2.2. *Na^+ fluxes into luminal-membrane vesicles*

Measurements of the Na^+ fluxes into the vesicle preparations were performed essentially as the protocol described by Garty et al. [2]. Dowex 50W-X8 columns were prepared in 1 ml tuberculin syringes. The resin was washed with 1 M Tris to bring it on Tris form, followed by 5–6 vols of 298 mM sucrose, 27 mM Hepes/Tris, pH 7.4, containing 1 mg/ml human 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 EDTA, 55 mM NaCl (or 55 mM KCl, LiCl, Na-gluconate or 27.5 mM Na_2SO_4), 27 mM Hepes/Tris, pH 7.4, for 1.5 h on ice. The vesicles were collected by centrifugation (25000 × g for 30 min at 2°C) and resuspended in the same buffer, approx. 700 μl, with a protein concentration of 5 mg/ml. A portion of 300 μl was applied to one column, another 300 μl to another column. Both samples were then centrifuged on a clinical centrifuge (1000 rpm) for 2 min. The pooled eluates were diluted to 2.0 ml with 298 mM sucrose, 27 mM Hepes/Tris, pH 7.4, buffer, and divided in 2 portions of 1.0 ml, one with amiloride (350 μM), the other without addition of amiloride. The experiment was initiated by adding 25 μl $^{22}Na^+$ (122 kBq; 0.5 nmol). Samples of 140 μl were removed at various times (1, 3, 5, 7, 10, 15 and 60 min) and uptake was stopped by passing the samples through the Dowex columns by centrifugation. The eluates were diluted to 500 μl and used for protein and radioactivity determinations. 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 7.4, containing 3.75 mM EGTA and 1.5–3.85 mM $CaCl_2$ to give the desired concentration of free Ca^{2+} before adding tracer $^{22}Na^+$. The free Ca^{2+} in the EGTA-Ca^{2+} buffer system was calculated according to Pershadsingh and McDonald [7].

3. RESULTS AND DISCUSSION

3.1. *Characteristics of $^{22}Na^+$ uptake in luminal-membrane vesicles*

Fig.1A shows the uptake pattern of $^{22}Na^+$ into highly purified luminal-membrane vesicles from pars recta loaded with NaCl, in the absence (curve 1) or presence of amiloride (curve 2). It is seen that the uptake of isotope against the opposing Na^+ concentration gradient rose to a peak value at about 10 min and then decreased towards the equilibrium value. In the presence of amiloride uptake was much reduced and the transient isotope accumulation phenomenon ('overshoot') was not observed. These were the characteristics expected

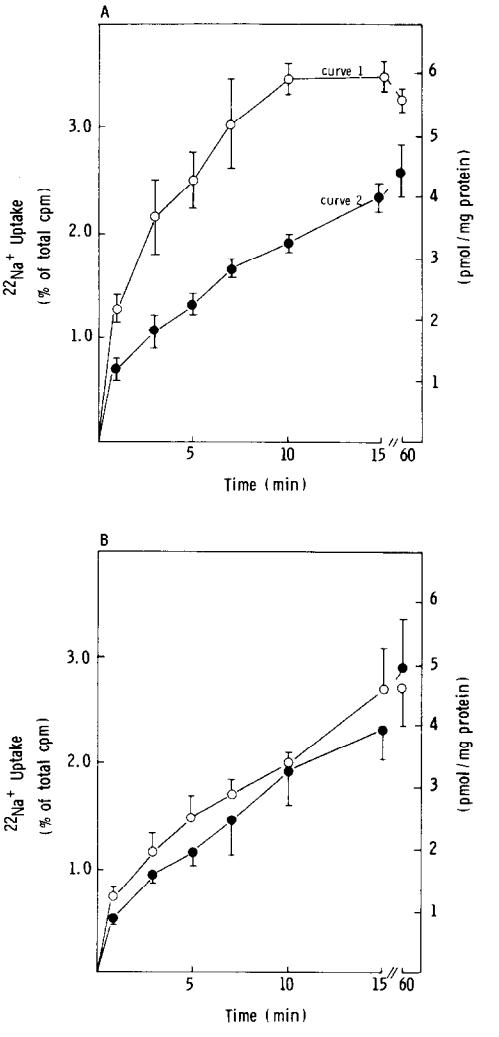

Fig.1. Time course of $^{22}Na^+$ uptake into luminal-membrane vesicles from rabbit proximal tubules. 100 μl of vesicles (protein conc. 1.5 mg/ml), Na_{in}^+ = 55 mM, Na_{out}^+ = 0 mM, were incubated at room temperature with $^{22}Na^+$ (122 kBq; 0.5 μM) in the absence (curve 1, ○) and presence (curve 2, ●) of 0.35 mM amiloride. Means ± SD of four independent experiments are shown. (A) Luminal-membrane vesicles obtained from pars recta; (B) luminal-membrane vesicles obtained from pars convoluta. The uptake is expressed as a percentage of the total counts present in the transport assay.

for a conductive $^{22}Na^+$ flux via an amiloride-inhibited Na^+ channel.

Fig.1B depicts the time course of $^{22}Na^+$ uptake by vesicles from pars convoluta loaded with NaCl. No overshoot was observed under these experimental conditions. Furthermore, it appears from the figure that addition of amiloride to the incubation medium did not significantly influence the uptake of $^{22}Na^+$. These results suggest that Na^+ channels only exist in straight segments (pars recta) of rabbit proximal tubule. The experiments described in the following sections are therefore performed with luminal-membrane vesicles isolated from pars recta of rabbit proximal tubule.

3.2. *Electrical nature*

Table 1 provides direct evidence that the amiloride-inhibited $^{22}Na^+$ accumulation is driven by an electrical potential. In these experiments the luminal-membrane vesicles from pars recta were loaded with either 55 mM NaCl, 27.5 mM Na_2SO_4 or 55 mM Na-gluconate, and the uptake of $^{22}Na^+$ was studied in the absence and presence of amiloride. It is apparent from table 1 that the rate of uptake of isotope was highest for gluconate, intermediate with sulfate medium and lowest with chloride. The results can be explained as follows. It is known that the passive permeability of the vesicle membrane for gluconate is lower than that for sulfate while the permeability of the divalent anion sulfate is lower than that for chloride [8,9]. The cation diffusion potential should therefore be progressively short-circuited by anions of increasing permeability, i.e., in the order $Cl^- > SO_4^{2-} >$ gluconate, and hence the rate of the $^{22}Na^+$ uptake should follow the observed order $Cl^- < SO_4^{2-} <$ gluconate. The amiloride-insensitive flux was not affected by anion (not shown) and is therefore probably electroneutral.

The above-mentioned theory was further tested by examining the effect of K^+ ionophore valinomycin [10] on the uptake of $^{22}Na^+$ in KCl-loaded vesicles. In these experiments luminal-membrane vesicles from pars recta were loaded with 55 mM KCl for 90 min at 0°C. These vesicles were then passed through a Tris-equilibrated Dowex column as usual, and aliquots were incubated for 1 min with valinomycin, or with ethanol (2%) and $^{22}Na^+$ uptake was measured. Fig.2 shows that the valinomycin-induced K^+ diffusion potential enhanced the influx of isotope (cf. curve 1 with 3) in these vesicle preparations. These results thus established that the amiloride-sensitive $^{22}Na^+$ flux is driven by an electrical diffusion potential. Curves 2 and 4 serve as a control and show the influx of $^{22}Na^+$ into NaCl-loaded vesicles in the absence and presence of amiloride, respectively.

Table 1

Evidence of electrogenic Na^+ channels in luminal-membrane vesicles from pars recta

Loaded with	$^{22}Na^+$ uptake (pmol/mg protein per 10 min)
NaCl (55 mM)	10.2
Na_2SO_4 (27.5 mM)	14.9
Na-gluconate (55 mM)	15.5

The vesicles were loaded as described in the text and uptake assayed for 10 min. Results are mean values of two independent experiments

3.3. *Ion selectivity*

Fig.3 summarizes information on the monovalent cation selectivity of the vesicles containing the amiloride-sensitive Na^+ channel. The selectivity to cation was tested by loading vesicles with different cations and examining the ability of opposing gradients of various cations to sustain amiloride-sensitive $^{22}Na^+$ accumulation. The observed ranking order $Li^+ > Na^+ > K^+$ is a reflection of the decreasing electrical potential driving $^{22}Na^+$ accumulation and therefore of the relative permeability of the membrane vesicles of interest to these cations. The result could mean that the Na^+ channels themselves show this order of selectivities, but it is more probable that the Na^+ channels show a higher selectivity to Na^+ than to say K^+, and in addition the membrane vesicles are somewhat selective for all cations compared to anions.

3.4. *Role of intravesicular Ca^{2+}*

It has previously been reported that incubation with a high concentration of EGTA and the Ca^{2+} ionophore A23187 in combination are required for depletion of Ca^{2+} and reduction of K^+ channel activity in luminal-membrane vesicles isolated from thick ascending limb of Henle's loop of the rabbit

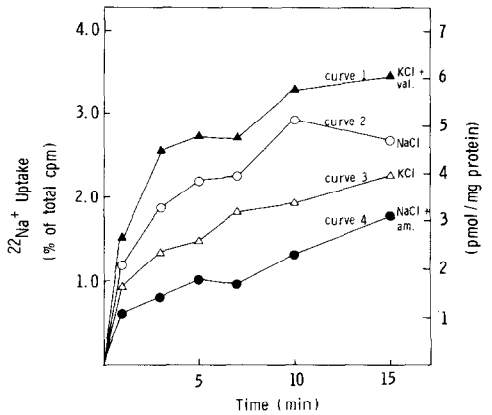

Fig.2. Time course of $^{22}Na^+$ uptake into luminal-membrane vesicles from pars recta. NaCl-loaded vesicles (55 mM) incubated in the absence (curve 2, ○) and presence of 0.35 mM amiloride (am.) (curve 4, ●). KCl-loaded vesicles (55 mM) in the absence (curve 3, △) and presence of 10 μM valinomycin (val.) (curve 1, ▲). The experimental protocol was as described in the legend to fig.1. Results are mean values of two independent experiments.

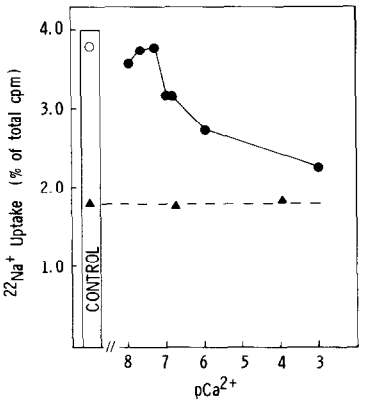

Fig.4. The effect of Ca^{2+} on the $^{22}Na^+$ uptake into luminal-membrane vesicles from pars recta. The vesicles were depleted of Ca^{2+} by treatment with 3.75 mM EGTA and 10 μM Ca^{2+} ionophore A23187 for 20 min at 20°C before loading the vesicles with 55 mM NaCl. The vesicles were incubated for 1 min at 20°C with 298 mM sucrose, 27 mM Hepes/Tris, pH 7.4, containing 3.75 mM EGTA and 1.5–3.85 mM $CaCl_2$ to give the desired concentration of free Ca^{2+} before adding tracer $^{22}Na^+$. As references, vesicles loaded with NaCl (55 mM) and incubated in the absence (○) and presence of 0.35 mM amiloride (▲) are shown.

kidney [11]. Exactly the same procedures have been used in this study, to remove intravesicular Ca^{2+} completely. Fig.4 illustrates the effect of increasing Ca^{2+} concentration on the uptake of $^{22}Na^+$ by luminal-membrane vesicles from pars recta. It appears from fig.4 that at low intravesicular concentrations of Ca^{2+} the influx of

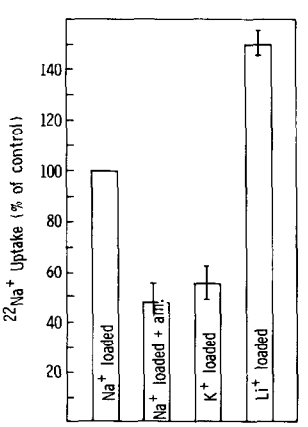

Fig.3. $^{22}Na^+$ uptake into luminal-membrane vesicles from pars recta loaded with either 55 mM NaCl in the absence or presence of 0.350 mM amiloride (am.) or 55 mM KCl or 55 mM LiCl. The uptake was measured for 10 min. The experimental protocol was as described in text to fig.1.

radioactive Na^+ is not significantly different, but at relatively high Ca^{2+} concentrations the influx of $^{22}Na^+$ gradually decreased. The concept of intracellular Ca^{2+} as a regulator of luminal-membrane Na^+ channel activity has intuitive appeal. Ca^{2+} has been established as a regulator of other channels. In this connection, it is of interest to note that Garty and Lindemann [12] provided evidence that the decrease in luminal-membrane Na^+ conductance in the frog skin is dependent on serosal Ca^{2+} concentration and is accelerated by high mucosal Na^+ concentrations. In agreement with Taylor and Windhager [13], they proposed that Ca^{2+} is taken up across the basolateral-membrane via a Na^+-Ca^{2+} antiport mechanism and that Ca^{2+} causes feed-back inhibition of the luminal-membrane Na^+ channels. Direct evidence for the existence of similar regulatory mechanism in the mammalian renal proximal tubule has not been provided in the literature. However, Gmaj et al. [14], in their study on the Ca^{2+} transport into brush-border membrane vesicles and basolateral-membrane vesicles isolated from rat renal cortex, observed the presence of an Na^+/Ca^{2+} exchange system in basolateral-membrane vesicles, but not

in the brush-border-membrane vesicles, These findings together with the results presented in this communication suggest a possible role of Ca^{2+} in the regulation of luminal-membrane Na^{+} channels in pars recta.

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