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Sodium-Hydrogen Exchange System in Brush Border Membranes from Cortical and Medullary Regions of the Proximal Tubule

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The Na^+/H^+ exchange system was studied in brush border membrane vesicles isolated from cortical and medullary regions of the proximal tubule of rabbit kidney. The activity of the exchanger was assessed by measuring hydrogen influx (monitored by acridine orange fluorescence), ^{22}Na influx and the sensitivity of these fluxes to amiloride and its analogue ethylisopropyl amiloride. In contrast to previously published data (indicating the absence of pH-gradient driven and amiloride sensitive ^{22}Na-influx in medullary site vesicles (13,15)), Na^+/H^+ exchange activity could be detected in both membrane preparations by sodium tracer and fluorescence detection of hydrogen influx. Amiloride inhibition of ^{22}Na influx was more effectively protected by increasing sodium concentration in cortical than in medullary vesicles, suggesting differences in the action of amiloride in these preparations.

The Na^+/H^+ exchanger is shown to exist in all mammalian tissues investigated (1). This transport system has been implicated in a variety of cellular functions such as: cell proliferation (2,3,4), egg fertilization(5), volume regulation (6) and urine acidification (7). The acidification of urine is primarily mediated by the Na^+/H^+ exchange in the proximal part of the tubule in the mammalian kidney (8). The proximal tubule is known to divide into three functional segments: S1, S2 and S3 (9). The properties of various transport systems change along the length of the proximal tubule (10). A "classic" example of this is the sodium-coupled glucose transport, which is carried out by two distinctly different transporters in the proximal tubule. These carriers differ not only in their affinity to the substrate but also in their sugar specificity and sodium/glucose stoichiometry (11,12).

Utilizing ^{22}Na-tracer fluxes in isolated brush border membrane preparations, it was reported that the Na^+/H^+ exchange is not expressed in the S3 segment of the proximal tubule in the rabbit (13). In contrast, in perfused tubule, it was shown that the recovery of cells from acid load in this part of the nephron is sodium dependent and amiloride sensitive, indicating the existence of Na^+/H^+ exchanger in this segment (14). Recently, the group which originally reported the absence of this transporter in the S3 segment was able to measure the activity of the exchanger by employing the AO fluorescence technique. Again, ^{22}Na-influx measurements did not provide evidence for Na^+/H^+ exchange activity (15).

Abbreviations: AO, Acridine Orange; FCCP, carbonyl cyanide p-trifluoromethoxyphenyl hydrazone; TMA, tetramethylammonium; AU, arbitrary units; EIPA, ethylisopropyl amiloride; MES, 2-((N-morpholino)ethanesulfonic acid); HEPES, N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid.

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In the present paper the activity of the Na^+/H^+ exchanger was determined in brush border membranes isolated from outer cortex (S1) and outer medullary (S3) sites of rabbit kidney. In contrast to what has been reported previously, activity of the Na^+/H^+ exchanger in both preparations could be detected with by two different experimental procedures, Na-influx and AO fluorescence measurements.

MATERIALS

$^{22}NaCl$ was obtained from New England Nuclear (Switzerland). AO was obtained from Eastman Kodak (Rochester, NY). Valinomycin and FCCP were purchase from Boehringer (Mannheim, FRG). All other reagents were of the highest purity commercially available. TMA-gluconate, which substitutes for NaCl in the flux experiments, was made by titrating a solution of TMA-hydroxide with gluconic acid. EIPA was obtained from T. Friedrich, Max-Planck Institute for Biophysics, Frankfurt (FRG).

METHODS

Membrane vesicle preparation: Rabbit kidneys were taken out of their capsules and cut tangentially with a razor blade to collect the outer cortical part of the kidney. The remains of the kidneys were then cut transversely and dissected under binoculars to obtain the outer medullary region of the proximal tubule as previously described (11). Membrane vesicles were prepared as previously described (16). Tissue slices were homogenized using a polytron for 2 minutes at setting 5. 12 mM $MgCl_2$ was added for 15 min on ice. The homogenate was then centrifuged at 3000g for 15 min. The pellet was discarded and the supernatant was centrifuged at 27000g for 30 minutes. The pellet was homogenized by teflon pestle (20 strokes). $MgCl_2$ was added as before and the procedure was repeated with low and high speed spins. The last pellet was collected, suspended in transport buffer (300 mM mannitol, 50 mM MES pH 5.5 and 50 mM K-gluconate) and frozen in liquid nitrogen until use. Similarly to results described in reference 11, apical membrane markers (alkaline phosphatase and gamma-glutamyl transferase) were enriched more than 10 times while no enrichment was observed in the activity of basolateral enzyme marker (Na^+/K^+ ATPase; data not shown).

Transport assays: sodium influx was measured as previously described using a rapid filtration assay (17). Membranes were incubated for the appropriate periods of time with incubation medium containing the radiolabelled sodium (^{22}Na). At the end of the incubation the vesicles were separated from the incubation medium on Millipore filter (0.65 μ) under light suction. Subsequent washes followed the filtration, then filters were counted in a scintillation counter. The amiloride insensitive component of the sodium influx was defined as the influx in the presence of 1 mM amiloride.

Fluorimetric measurements: Hydrogen ion influx into vesicles loaded with NaCl were measured by continuous recording of AO fluorescence at 37°C as previously described (17,18). Briefly, a spectrofluorimetric cuvette was filled with 2.03 ml of buffer containing 6 μM of AO. Ionophores were added from ethanolic stock solution (final alcohol content never exceeded 0.5%). Fluorescence was measured with excitation at 493 nm and emission at 535 nm with a Shimadzu RF510 fluorimeter. To allow comparison of the fluorescence signal under different conditions, gain was altered to give an emission reading in the absence of vesicles of 90 AU, then remained constant throughout the experiment. To initiate each trial, 20 μl of vesicles in suspension, adjusted to give a final protein concentration of 350 μg/ml, were injected into the cuvette. EIPA was added to final concentration of 1 μM. At this concentration EIPA is known to block the activity of the Na^+/H^+ exchanger but does not have any effect on the fluorescence signal (in contrast, amiloride, at the concentration needed to block the exchanger, quenches the fluorescence signal).

Data presentation: Representative experiments are shown. Each point of the tracer uptake measurements represents a mean and standard deviation from at least 3 filters. All experiments were repeated at least three times with the same qualitative results, although with some quantitative differences in the transport rates between the different experiments and vesicle preparations.

RESULTS

Sodium uptake as a function of time is depicted in figure 1. In the presence of a pH gradient (pH 7.4 out vs. pH 5.5 in), a typical "overshoot" (increase intravesicular concentration above the equilibrium volume) was observed in both the medullary and the cortical vesicle preparations. No such "overshoot" could be seen in the absence of pH gradient, which is to be expected if sodium is driven into the vesicles by the electrochemical gradient of hydrogen ions (1). The peak of the overshoot was higher in the cortical preparation compared to the medullary preparation. Figure 2

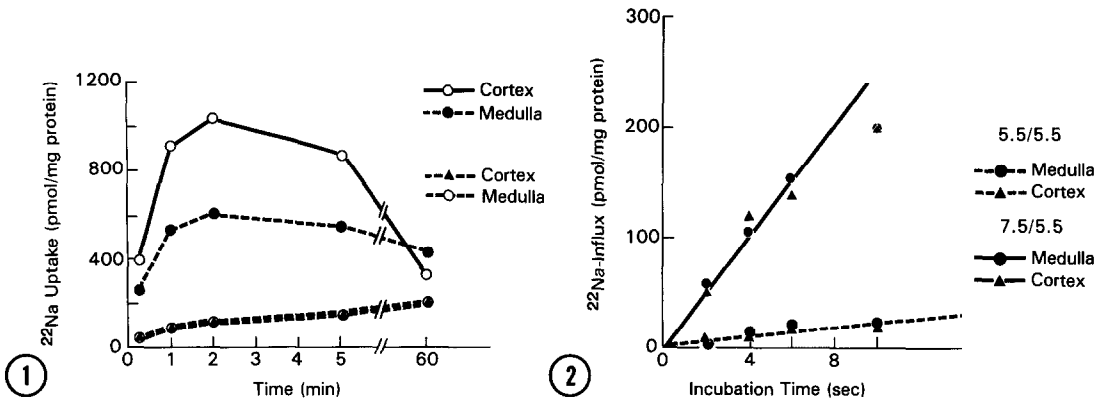

Figure 1 Time dependence of ^{22}Na uptake into brush border vesicles isolated from cortical (o,▲) and medullary (●,o) sites. Vesicles were preloaded with 300 mM manitol, 20 mM K-gluconate and 50 mM MES pH 5.5. Incubation media contained 300 mM mannitol, 1 mM NaCl, 20 mM K-gluconate, and adjusted to pH 5.5 (▲,o) or 7.5 (●,o) with 50 mM MES or Tris-HEPES, respectively.

Figure 2 Time dependence of uptake of ^{22}Na into brush border vesicles isolated from cortical (▲) and medullary (●) sites in the presence and absence of pH gradient. Intra- and extravesicular solutions are as described in figure 1.

shows, on an expanded time scale, the linear portion of the sodium influx into vesicles prepared from medullary and cortical sites in the presence and absence of a pH gradient.

Taken together, figures 1 and 2 manifest the activity of the exchanger at the two sites of the proximal tubule. Since a similar rate of sodium influx per milligram of vesicle protein was observed in each preparation, it is unlikely that cortical contamination accounts for the Na^+/H^+ activity measured in the medullary preparation. Furthermore, as similar equilibrium values were reached, we assume that the vesicular size is similar in the two preparations. This latter conclusion is also supported by control experiments where no differences between the two preparations were found for the equilibrium space for 0.1mM D-glucose (not shown).

The effect of amiloride on the initial rate of sodium influx (4 sec) into cortical or medullary vesicles at 0.1 and 10 mM sodium is depicted in figure 3. Half maximal amiloride inhibition was detected at about 50 to 100 μM, as has been reported for the effect of amiloride on Na^+/H^+ exchange in other systems (19). A semiquantitative comparison of the relative potency of amiloride (at an arbitrary concentraion of 50 μM) to inhibit ^{22}Na-influx at two different sodium concentrations is presented for the two membrane preparations in Table 1. This analysis suggests differences in the amiloride action on the Na^+/H^+ exchanger in the two vesicle preparations. In the cortex amiloride seems to be more effective though its action was more sensitive to an increase in the sodium concentration than in the medulla. This finding is consistent with the notion that at the medullary region amiloride and sodium do not compete for the same site (1).

Figure 4 depicts the changes in fluorescence with time of cortical vesicles loaded with AO and NaCl and bathed in sodium free medium. As expected for vesicles containing the Na^+/H^+ exchanger, there was a gradual acidification of the intravesicular space due to the exchange of sodium with hydrogen (broken line). When the cuvette contained 1 μM of EIPA (dashed and full lines) the acidification was blocked and only a minor change in fluorescence was observed (this is due to the quench of the dye by the vesicles (18)). As expected from a neutral transport system such as the Na^+/H^+ exchanger, clamping the membrane potential to zero (by exposing the vesicles

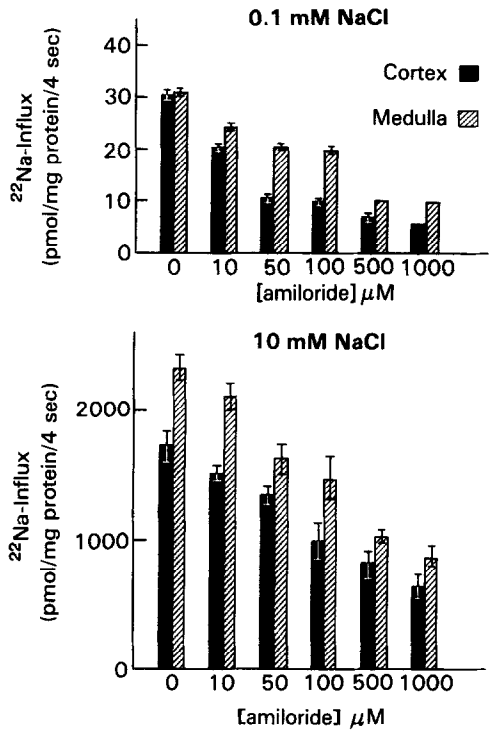

Figure 3 Concentration dependence of amiloride inhibition of the initial (4 sec) ^{22}Na-uptake into brush border membranes isolated from cortical (filled) and medullary (hatched) sites. Vesicles were loaded as described in Figure 1 containing 50 mM MES pH 5.5. Incubation media were similar to the incubation media described in figure 1 containing 50 mM Tris-HEPES pH 7.5, 0.1 mM (upper panel) or 10 mM sodium (lower panel).

to valinomycin in the presence of 50 mM K-gluconate on either side of the membrane) did not affect the rate of acidification (dashed-dotted line). In the presence of a sodium gradient the intravesicular space remained acidic. Collapse of this gradient by addition of sodium chloride to the external solution (arrow a) caused rapid alkalinization. Addition of an equimolar concentration of TMA to the external solution (arrow b) did not change the fluorescence. Figure 5 shows that the same phenomena depicted in figure 4 for vesicles isolated from the cortical site occured when vesicles isolated from the medullary site were injected into the fluorimetric cuvette. Although the magnitude of the signal was somewhat smaller, there was no qualitative difference between the two systems.

Table I

Inhibition of the ^{22}Na-influx in the presence of 50 μM amiloride as a function of Na^+ concentration

Sodium concentration	% of inhibition 0.1 mM	% of inhibition 10mM	Ratio 0.1mM/10mM
Cortex	63+6	25+4	2.50+0.3
Medulla	33+5	32+4	1.03+0.2

The data represent the mean + SE of 3 separate experiments performed each in triplicate. In each experiment (performed as in figure 3) inhibition was calculated as percent of control.

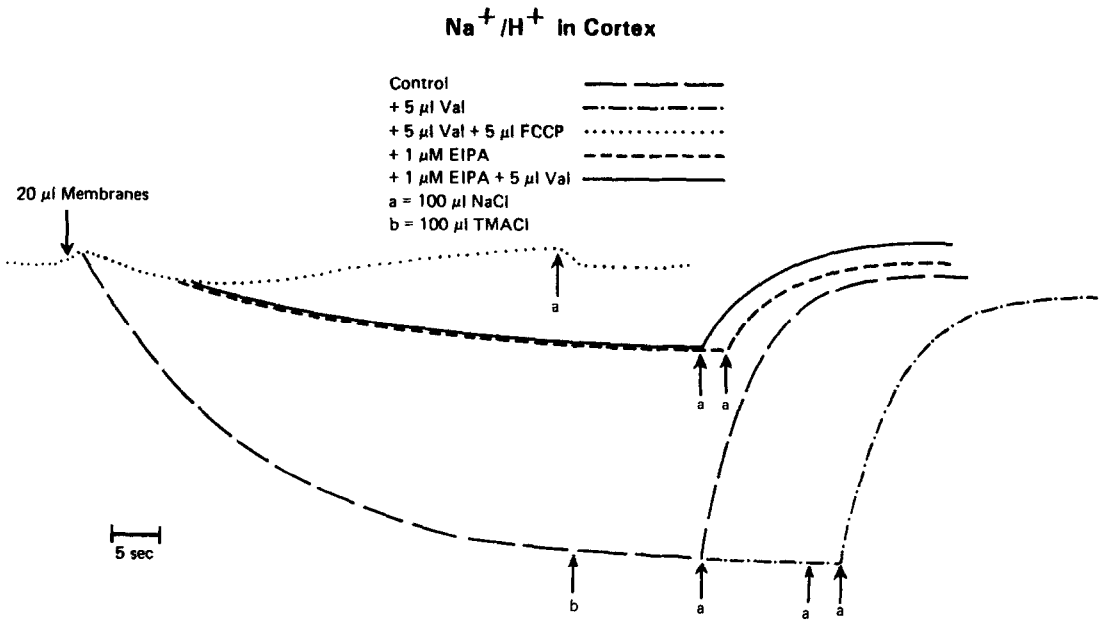

Figure 4 Changes in pH in vesicles isolated from the cortical site with time under various conditions. Vesicles were preloaded with 100 mM mannitol, 100 mM NaCl, 50 mM KCl and 5 mM HEPES-Tris pH 7.4. 20 µl of vesicles were injected into 2.03 ml of buffer (100 mM mannitol, 50 mM KCl, 100 mM TMA-Cl and 5 mM HEPES-Tris pH 7.4) containing (-.-) 2 µM valinomycin, (....) 2 µM valinomycin + 8 µM FCCP, (---) 1 µM EIPA, (—) 1 µM EIPA + 2 µM Valinomycin. 100 µl of NaCl 1 M was injected at (a) and 100 µl of TMA-Cl 1 M was injected at (b).

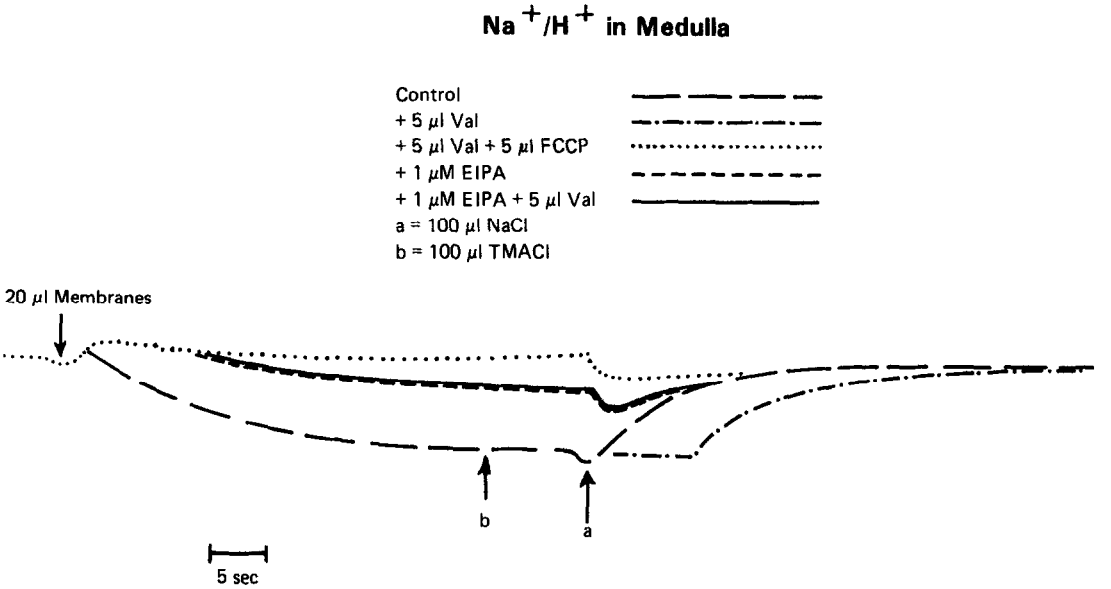

Figure 5 Changes in pH in vesicles isolated from the medullary site with time under various conditions. Vesicles were preloaded with 100 mM mannitol, 100 mM NaCl, 50 mM KCl and 5 mM HEPES-Tris pH 7.4. 20 µl of vesicles were injected into 2.03 ml of buffer (100 mM mannitol, 50 mM KCl 100 mM TMA-Cl and 5 mM HEPES-Tris pH 7.4) containing (-.-) 2 µM valinomycin, (....) 2 µM valinomycin + 8 µM FCCP, (---) 1 µM EIPA, (—) 1 µM EIPA + 2 µM Valinomycin. 100 µl of NaCl 1 M was injected at (a) and 100 µl of TMA-Cl 1 M was injected at (b).

Our data do not offer an explanation for the smaller signal in the medullary site vesicles as compared to the cortical membrane preparation. In view of our tracer flux measurements (Figures 1-3), we have no reason to believe that this difference in transport rates (as measured by the AO technique) reflects actual differences in the activity of the exchanger in the two preparations.

DISCUSSION

In the present study we have investigated the activity of the Na^+/H^+ exchange system in vesicle preparations made from two distinct parts of the rabbit nephron: the outer cortical and the outer medullary regions of the proximal tubule. The activity of the exchanger was evaluated in vesicles made from both segments by measuring sodium influx, using a rapid filtration assay and by monitoring hydrogen influx, using AO fluorescence.

An appropriate sodium gradient across the vesicle membrane resulted in hydrogen influx which was blocked by 1 μM EIPA. The change in intravesicular pH due to the activity of the exchanger was prevented in the presence of the protonophore FCCP. The Na^+/H^+ exchange activity was independent of membrane potential; the addition of valinomycin, when $[K^+]_{in} = [K^+]_{out}$, did not change the observed hydrogen influx into either of the vesicle preparations.

Differences were detected in tracer flux measurements when the interactions of amiloride and sodium were investigated in the two vesicle preparations (table 1). 10 mM sodium was more effective in lowering the inhibitory effect of an arbitrary amiloride concentration (50 μM) on the transporter in the outer cortical preparation as compared to the outer medullary vesicles. It is conceivable that less competition between sodium and amiloride occurs at the outer medullary site than at the outer cortical site. This would suggest a different inhibitory action of amiloride at the two proximal tubule sites.

Previous attempts to evaluate the activity of the exchanger in these two parts of the nephron, by tracer flux measurements, detected little or no activity of the exchanger in the outer medullary site (13,15). We have no explanation why in these previous studies Na^+/H^+ exchange activity could be detected in membranes from pars recta only by AO measurements and not by tracer flux studies. In our hands, both techniques provided evidence for Na^+/H^+ exchange activity from membranes derived from either site of the nephron with AO, being a signal smaller in pars recta membranes.

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