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Solubilization and Reconstitution of Renal Brush Border Na^+-H^+ Exchanger

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Summary. In order to permit future characterization and possible isolation of the Na^+-H^+ exchanger from the apical membrane of proximal tubular cells, studies were performed to solubilize and reconstitute this transporter. Rabbit brush border membranes were prepared by a magnesium aggregation method, solubilized with the detergent octyl glucoside, and reconstituted into artificial phospholipid vesicles. In the presence of a pH gradient (pH_{in} 6.0, pH_{out} 8.0), the uptake of 1 mM $^{22}Na^+$ into the proteoliposomes was five- to sevenfold higher than into liposomes. Amiloride (2 mM) inhibited proton gradient-stimulated uptake of sodium by 50%. As compared to proton gradient conditions, the uptake of sodium was lower in the absence of a pH gradient but was significantly higher when the outside and inside pH was 6.0 than 8.0. The K_a for sodium in reconstituted proteoliposomes studied under pH gradient conditions was 4 mM. The uptake of sodium in proteoliposomes prepared from heat-denatured membrane proteins was significantly decreased. These studies demonstrate that proteoliposomes prepared from octyl glucoside-solubilized brush border membrane proteins and asolectin exhibit proton gradient-stimulated, amiloride-inhibitable, electroneutral uptake of sodium. The ability to solubilize and reconstitute the Na^+-H^+ exchanger from the apical membrane of the proximal tubule will be of value in isolating and characterizing this transporter.

Key Words renal electrolyte transport · sodium transport · hydrogen ion transport · renal proximal tubule · Na^+/H^+ exchange · rabbit kidney

Introduction

The brush border membrane of the proximal convoluted tubule contains a transport protein(s) that exchanges sodium ions in the lumen for hydrogen ions in the cells [1, 9, 15]. This electroneutral cation exchanger is important in the reclamation of filtered bicarbonate and in the regulation of the acid-base balance of the organism. Recent evidence has been advanced that a sodium-proton exchange transport system is present in a variety of other cells. Operation and activation of the Na^+-H^+ exchanger has been suggested to be involved in the defense of cell volume, cell growth and tumor formation, and in the generation of systemic hypertension [14]. A number of recent studies have examined the factors that modulate the activity of this transporter protein [5].

In renal brush border membranes, the presence of a Na^+-H^+ exchanger has been inferred from the findings that the uptake of $^{22}Na^+$ into brush border membrane vesicles is stimulated by the presence of an oppositely directed proton gradient [9, 15]. This stimulation is not due to electrical coupling and can be inhibited by amiloride in millimolar concentrations. While the functional characteristics of the renal Na^+-H^+ exchanger have been defined, little is known about the nature of the transport protein itself [4, 18]. Study of the Na^+-H^+ exchanger would be facilitated greatly by development of methodology to solubilize the transporter and to examine its activity in a reconstituted system. The present communication reports results of studies designed to achieve these aims.

Materials and Methods

Brush border membranes were obtained from the rabbit kidney by a magnesium precipitation method as previously described [7, 8]. As assessed by comparison of alkaline phosphatase activity in the brush border membranes and the whole homogenate, the membrane preparation was enriched 10-fold or more.

Brush border membrane proteins were extracted by mixing one part of the membrane preparation (5 mg/ml) with 1.25 parts of 8% octyl glucoside at pH 6.0 for 15 min at 0°C. In the reaction mixture, the protein concentration was 2.2 mg/ml and the concentration of octyl glucoside was 4.4%. The membrane-detergent mixture was centrifuged at 100,000 × g for 30 min. 1.6 parts of the supernatant was then mixed with one part of asolectin (35 mg/ml) (crude soybean phospholipid) (Associated Concentrates, Woodside, L.I., N.Y.) which had been sonicated to translucency for 10 min at 22°C. The final measured concentration of the proteoliposomes-octyl glucoside mixture was 0.5 mg/ml protein. The final mixture contained 2.73% octyl glucoside, and 13.5 mg/

ml asolectin. The proteoliposomes were then dialyzed for 18 hr at 0°C using 6,000–8,000 dalton cutoff dialysis membranes. The dialysis solution was changed at 12 hr. The dialyzed proteoliposomes were used for transport studies. In the assayed samples there were 17 μg of protein and 459 μg of asolectin.

The original brush border membranes were suspended in a solution containing 254 mM mannitol, 10 mM Tris, 16 mM HEPES and 10 mM $MgSO_4$ (pH 7.6). The $MgSO_4$ was removed by washing and resuspending the brush border pellet to a final concentration of 5 mg/ml in 274 mM mannitol, 10 mM Tris, and 16 mM HEPES (pH 7.6). The 8% octyl glucoside and the sonicated asolectin (35 mg/ml) were prepared in the dialysate buffer which contained 250 mM mannitol, 50 mM MES/Tris (pH 6.0). The dialysis procedure removed the detergent and set the internal pH of the proteoliposomes. In studies where the internal pH was other than 6.0, the dialysate was adjusted to obtain the desired pH.

The uptake of $^{22}Na^+$ was determined in the proteoliposomes and liposomes. Except where indicated otherwise, the final uptake solution contained 1 mM Na^+, 250 mM mannitol, 50 mM Tris/MES (pH 8.0). 100 μl of the reaction mixture was applied to 1 ml Dowex 50 × 8 (Tris), 100-mesh column and rapidly eluted with vacuum suction with 1 ml of 300 mM sucrose (pH 8.0) at 0°C. The eluent containing the vesicles was collected directly into scintillation fluid and the radioactivity determined. To monitor the changes in intravesicular pH, liposomes and proteoliposomes were incubated in solutions containing acridine orange (6 μM). After the pH-sensitive dye partitioned into the intravesicular space, the change in fluorescence in response to different extravesicular solutions was monitored as a function of time. The data were fitted by computer analysis to the general equation

$$\Delta F = C \cdot e^{-kt}$$

where ΔF is the difference between final fluorescence and fluorescence at time t after mixing, and C and k are constants. The value k was taken to represent the first-order rate constant for dissipation of the hydrogen ion gradient. Protein concentrations were determined by the method of Lowry et al. [13]. Statistical comparisons were performed using Peritz analysis of variance [6].

Results

In initial experiments, brush border membranes were incubated in varying concentrations of octyl glucoside, and the partition of membrane proteins into the soluble and particulate fractions was determined. When studied under proton gradient conditions, the uptake of $^{22}Na^+$ into proteoliposomes prepared with the solubilized membrane proteins was 16.0 nmol · 2 min^{-1} · mg $protein^{-1}$ when the octyl glucoside concentration was 4.4%, 14.1 nmol · 2 min^{-1} · mg $protein^{-1}$ when the octyl glucoside concentration was 2%, and 4.3 nmol · 2 min^{-1} · mg $protein^{-1}$ min at octyl glucoside concentrations of 0.5 and 1%. Exposure of the brush border membranes to 4.4% octyl glucoside resulted in solubilization of 70% of the membrane protein. Based on initial rates of amiloride inhibitable, proton gradient-stimulated sodium uptake in natural brush border membranes from the rabbit kidney and in proteoliposomes reconstituted with detergent solubilized proteins from these membranes, approximately 70% of Na^+-H^+ exchange activity was recovered in the detergent solubilized proteins. As compared to proton gradient-stimulated (pH_{in} 6.0, pH_{out} 8.0), amiloride-inhibitable, sodium uptake in natural brush border membranes, 60 to 80% of transport activity was recoverable in the solubilized fraction assayed in reconstituted proteoliposomes. In all subsequent studies, the brush border membranes were solubilized in 4.4% octyl glucoside and the solubilized proteins used to prepare the proteoliposomes.

Under proton gradient conditions (pH_{in} 6.0, pH_{out} 8.0), the uptake of sodium by the proteoliposomes was linearly related to the concentration of protein up to protein concentrations of 1 mg/ml. All remaining studies were performed using a protein concentration of 0.5 mg/ml. If the proteoliposomes formed after overnight dialysis were left at 0°C for an additional 24 hr, there was a significant loss of Na^+-H^+ exchange activity. If the solubilized proteins, in the presence of octyl glucoside, were left for 24 hr at 0°C, there was also a significant loss of Na^+-H^+ exchange activity. However, if the solubilized proteins were dialysed overnight to remove the detergent, subsequent reconstitution of these proteins into proteoliposomes resulted in demonstrable transport activity. If the solubilized proteins, in the presence of octyl glucoside, were frozen at −80°C, activity remained at the same or higher levels as freshly prepared proteoliposomes for at least two weeks. Subsequent studies were performed using either freshly prepared proteins or rapidly frozen membrane proteins stored at −80°C.

Figure 1 is the time course of uptake of $^{22}Na^+$ from a 1 mM sodium solution into proteoliposomes and liposomes studied under proton gradient conditions. The results are expressed as pmol/mg lipid to permit direct comparison between proteoliposomes and liposomes. Also shown is the effect of amiloride (2 mM) on sodium uptake. As compared to liposomes, the uptake of sodium in the proteoliposomes was five- to sevenfold higher. The presence of amiloride resulted in slightly higher rates of sodium uptake in the liposomes. Amiloride inhibited the uptake of sodium into the proteoliposomes at two minutes by approximately 50%. If the results are corrected for the effect of amiloride on the liposomes, the inhibitory effect of amiloride in proteoliposomes was over 70%. The uptake of sodium in the presence of amiloride in proteoliposomes approximated that observed in the liposomes studied under similar conditions.

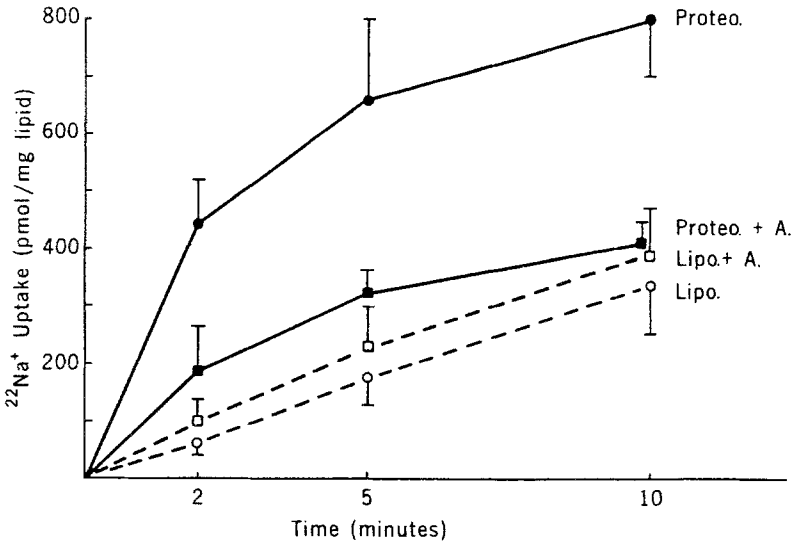

Fig. 1. The time-dependent uptake of $^{22}Na^+$ in proteoliposomes (*Proteo.*) and liposomes (*Lipo.*), in the absence and presence (+*A*) of amiloride (2 mM). The extravesicular sodium concentration was 1 mM. Sodium uptake is expressed as pmol/mg lipid. The protein concentration of the proteoliposomes was 0.5 mg/ml. Studies were performed under proton gradient conditions (pH_{in} 6.0, pH_{out} 8.0). Values are means ± SEM for seven preparations

Apparent equilibrium was achieved at 180 min of incubation when sodium uptake averaged 1280 ± 271 pmol/mg lipid in the absence of amiloride and differed by less than 10% in the presence of amiloride. The above uptakes, however, did not represent true equilibrium values as sodium uptake continued to increase over extended time periods up to and beyond 24 hours. The kinetics of these processes would argue against their being due to relevant transporters and were not explored further. To determine the volume of proteoliposomes and to exclude the possibility that the inhibitory action of amiloride was the consequence of a nonspecific effect on the integrity of the vesicles, the uptake of rubidium was determined in proteoliposomes. The uptake of rubidium was determined under proton gradient conditions where $K_{in} = K_{out} = 33$ mM and in the presence of valinomycin. Equilibrium uptake of rubidium was attained by 30 min and was not affected by the presence of amiloride (2 mM). The vesicular volumes, calculated from the rubidium uptake at equilibrium, averaged 1.57 ± 0.07 μl/mg lipid in the absence of amiloride and 1.52 ± 0.18 μl/mg lipid in the presence of amiloride.

Figure 2 is the dose-response relation between the uptake of 1 mM $^{22}Na^+$ and the concentration of amiloride. The uptake of sodium is expressed as nmol · 2 min^{-1} · mg $protein^{-1}$. Amiloride inhibited the proton gradient-stimulated uptake of sodium in a dose-dependent manner. Maximum inhibition of sodium uptake was observed at concentrations of amiloride of 2 to 4 mM. The K_i for amiloride was 0.75 mM. In all subsequent studies in proteoliposomes, amiloride was used in a concentration of 2 mM.

To examine the possibility that the higher K_i for

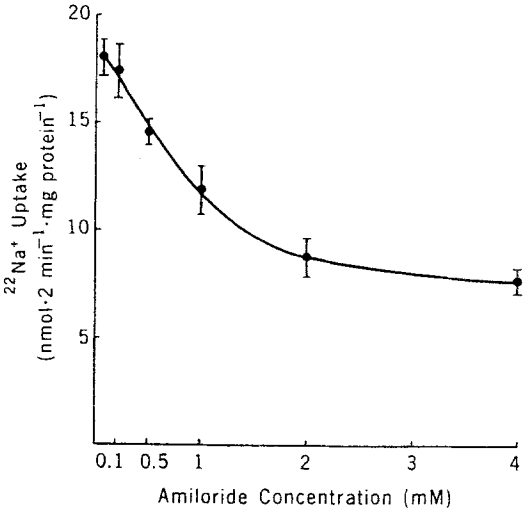

Fig. 2. Effect of amiloride on proton gradient (pH_{in} 6.0, pH_{out} 8.0) stimulated uptake of $^{22}Na^+$ in proteoliposomes. The extravesicular sodium concentration was 1 mM. Sodium uptake is expressed as nmol · 2 min^{-1} · mg $protein^{-1}$. Values are the means ± SEM for three preparations

amiloride in reconstituted proteoliposomes as compared to natural brush border membranes was the secondary consequence of partitioning of amiloride into a vesicular space with a reduction in the external concentration of the drug, additional studies were performed. The K_i for amiloride was determined in natural brush border membranes and in brush border membranes to which liposomes were added in the same concentration as in the proteoliposome studies. The uptake of $^{22}Na^+$ was determined under pH gradient conditions (pH_{in} 6.0, pH_{out} 8.0) using Dowex columns. Uptake was measured at 10

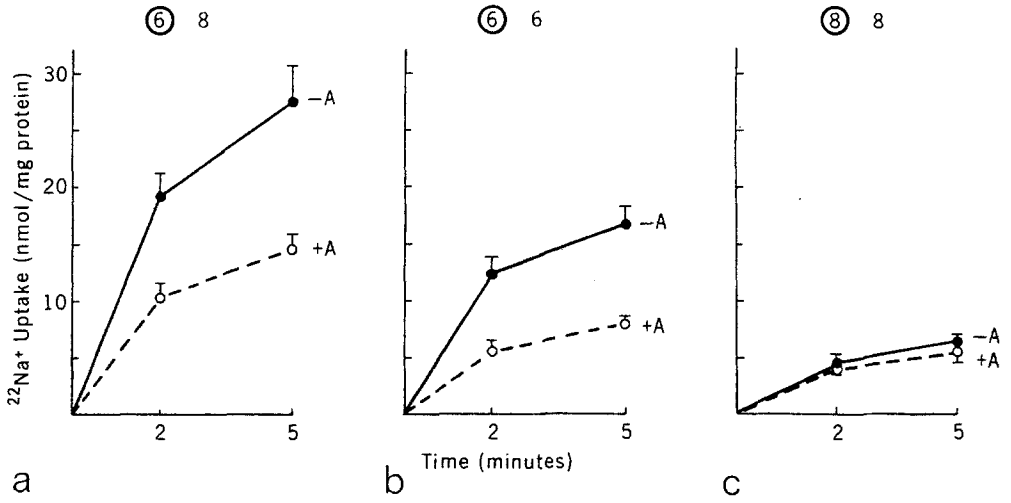

Fig. 3. The pH dependence of $^{22}Na^+$ uptake into proteoliposomes, in the absence ($-A$) and presence ($+A$) of amiloride (2 mM). The extravesicular sodium concentration was 1 mM. Values are means ± SEM for four preparations. The internal and external pH's are indicated above each panel

sec in the absence and presence of a range of concentrations of amiloride from 1 μM to 2 mM. Under the conditions of study, the K_i for amiloride averaged 37 μM in natural membranes and 62 μM in membranes containing added liposomes (n = 2).

To explore further the relation between the Na^+-H^+ exchanger in reconstituted proteoliposomes and amiloride, studies were performed using amiloride analogues with greater or less affinity for the exchanger as determined in other systems [17]. Phenamil, an analogue 100-fold more potent than amiloride for inhibition of the sodium channel but 10 times less potent for inhibition of Na^+-H^+ exchanger, did not affect the proton gradient-stimulated sodium uptake in the reconstituted proteoliposomes in concentrations up to 1 mM. 6-iodoamiloride and 5-(N-ethyl-N-isopropyl) amiloride both have greater affinity for the Na^+-H^+ exchanger than amiloride but differ in their effect on sodium channels. 6-iodoamiloride inhibits sodium channels with a K_i similar to amiloride while 5-(N-ethyl-N-isopropyl) amiloride is 100 times less potent. In reconstituted proteoliposomes, both 6-iodoamiloride and 5-(N-ethyl-N-isopropyl) amiloride in maximal concentrations, inhibited the proton gradient-stimulated sodium uptake to a degree equal to that of amiloride. The K_i for 6-iodoamiloride was 40 μM. The K_i for 5-(N-ethyl-N-isopropyl) amiloride was 10 μM. Thus, the rank order of potency was 5-(N-ethyl-N-isopropyl) amiloride >6-iodoamiloride > amiloride > phenamil.

Figure 3 summarizes the results of studies designed to determine the effect of a pH gradient, *per se*, on the uptake of sodium. Figure 3*a* is the uptake of sodium in the presence of a pH gradient (pH_{in} 6.0, pH_{out} 8.0). Figure 3*b* and *c* are the uptakes of sodium in the absence of a pH gradient where the internal and external pH was either 6.0 (Fig. 3*b*) or 8.0 (Fig. 3*c*). The uptake of sodium is higher in the presence of a pH gradient than in its absence. The uptake of sodium was higher in the presence of an acidic environment (pH 6.0) than in an alkaline environment (pH 8.0).

Additional studies were performed to determine if the pH gradient-stimulated uptake of sodium was occurring by a Na^+-H^+ exchange mechanism or was the secondary consequence of a voltage coupling due to the diffusion of hydrogen ions from the intravesicular space. The uptake of $^{22}Na^+$ was measured under voltage clamped conditions whereby the internal and external potassium concentration was 30 mM and valinomycin (10 μg/ml) was present. When the internal and external pH was 8.0, $^{22}Na^+$ uptake averaged 7.6 ± 0.4 nmol · 2 min^{-1} · mg $protein^{-1}$ (n = 4). As compared to results obtained at internal and external pH 8.0, sodium uptake was 100.6 ± 17.7% higher when the internal and external pH was 6.0, and 303.2 ± 17.9% higher under pH gradient conditions (pH_{in} 6.0, pH_{out} 8.0). Thus, under presumed voltage clamped conditions, the presence of an acidic intravesicular solution stimulated the uptake of sodium. To explore further the possible role of voltage coupling on sodium uptake under pH gradient conditions, $^{22}Na^+$ uptake was measured in the presence of 4 μM FCCP to create an intravesicular negative potential difference. $^{22}Na^+$ uptake averaged 22.3 ± 0.6 nmol · 2 min^{-1} · mg $protein^{-1}$ and 24.5 ± 0.5 in the absence and presence of FCCP, respectively (P = NS, n = 3).

Acridine orange fluorescence measurements

were made to determine if sodium in the bathing media could increase the efflux of protons from the intravesicular space. In a manner analogous to that observed using natural renal brush border membrane vesicles, the addition of proteoliposomes at pH 6.0 to a solution containing acridine orange at pH 8.0, resulted in rapid quenching of acridine orange fluorescence followed by a recovery phase as protons exited the intravesicular space. Under pH gradient conditions (pH_{in} 6.0, pH_{out} 8.0) and in the presence of potassium (K_{in} = K_{out} = 30 mM) and valinomycin, the rate constant for dissipation of the proton gradient was 216 ± 7% higher when the outside solution contained sodium (50 mM) as compared to N-methylglucamine gluconate (NMG) (50 mM) ($P < 0.01$) The rate constant for dissipation of the proton gradient (pH_{in} 6.0, pH_{out} 8.0) using acridine orange fluorescence was also used to explore other aspects of the conductive properties of the reconstituted proteoliposomes (n = 4). In separate experiments and in the absence of a voltage clamp, the rate constant for dissipation of the proton gradient averaged 0.048 ± 0.005 sec^{-1} when the outside solution contained 100 nM NMG and 0.062 ± 0.005 when the outside solution contained 100 mM potassium gluconate ($P < 0.05$). The increase in the rate constant for dissipation of the proton gradient when the outside solution contained potassium would suggest the presence of a potassium conductive pathway in the proteoliposomes. In the presence of the proton ionophore FCCP, the rate constant for dissipation of the proton gradient when NMG was present in the external solution was 0.050 ± .006 sec^{-1}; a value not significantly different from that observed in the absence of the ionophore. In the presence of FCCP and 100 mM potassium in the external solution, the rate constant was significantly increased to 0.102 ± 0.014 sec^{-1}. This result confirms the presence of a significant potassium conductance in the proteoliposomes. The failure of FCCP to increase the rate of hydrogen ion movement out of the vesicle was probably the result of a negative intravesicular potential difference generated by proton diffusion. When the potential difference was shunted by imposition of an oppositely directed potassium gradient, the rate of dissipation was significantly accelerated. Thus, FCCP was behaving as a proton ionophore and, in the absence of potassium, resulted in an intravesicular negative potential difference. In the presence of FCCP, valinomycin, and potassium in the external solution, the rate of dissipation of the proton gradient was increased further and averaged 0.320 ± 0.021 sec^{-1}. To examine in greater detail the proton conductive properties of the reconstituted proteoliposomes, the rate constant for dissipation of the proton gradient

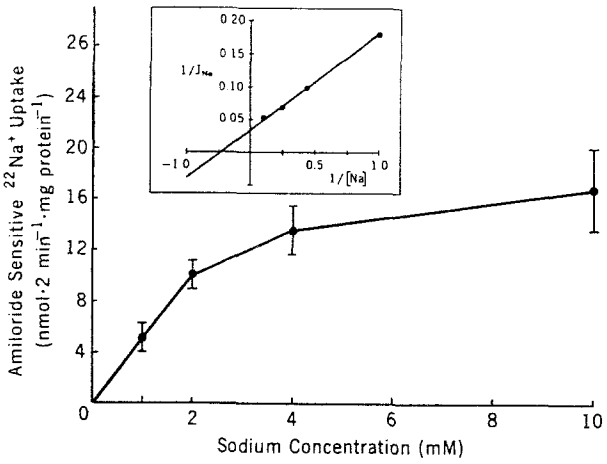

Fig. 4. Effect of external sodium concentration on the amiloride (2 mM) inhibitable component of $^{22}Na^+$ uptake studied under proton gradient conditions. Values are the means ± SEM for four preparations. The inset is a double reciprocal plot of sodium concentration and amiloride-sensitive $^{22}Na^+$ uptake

was determined in the presence of a voltage clamp and in the presence of an inwardly directed potassium gradient. As compared to control conditions with 100 mM NMG in the outside solution (0.048 ± 0.005 sec^{-1}), the rate constant was significantly higher in the presence of valinomycin and equal concentrations of potassium (100 mM) inside and outside the vesicles (0.140 ± 0.020 sec^{-1}). In the presence of valinomycin and potassium in the outside solution only, circumstances designed to induce an intravesicular positive potential difference, the rate constant for dissipation of the proton gradient averaged 0.278 ± 0.030 sec^{-1}.

Figure 4 plots the relation between the concentration of sodium in the external uptake solution and the amiloride-sensitive component of sodium uptake. The uptake of sodium from an external solution containing 1 or 20 mM sodium was linear for at least 2 min. The uptake of sodium at 2 min, therefore, was taken as an approximation of an initial rate. A double reciprocal plot of sodium concentration and sodium uptake is shown in the inset. The K_a for sodium was 4 mM. Additional studies were performed to determine if other monovalent cations affected the amiloride-sensitive uptake of 1 mM $^{22}Na^+$ under proton gradient conditions (n = 4). Studies were performed in the presence of potassium (30 mM) in the internal and external solutions and valinomycin. To the 1 mM $^{22}Na^+$ uptake solution, lithium, sodium, or tetramethylammonium (TMA) as the chloride salts, were added in concentrations of 5, 10, or 20 mM. Under the conditions of study, TMA did not affect the uptake of $^{22}Na^+$. At all concentration of TMA tested, $^{22}Na^+$ uptake av-

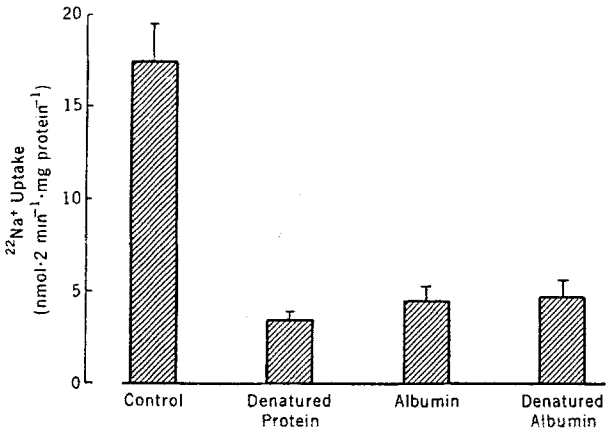

Fig. 5. The specificity of proton gradient-stimulated uptake of $^{22}Na^+$ into proteoliposomes made with solubilized brush border membrane proteins (*Control*), heat denatured membrane proteins, albumin, and heat denatured albumin. The extravesicular sodium concentration was 1 mM. Values are means ± SEM for four preparations

eraged 105 ± 8% of control values. Unlabeled sodium or lithium in the external solution, inhibited the uptake of isotope in a concentration-dependent manner. There were no differences between the effects of lithium or sodium. The uptake of $^{22}Na^+$, expressed as a percent of control values, averaged 44.7 ± 1.7, 34.3 ± 0.4, and 21.3 ± 4.3% when the external solution contained 5, 10, or 20 mM unlabeled sodium, respectively. When the external solution contained 5, 10, or 20 mM lithium, the uptake of $^{22}Na^+$ averaged 48.5 ± 2.5, 31.5 ± 3.5 and 17.0 ± 3.7% of control values, respectively. To provide an additional control for the effect of the differences in the internal and external osmolalities of the above solutions and to examine if the proteoliposomes responded to osmotic differences, additional studies were performed under voltage clamped conditions whereby additional mannitol was added to the uptake solution to increase the osmolality by an additional 12.5 to 200 mOsm/kg H_2O. The addition of mannitol to increase the osmolality of the external solution by up to 100 mOsm/kg H_2O had no effect on sodium uptake. Increases in the osmolality of the external solution by an additional 200 mOsm/kg H_2O resulted in only a 10% decrease in sodium uptake.

To determine if transport was occurring into an intravesicular space, liposomes and proteoliposomes, with and without amiloride, were incubated in 1 mM $^{22}Na^+$ under proton gradient conditions for 60 min and then exposed to octyl glucoside (5.3%) to completely solubilize the lipid. As compared to the uptake into the proteoliposomes and liposomes, only 5% or less of the total number of counts were present after exposure to the detergent. The binding of sodium to the extracted proteins was determined by dialysing the octyl glucoside-solubilized proteins and comparing $^{22}Na^+$ eluting from the Dowex column after incubation of the proteins for 2 min in the 1 mM Na^+ uptake solution. Virtually no $^{22}Na^+$ was recovered in the eluent, indicating the absence of direct binding of sodium to the proteins. To determine the thermal lability of the transporter, the solubilized proteins were placed in a boiling water bath for 10 min and then rapidly cooled on ice prior to incorporation into proteoliposomes (Fig. 5). The process of boiling and cooling resulted in rates of $^{22}Na^+$ uptake into proteoliposomes of 3.3 ± 0.5 nmol · 2 min^{-1} · mg protein^{-1}. Proteoliposomes prepared with nondenatured brush border proteins had uptakes of 18.0 ± 1.7 nmol · 2 min^{-1} · mg protein^{-1}. A final series of reconstitutions were performed using bovine serum albumin instead of octyl glucoside-solubilized brush border membrane proteins. The uptake of sodium under proton gradient conditions in the albumin-containing proteoliposomes averaged 4.9 ± 0.6 nmol · 2 min^{-1} · mg protein^{-1}. If the albumin was heat denatured, the uptake of sodium averaged 4.9 ± 1.1 nmol · 2 min^{-1} · mg protein^{-1}. These values are significantly lower than those obtained in proteoliposomes prepared with proteins from renal brush border membranes. Amiloride had no significant effect on the uptake of sodium in proteoliposomes prepared with the denatured membrane proteins, albumin, or denatured albumin.

Discussion

The Na^+-H^+ exchanger in the brush border membrane of the kidney has been well characterized in terms of its transport properties and its regulation [9, 15]. A full understanding of the nature of the transporter itself awaits isolation of the specific protein or protein complex which mediates this exchange process. As a step toward isolation of the Na^+-H^+ countertransporter, it was considered important to develop a functional assay for the activity of the solubilized transporter by reconstitution into artificial membranes.

Renal brush border membrane vesicles, prepared from the kidney of the rabbit by a magnesium aggregation technique, were utilized as the source of the Na^+-H^+ exchanger. Many prior studies have indicated the presence of electroneutral, proton gradient-stimulated, amiloride-inhibitable sodium uptake in these membranes [8, 9, 15]. The membranes were extracted with 4.4% octyl glucoside which resulted in solubilization of approximately 70% of the

brush border membrane proteins. The solubilized proteins were then reconstituted into artificial liposomes of asolectin. After dialysis to remove the detergent, the rate of $^{22}Na^+$ uptake was measured under a variety of experimental conditions. As shown in Fig. 1, the formation of proteoliposomes using solubilized membrane proteins from renal brush border membranes resulted in uptakes of sodium that are five- to sevenfold higher than that observed in liposomes studied under the same experimental conditions. The uptake of sodium represents transport into a vesicular space since dissolution of the artificial vesicles with detergent reduced the sodium uptake by more than 95%. This method of determining that sodium was transported into a vesicular space was chosen in lieu of the more traditional method of osmotic shrinkage used in natural membranes, based on the evidence that asolectin proteoliposomes were resistant to changes in volume consequent to alterations in the external osmolality. There was virtually no binding of sodium to the extracted proteins. Several additional lines of evidence suggest that the uptake of sodium occurred by a mediated transport process. The uptake of sodium was significantly decreased if the solubilized proteins were denatured by boiling followed by rapid cooling. Albumin proteoliposomes had only low rates of sodium transport. These findings indicate that the soluble extract contains heat labile proteins which result in high rates of sodium transport. The kinetics of sodium uptake when studied under proton gradient conditions demonstrated first-order saturation kinetics. Under the conditions of study, the K_a for sodium was approximately 4 mM. The uptake of sodium was inhibited by amiloride and some amiloride analogues in a dose-dependent fashion. While amiloride may have several independent effects, it is also known to inhibit Na^+-H^+ exchange transport and sodium channels [2, 3, 9]. Collectively, these experiments support the conclusion that sodium uptake into the reconstituted proteoliposomes represent movement into a vesicular space by a mediated transport process.

In the absence of other known co-transported or countertransported substances in the intravesicular space or in the uptake solution, the mediated uptake of sodium could be occurring by a Na^+-H^+ countertransporter or by a conductive pathway for sodium. The uptake of sodium was higher in the presence of an outwardly directed proton gradient than in the absence of a proton gradient. This result could be explained by Na^+-H^+ exchange or by voltage coupling if the proteoliposomes contain a conductive pathway for proton exit from the vesicular space. From the studies with acridine orange, it would appear that the proteoliposomes do have a conductive pathway for protons. In addition, the proteoliposomes have a conductive pathway for potassium. The presence of these leak pathways are important in considering the mechanism by which a proton gradient stimulates the uptake of sodium. The uptake of sodium was higher when the internal and external pH was 6.0 than 8.0. In the absence of a gradient for hydrogen ions, a circumstance in which there will be no diffusion potential generated by protons, the presence of an intravesicular acid environment stimulated the uptake of sodium. In the presence of equal concentrations of potassium in the internal and external solutions and valinomycin, presumed voltage clamped conditions, sodium uptake was stimulated when a pH gradient was imposed and was higher when the internal and external pH was 6.0 than 8.0. Incubation of the proteoliposomes with FCCP, a proton ionophore, resulted in only a small increase in the apparent permeability to protons. The effect of FCCP on dissipating the proton gradient was increased greatly when the external solution contained potassium and valinomycin. It is probable that in the presence of FCCP alone, the intravesicular negative potential generated by proton diffusion limited the exit of protons as monitored by acridine orange fluorescence. When this potential difference was shunted by potassium and valinomycin, FCCP greatly accelerated the rate of dissipation of the proton gradient. FCCP did not affect the uptake of sodium when studied under proton gradient conditions. It seems reasonable to conclude, therefore, that the presence of an intravesicular negative potential difference did not affect the uptake of sodium. Taken together, despite the presence of a hydrogen ion conductive pathway in the reconstituted proteoliposomes, the proton gradient-stimulated uptake of sodium does not appear to be the consequence of indirect electrical coupling, but rather represents the operation of a Na^+-H^+ exchanger.

Based on the evidence presented, it would appear that artificial proteoliposomes prepared from solubilized brush border membrane proteins of the rabbit kidney exhibit proton gradient stimulated, electroneutral, amiloride-inhibitable sodium uptake. The K_a for sodium is 4 mM. These findings would appear to define, functionally, a sodium for proton exchange transport system. While there are obvious similarities between the characteristics noted above and those reported in natural renal brush border membranes, there are also some differences. In natural membranes, it is not possible to demonstrate stimulation of sodium uptake by protons in the absence of a pH gradient and the K_i for amiloride in the reconstituted proteoliposomes is higher than that observed in natural membranes [9].

Several aspects of the effect of amiloride are worthy of some additional comment. In natural brush border membrane vesicles, the K_i for amiloride is at least 10-fold lower than in the reconstituted proteoliposomes. In other systems, however, the K_i for amiloride inhibition of Na^+-H^+ exchange has been reported to be in the range observed in the present studies [2, 10]. In an attempt to determine the reason for the shift in amiloride sensitivity in the reconstituted proteoliposomes, studies were performed to explore the possibility that amiloride, a lipophilic base, may have diffused across the proteoliposomes and liposomes with a consequent reduction in the free concentration of drug in the external solution. This proved not to be a valid explanation for the decreased amiloride sensitivity in the reconstituted proteoliposomes, given the findings that the K_i for amiloride in natural brush border membranes was only minimally changed when the membranes were mixed with liposomes. It is likely, therefore, that other factors, such as the differences in lipid composition, specific protein composition, orientation of the transporter, and/or conditions of study, may account for the altered amiloride sensitivity in proteoliposomes as compared to natural membranes. Being a weak base, amiloride in high concentrations may decrease sodium uptake, when studied under proton gradient conditions, by dissipating the proton gradient [3]. While a possible contribution of this property of amiloride to the inhibition of sodium uptake in the present experiments is not definitely excluded, a number of considerations render this mechanism unlikely. First, if it is assumed that sodium uptake in the liposomes is the result of sodium diffusion by virtue of the sodium gradient and the intravesicular negative potential generated by proton diffusion, amiloride, if it were to have any effect, should decrease sodium uptake. Under the conditions of study, however, amiloride did not inhibit sodium uptake in liposomes, but rather resulted in a small but consistent increase in sodium uptake. Second, amiloride significantly inhibited sodium uptake when studies were performed at pH 6.0 in the absence of a pH gradient. Amiloride, under these conditions, would be predicted to be more protonated that at pH 8.0 and, hence, less likely to diffuse into and be trapped in the intravesicular space. It would seem more likely that amiloride was directly inhibiting the Na^+-H^+ exchanger.

To date, there have been relatively few successful attempts to solubilize and reconstitute Na^+-H^+ exchange activity [10–12, 16]. In bacteria for example, the Na^+-H^+ or related proton/cation exchange systems are important in regulation of cell pH, and Na^+-H^+ exchange activity has been reconstituted [16]. It is not clear at the present time, however, whether or not such exchange systems are identical with that present in the apical membrane of the proximal tubule of the kidney. LaBelle and co-workers have reported solubilization and reconstitution of an amiloride-inhibitable sodium transporter from the kidney [10–12]. These latter studies, while consistent with some of the findings of the present investigations, differ in a number of important aspects. First, the starting material in the studies of LaBelle were microsomes obtained from the medulla of the kidney. Na^+-H^+ exchange activity was present in these medullary microsomes. The functional significance and cellular location of this transport system is uncertain. By contrast, the present studies employed renal brush border membranes where Na^+-H^+ exchange activity has been well established and characterized. Second, LaBelle and co-workers were unable to demonstrate that the imposition of a proton gradient stimulated the uptake of sodium in the reconstituted proteoliposomes. The effect of a proton gradient on sodium uptake is clearly demonstrated in the present studies. The reasons for the differences between the two studies are not known but may be related, at least in part, to differences in the types of proteins reconstituted into the asolectin liposomes.

In the current experiments, octyl glucoside was used to solubilize the brush border membranes and asolectin used to prepare the proteoliposomes. Future studies will examine the applicability of other detergents and the role of other phospholipids in solubilizing and reconstituting Na^+-H^+ exchange activity from renal brush border membranes. The ability to solubilize and reconstitute the Na^+-H^+ exchanger enables future efforts to isolate and characterize this transporter.

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