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RECONSTITUTED AMILORIDE-INHIBITED SODIUM TRANSPORTER FROM RABBIT KIDNEY MEDULLA IS RESPONSIBLE FOR Na^+-H^+ EXCHANGE

EDWARD F. LaBELLE

Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX 77550 (U.S.A.)

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Microsomes formed from rabbit kidney medulla and reconstituted proteoliposomes formed from these microsomes were capable of amiloride-inhibited Na^+ transport that was insensitive to valinomycin either with or without K^+. This indicated that the Na^+ transport process was electroneutral. This Na^+ transport process was insensitive to extravesicular Cl^- or HCO_3^- and not stimulated by high intravesicular gradients of K^+, Ca^{2+} or Mg^{2+}, which indicated that the process did not require NaCl or $NaHCO_3$ co-transport or Na^+/K^+, Na^+/Ca^{2+} or Na^+/Mg^{2+} counter-transport. Na^+ uptake into microsomes or proteoliposomes was inhibited by extravesicular K^+, Ca^{2+}, Mg^{2+} or La^{3+}, which indicated that these ions interacted with the Na^+-binding site on the transport protein. Na^+ uptake into microsomes was stimulated by intravesicular protons and inhibited by extravesicular protons. This suggested that microsomes were capable of Na^+-H^+ exchange and this was confirmed when Na^+ was shown to stimulate H^+ efflux from microsomes. The amiloride-inhibited Na^+ transporter from medulla microsomes which has been reconstituted into proteoliposomes is most likely a Na^+-H^+ exchanger. This Na^+ transporter was totally insensitive to the uncoupler 1799, either in the presence or absence of valinomycin plus K^+ and less sensitive to NH_3 than to amiloride. This indicated that amiloride inhibited Na^+ transport not merely by acting as a weak-base uncoupler but by directly interacting with the protein responsible for Na^+-H^+ exchange.

Introduction

Membrane vesicles have been isolated from either toad urinary bladder or rabbit kidney medulla that are capable of amiloride-inhibited Na^+ transport [1,2]. The protein responsible for amiloride-inhibited Na^+ transport into the kidney vesicles has been extracted from the native membranes with the aid of octylglucoside, mixed with purified phospholipid, and reconstituted into proteoliposomes capable of amiloride-inhibited Na^+ transport [3]. The concentration of amiloride required for optimal inhibition of Na^+ transport into either the toad bladder or rabbit kidney membrane vesicles or into the reconstituted proteoliposomes was fairly high (10^{-3} M) and there was some doubt concerning the nature of the transport system that was being studied [1–3]. Amiloride is a well-known inhibitor of an electrogenic Na^+ conductance across the apical membranes of such tight epithelia as toad urinary bladder, frog skin, rabbit urinary bladder, or cortical collecting tubule of mammalian kidney [4–7]. However, this Na^+ conductance is normally sensitive to extremely low (10^{-7}–10^{-6} M) concentrations of amiloride [4,6,8]. Another amiloride-sensitive Na^+ transporter has

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Hepps, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Mes, 4-morpholineethanesulfonic acid; 1799, bis(hexafluoracetonyl) acetone.

been reported that catalyzes electroneutral Na^+-H^+ exchange and this transporter is usually sensitive to much higher amiloride concentrations (10^{-3} M). This amiloride-inhibited Na^+-H^+ antiporter has been found in brush border membrane vesicles formed from either kidney cortex or small intestine of either rat or rabbit [9–11]. It has also been demonstrated to exist in surface membranes of cultured dog kidney (MDCK) cells [12,13], frog and mouse skeletal muscle cells [14,15], cultured human, mouse and hamster fibroblast cells [16–18], cultured mouse neuroblastoma cells [19], *Amphiuma* red blood cells [20], and rat liver cells [21]. The amiloride-inhibited Na^+-H^+ antiporter has been shown to play an important role in cell volume regulation [20,22], intracellular pH regulation [15,19], the regulation of cell division [16–18,21], and in acid secretion by the kidney [9,10]. The Na^+-H^+ transporter is also believed to be inhibited by extracellular Ca^{+2} [23,24]. The data below indicate that Na^+ transport into kidney medulla microsomes is insensitive to alterations in membrane potential produced by valinomycin and, therefore, most likely represents an electroneutral flux. We have also determined that Na^+ uptake into either microsomes or reconstituted proteoliposomes formed from microsomes is totally insensitive to either extravesicular Cl^- or HCO_3^- and is not stimulated by intravesicular cations such as K^+, Mg^{2+}, or Ca^{2+}. Yet Na^+ influx into the kidney medulla microsomes was stimulated by high levels of intravesicular protons and inhibited by high levels of extravesicular protons. Extravesicular sodium was also shown to stimulate proton efflux from the microsomes. These data indicate that the process most likely represents Na^+-H^+ exchange. Amiloride-inhibited Na^+ uptake into either microsomes or reconstituted proteoliposomes is also inhibited by extravesicular K^+, Ca^{2+}, or Mg^{2+} at low Na^+ concentrations indicating that these ions interact with the Na^+-binding site on the transport protein.

Materials and Methods

Materials

New Zealand white rabbits were obtained from Nichols Rabbitry, Lumberton, TX. Amiloride-HCl (*N*-amidino-3,5-diamino-6-chloropyrazine carboxamide) was the generous gift of Dr. Clement A. Stone of Merck, Sharp and Dohme Research Laboratories, West Point, PA. Dowex 50X8-100 (H^+ form), Hepes, Hepps, Tris, Mes, octylglucoside, acridine orange, and valinomycin, were from Sigma Chemical Co., St. Louis, MO. ^{22}Na was obtained from Amersham, Arlington Heights, IL. Asolectin was obtained from Associated Concentrates, Woodside, NY and uncoupler 1799 was the generous gift of Dr. P.G. Heytler of Dupont, Wilmington, DE.

Methods

Rabbit kidney medulla microsomes. Rabbit kidney medulla microsomes were formed by a modification of the procedure of Barnes et al. [25] as described in LaBelle and Lee [2]. Protein concentrations were measured by the procedure of Bradford [26].

Reconstituted proteoliposomes. Reconstituted proteoliposomes were formed from kidney medulla microsomes by a modification of the procedure of Racker [27] as described in LaBelle and Lee [3]. Briefly, the microsomes were extracted with octylglucoside, the extract treated with soy phospholipid (asolectin) and the lipid-protein-detergent mixtures dialyzed overnight to form the proteoliposomes.

$^{22}Na^+$ uptake determination. Na^+ transport into either microsomes or proteoliposomes was measured by a modification of the method of LaBelle and Valentine [1] as described in LaBelle and Lee [3]. To measure $^{22}Na^+$ transport, the microsomes, proteoliposomes or lipid vesicles were incubated briefly with the isotope and then applied to small (1 ml) Dowex columns (pre-equilibrated with 0°C sucrose (0.25 M)) and eluted with sucrose (0°C) (0.25 M) into scintillation vials and the radioactivity in the vials determined [3]. The release of $^{22}Na^+$ from either microsomes or proteoliposomes was measured by a modification of the procedure of LaBelle and Valentine [1]. The microsomes or proteoliposomes were incubated with $^{22}Na^+$ and various compounds and then applied to 1 ml columns of Dowex 50X8 (Tris) 100 mesh and eluted with 1 ml sucrose (0.25 M) and the radioactivity eluted from the columns measured as described by LaBelle and Valentine [1].

Phospholipid vesicle formation. Phospholipid

vesicles were formed from soy phospholipid (asolectin) as described in LaBelle and Valentine [1].

Acridine orange uptake into microsomes. Microsomes were incubated with acridine orange in pH 6 or pH 8 buffer solutions (composition described in the legend of Table I), and then the incubation mixtures were applied to small columns of Dowex 50X8-100 (Tris form)-100 mesh (1 ml) (preequilibrated with 0°C sucrose (0.25 M)) and eluted with 1 ml sucrose (0.25 M) (0°C). The eluted microsomes were sonicated briefly and the fluorescence of the samples was measured with an Aminco-Bowman Spectrophotofluorimeter. The excitation wavelength was 470 nm and the emission wavelength was 550 nm. These were not the optimal wavelengths for acridine orange but were used to minimize effects of light scattering from the microsomes on the measurements. The fluorescence of standard solutions of acridine orange was determined in order to quantitate the data obtained from the experiments.

Results

Effect of valinomycin on Na^+ influx into kidney microsomes

In order to elucidate the mechanism of amiloride-inhibited Na^+ transport into rabbit kidney medulla microsomes, the sensitivity of this process to valinomycin was examined. Valinomycin failed to affect Na^+ transport into the microsomes when used at concentrations less than 8 μg/ml whether in the presence or absence of K^+ (Table II), but a slightly higher concentration of valinomycin (20 μg/ml) stimulated Na^+ transport into the microsomes by a slight but reproducible extent in the absence of added K^+ (Fig. 1). This suggested that the microsomes might have been formed from the rabbit kidney medulla with a small amount of internal K^+, and that upon dilution into $^{22}Na^+$-containing solution a K^+ gradient (high inside) was produced and this gradient together with valinomycin, produced a transient membrane potential (outside positive) that enhanced Na^+ influx through an electrogenic channel. Evidence for such an electrogenic channel was of considerable interest. However, we have found that at sufficiently high concentrations (20 μg/ml)

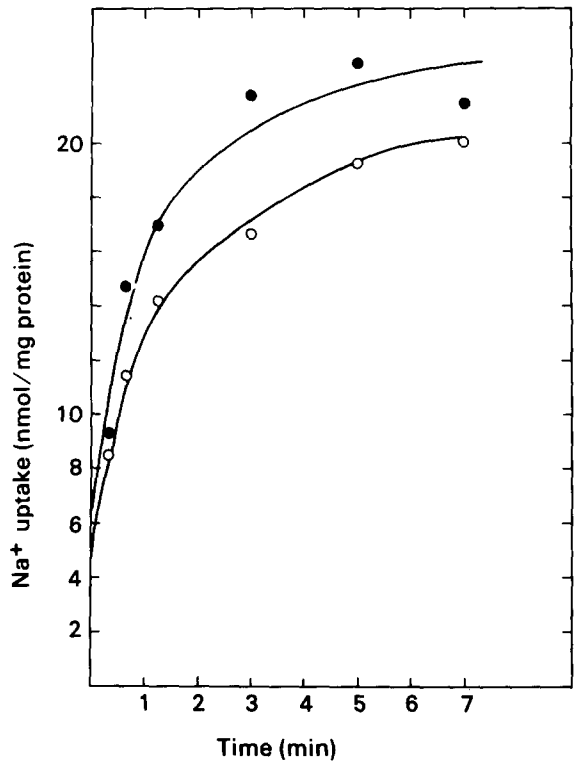

Fig. 1. Effect of valinomycin on sodium uptake into rabbit kidney medulla microsomes. Rabbit kidney medulla microsomes (0.09 mg protein) were incubated for the times indicated at 20°C with ^{22}Na-Hepps (0.5 μCi, 5.9 mM, pH 8.2) and sucrose (0.25 M) in a final volume of 0.25 ml both with (●) or without (○) valinomycin (5 μg) and then the incubation mixtures were applied to Dowex columns and eluted as described [2].

valinomycin is capable of carrying $^{22}Na^+$ into pure phospholipid vesicles in the absence of any K^+ (data not shown). These data indicated that valinomycin could also carry a small amount of $^{22}Na^+$ into microsomes if used in high enough amounts. This was proven by an experiment in which microsomes were preincubated in $^{22}Na^+$-containing solution for 30 min in order to load them with isotope. Next the microsomes were diluted into K^+-free, $^{22}Na^+$-free solution with and without valinomycin for 2 min and applied to Dowex columns. If the microsomes contained any K^+, the dilution procedure should have produced a K^+ gradient (high inside the microsomes) which would have produced an outside-positive potential in the presence of valinomycin. Such a potential

would have slowed $^{22}Na^+$ efflux from the microsomes if potential effects of valinomycin alone were present. However, if valinomycin could carry $^{22}Na^+$ itself across the microsomal membrane, the ionophore ought to lower the amount of $^{22}Na^+$ remaining in the microsomes after dilution. When this experiment was performed, microsomes loaded with $^{22}Na^+$ and diluted into K^+-free, valinomycin-free solution retained 36.2 ± 0.9 nmol $^{22}Na^+$/mg protein, while microsomes diluted into K^+-free, valinomycin-containing (20 μg/ml) solution retained 30.4 ± 1.9 nmol $^{22}Na^+$/mg protein. Thus valinomycin induced $^{22}Na^+$ loss from the microsomes which could only be explained if the ionophore were carrying Na^+ directly at high concentrations.

Sensitivity of Na^+ transport to pH gradients

Since the above experiments indicated that Na^+ uptake into kidney medulla microsomes was electroneutral, it seemed most likely that we were observing Na^+-H^+ exchange. We were then able to show that sodium uptake into the microsomes could be markedly influenced by pH gradients across the microsomal membranes (Table I). When microsomes were preincubated at pH 6.0 for 45 min, and Na^+ uptake was measured in a pH 8.0 solution, amiloride-sensitive Na^+ transport was nearly doubled over the level of transport observed when both the preincubation and the incubation pH were 8.0. Likewise, microsomes preincubated at pH 8.0 and incubated with $^{22}Na^+$ at pH 6.0 showed 78% inhibition of transport. This suggested that microsomes loaded with protons accumulated Na^+ very well while the presence of high levels of external protons inhibited Na^+ influx (Table I). Only amiloride-inhibited Na^+ transport was affected by the proton gradients. No effects of such gradients were observed on amiloride-insensitive Na^+ transport.

Kidney medulla microsomes were preincubated for 30 min in the presence of the dye acridine orange, then incubated for 2 min and applied to Dowex columns to separate extravesicular acridine orange from intravesicular dye. The amount of acridine orange accumulated in the microsomes was determined by measuring the fluorescence of the microsomes after sonication to release trapped dye. Microsomes preincubated at pH 6 and incubated at pH 8 were shown to accumulate 0.311 ± 0.032 nmol acridine orange/min per mg protein ($n = 4$) while microsomes preincubated at pH 8 and incubated at pH 6 accumulated only 0.151 ± 0.002 nmol acridine orange/min per mg protein. Since microsomes loaded at pH 6.0 accumulated twice as much acridine orange as microsomes loaded at pH 8.0, this reflected the increased proton concentration inside the vesicles. This indicated that vesicle exposure to different pH buffer solutions did indeed generate pH gradients across the vesicular membranes.

When reconstituted proteoliposomes formed from the microsomes were preincubated at either pH 6.0 or 8.0 and incubated with $^{22}Na^+$ at either pH, no significant effects of the apparent pH gradients on amiloride-sensitive Na^+ transport

TABLE I

EFFECT OF pH GRADIENT ON SODIUM TRANSPORT INTO RABBIT KIDNEY MEDULLA MICROSOMES

Aliquots (20 μl, 0.19 mg protein) of rabbit kidney medulla microsomes were preincubated for 45 min at 20°C in a total volume of 40 μl with either pH 6.0 buffer (45 mM Mes/14 mM Tris/7 mM Hepes/183 mM sucrose) or pH 8.0 buffer (22 mM Tris/25 mM Hepps/202 mM sucrose) and then diluted with solutions (0.21 ml) containing $^{22}NaCl$ (0.4 μCi, 4 mM), either with or without amiloride (2.0 mM) and either pH 8.0 buffer (28 mM Tris/31 mM Hepps/ 190 mM sucrose) or pH 6.0 buffer (56 mM Mes/9 mM Hepes/18 mM Tris/167 mM sucrose) and then incubated at 20°C for 2 min and applied to Dowex columns and eluted.

Preincubation pH (internal pH)	Incubation pH (external pH)	Na^+ uptake (mean ± S.D. ($n = 3$)) (nmol/min per mg protein)		
		− Amiloride	+ Amiloride	Difference
6.0	8.0	0.855 ± 0.112	0.263 ± 0.046	0.592
8.0	8.0	0.618 ± 0.026	0.263 ± 0.003	0.355
8.0	6.0	0.362 ± 0.085	0.283 ± 0.046	0.079

were observed (data not shown). When these proteoliposomes were preincubated at pH 6.0 in the presence of acridine orange and incubated at pH 8.0 the amount of acridine orange accumulated by the proteoliposomes was 0.411 ± 0.095 nmol/min per mg protein ($n = 4$), while proteoliposomes preincubated at pH 8.0 with acridine orange and incubated at pH 6.0 accumulated 0.384 ± 0.082 nmol acridine orange/min per mg protein. Since the same amount of dye was accumulated no matter what the orientation of the pH gradient, it seemed likely that these proteoliposomes were incapable of maintaining imposed pH gradients long enough to permit effects on the Na^+-H^+ exchange protein.

Control determinations indicated that when acridine orange was incubated without vesicles or vesicles incubated without acridine orange, and either mixture applied to Dowex columns, the amount of fluorescent material recovered from the columns are barely detectable.

Na^+-dependent proton transport by microsomes

Further evidence in support of the existence of Na^+-H^+ exchange across the microsomal membranes was provided by following the Na^+-dependent exclusion of acridine orange by the microsomes. When kidney medulla microsomes were incubated with acridine orange for 30 min at 20°C at pH 6.0, and diluted into a solution containing NaCl, the presence of the Na^+ gradient (high outside) decreased microsomal accumulation of the dye from 0.41 ± 0.05 nmol dye/min per mg protein ($n = 3$) observed in the absence of Na^+ to 0.165 ± 0.02 nmol dye/min per mg protein observed in the presence of Na^+ (50 mM). The exclusion of acridine orange by the microsomes in response to a Na^+ gradient indicated that the microsomes excluded protons in response to the Na^+ gradient. This provided direct evidence that the microsomes could transport H^+ in exchange for Na^+. The amount of acridine orange excluded from the microsomes in response to the Na^+ gradient was 0.245 nmol/min per mg protein (the difference between the two values above). This number was nearly the same as the amount of Na^+ accumulated by the microsomes in response to a pH gradient: 0.237 nmol/min per mg protein (the difference between the values 0.855 and 0.618 in Table I).

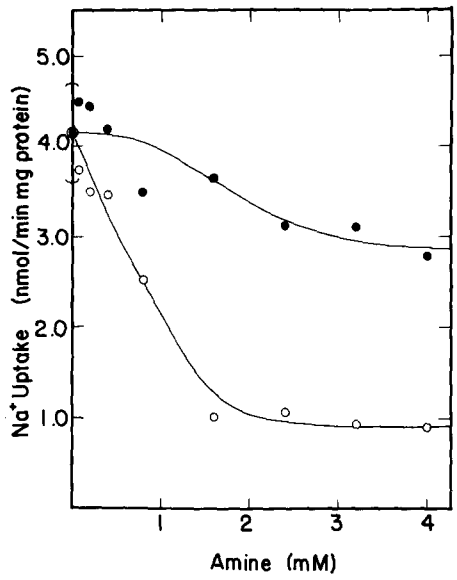

Fig. 2. Effect of NH_4^+ and amiloride on Na^+ uptake into microsomes. Aliquots of rabbit kidney medulla microsomes (0.19 mg protein) were incubated for 2 min at 20°C with sucrose (0.25 M), $^{22}Na^+$ (1 μCi, 10 mM), Cl (4 mM), and sufficient Hepps buffer (pH 8.2) to serve as a counterion for Na^+ in the presence of increasing concentrations of amiloride (○) or NH_4^+ (●), in a total volume of 0.25 ml. The incubations were terminated as described [2].

Sensitivity of microsomes to uncouplers

In order to determine whether amiloride was capable of inhibiting Na^+-H^+ exchange across microsomal membranes by uncoupling a preexisting H^+ gradient, the sensitivity of Na^+ transport to several compounds was measured. Sodium transport into the microsomes was more sensitive to amiloride than to ammonium and was totally unaffected by the proton ionophore 1799 (Fig. 2, Table II). The data in Table II are representative of the results of four experiments. The uncoupler 1799 also failed to influence Na^+ transport in the presence of the K^+ ionophore valinomycin together with 0.2 mM KCl. This suggested that Na^+ movement into the microsomes did not depend on the existence of a proton gradient inside the microsomes. Any such proton gradient ought to be extremely sensitive to the uncouplers employed in Table II. Likewise, 1799 failed to inhibit $^{22}Na^+$ transport into reconstituted proteoliposomes formed from medulla microsomes, either alone or in the presence of valinomycin and KCl. Am-

TABLE II

EFFECT OF UNCOUPLERS ON Na^+ UPTAKE INTO RABBIT KIDNEY MEDULLA MICROSOMES

^{22}Na uptake into rabbit kidney medulla microsomes was measured as described in the legend of Fig. 1 for 2 min in the presence of the compounds below.

Compound	Sodium uptake (nmol/min per mg protein) (mean ± S.D. ($n = 2$))
None	6.4 ± 0.3
Amiloride (0.6 mM)	2.9 ± 0.05
KCl (0.2 mM)	6.4 ± 0.0
1799 (8 μg/ml)	6.6 ± 0.1
Valinomycin (8 μg/ml)	7.7 ± 1.2
1799 + KCl + valinomycin	7.0 ± 0.4

monia did inhibit Na^+ uptake into the proteoliposomes slightly, but not as much as did amiloride (data not shown).

Effects of Cl^- and HCO_3^- on Na^+ transport

Inorder to determine if Na^+ transport into the microsomes was influenced by major physiological anions, Na^+ uptake into the microsomes was measured in the presence of increasing concentrations of Cl^- and HCO_3^-. As shown in Fig. 3A neither Cl^- nor HCO_3^- had any stimulatory or inhibitory effects on Na^+ transport into microsomes. Na^+ transport into the microsomes was also measured after atmospheric CO_2 had been removed from the incubation medium. This was accomplished by boiling the medium and then cooling it under an atmosphere of N_2. The microsomes were also equilibrated with N_2 for 30 min. Even after the removal of atmospheric CO_2 from the medium, Na^+ transport into the microsomes was completely normal (data not shown), indicating that the transport process did not require HCO_3^- in the medium (which would be produced in small amounts from dissolved CO_2). This indicated that the Na^+ transport process is not influenced by any directly coupled NaCl or $NaHCO_3$ transport. Likewise, Cl^- failed to either stimulate or inhibit Na^+ uptake into reconstituted proteoliposomes formed from rabbit kidney medulla microsomes (Fig. 3B).

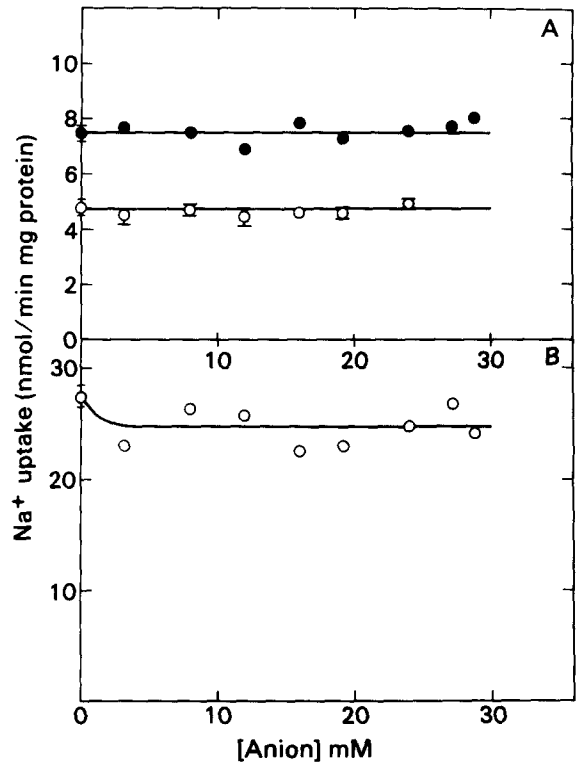

Fig. 3. Effects of Cl^- and HCO_3^- on sodium uptake into rabbit kidney medulla microsomes and reconstituted proteoliposomes. (A) Rabbit kidney medulla microsomes (0.55 mg protein) were incubated for 2 min at 20°C with $^{22}Na^+$ (0.8 μCi, 30 mM), sucrose (0.25 M), and increasing concentrations of either Cl^- (○) or HCO_3^- (●) in a total volume of 0.25 ml. The Hepps anion (pH 8.2) was used to replace either Cl^- or HCO_3^- in the incubation mixtures in order to keep the Na^+ concentration constant. After the incubations, the mixtures were applied to Dowex columns and eluted as described [2]. (B) Reconstituted proteoliposomes were formed from microsomes as described [3] and then ^{22}Na uptake into the proteoliposomes was measured in the presence of increasing concentrations of Cl^- as described above. Error bars indicate standard deviations of duplicate determinations.

Effects of K^+, Ca^{2+} or Mg^{2+} on Na^+ transport

The sensitivity of Na^+ transport to major physiological cations was also investigated in the experiments shown in Figs. 4–6. Microsomes were preincubated with increasing concentrations of K^+ and then diluted into K^+-free solutions in order to produce K^+ gradients across the microsomal membranes. The final internal K^+ concentration was 4.2-times higher than the external K^+ concentration as a result of this procedure, yet no stimula-

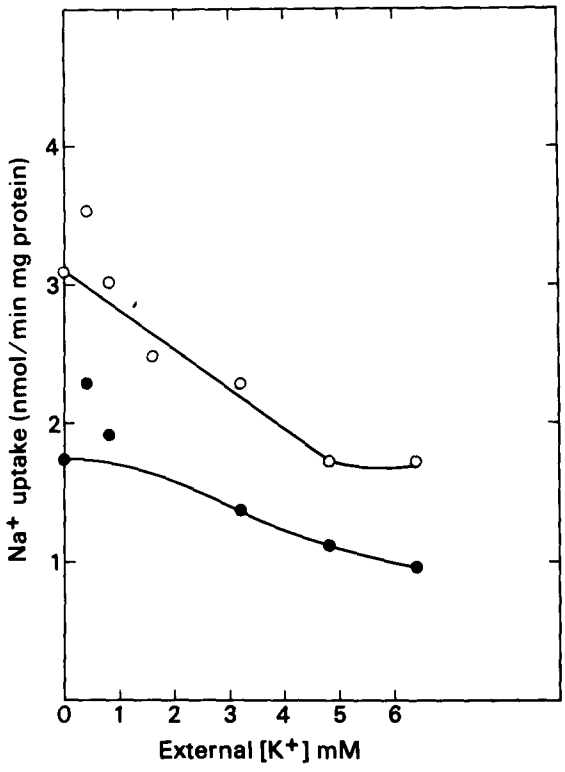

Fig. 4. Effects of internal and external K^+ on Na^+ uptake into rabbit kidney medulla microsomes. Aliquots (20 μl, 0.22 mg protein) of rabbit kidney medulla microsomes were either incubated with ^{22}Na-Hepps (0.4 μCi, 6.2 mM, pH 8.2), sucrose (0.25 M), and increasing concentrations (as indicated) of K-Hepps (pH 8.2) for 2 min at 20°C in a total volume of 0.25 ml (open symbols) and then applied to Dowex columns as described [2] or preincubated with sucrose (0.25 M) and increasing concentrations (4.2-times the indicated concentrations) of K-Hepps (pH 8.2) for 60 min at 20°C in a total volume of 60 μl, diluted for 2 min at 20°C with K^+-free solution (0.19 ml) containing ^{22}Na-Hepps (0.4 μCi, 6.2 mM, pH 8.2) and sucrose (0.25 M) (closed symbols), and then applied to Dowex columns and eluted as described [2].

tion of Na^+ uptake into the microsomes was provoked by the K^+ gradient (Fig. 4). K^+ actually decreased Na^+ uptake into the microsomes when present in the extravesicular solution, and this inhibition was not significantly altered by the presence of intravesicular K^+. The data in Fig. 4 are representative of the results obtained in three experiments.

When reconstituted proteoliposomes were preincubated with K^+ (31 mM) and then diluted to produce a 5-fold K^+ gradient across the vesicular membranes, the internal K^+ inhibited Na uptake and this inhibition was insensitive to the presence of a gradient. These experiments indicated that Na^+ transport into either microsomes or proteoliposomes was not stimulated by internal K^+ and hence was not complicated by any Na^+-K^+ exchange.

Further experiments were conducted to determine the effects of divalent cations on Na^+ transport. When microsomes were preincubated with increasing concentrations of either Ca^{2+} or Mg^{2+} and then diluted in nominally Ca^{2+}, Mg^{2+} free solutions containing $^{22}Na^+$, no stimulation of Na^+ transport was observed by either the Ca^{2+} or Mg^{2+} gradients, even though the final intravesicular divalent cation concentrations were 5-fold

TABLE III

EFFECTS OF K^+, Ca^{2+} AND Mg^{2+} ON Na^+ UPTAKE INTO RECONSTITUTED PROTEOLIPOSOMES

(A) Reconstituted proteoliposomes were formed from rabbit kidney medulla microsomes as described [3] and aliquots (20 μl) of the proteoliposomes were incubated with ^{22}Na-Hepps (0.4 μCi, 6.7 mM, pH 8.2), sucrose (0.25 M) and the compounds indicated below for 2 min at 20°C in a total volume of 0.25 ml, and then applied to Dowex columns and eluted as described [3].

Compounds added during incubation (incubation concentration)	Sodium uptake (mean ±S.D. ($n = 3$)) (nmol/min per mg protein)
None	25.0±0.9
K-Hepps (6.2 mM, pH 8.2)	16.2±0.6
$CaCl_2$ (0.48 mM)	11.3±0.2
$MgCl_2$ (0.48 mM)	10.3±0.5

(B) Aliquots (20 μl) of the reconstituted proteoliposomes were preincubated with sucrose (0.25 M) and the compounds below for 60 min at 20°C in a total volume of 50 μl, and then a solution (0.2 ml) containing ^{22}Na Hepps (0.4 μCi, 8.4 mM, pH 8.2) and sucrose (0.25 M) was added to the incubation mixtures and after 2 min further incubation at 20°C, the mixtures were applied to Dowex columns and eluted.

Compounds added during preincubation (preincubation concentration)	Sodium uptake (mean ±S.D. ($n = 3$)) (nmol/min per mg protein)
None	22.9±0.5
K-Hepps (31 mM, pH 8.2)	16.1±1.6
$CaCl_2$ (2.4 mM)	3.1±0.3
$MgCl_2$ (2.4 mM)	4.4±0.4

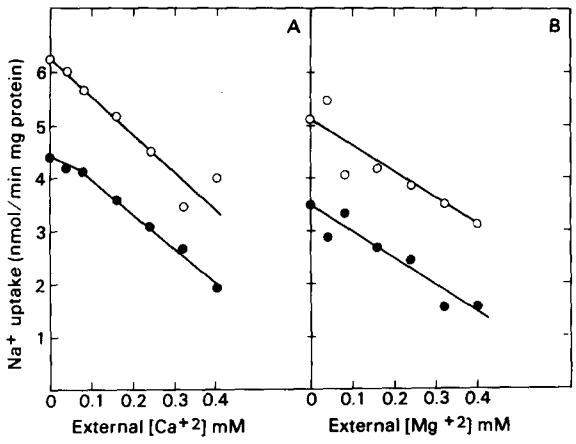

Fig. 5. Effects of internal and external Ca^{2+} and Mg^{2+} on Na^+ uptake into rabbit kidney medulla microsomes. (A) Sodium uptake into rabbit kidney medulla microsomes was measured as described in the legend of Fig. 4 except that $CaCl_2$ (indicated concentrations) was substituted for K-Hepps. (B) Same as A, except that $MgCl_2$ was substituted for $CaCl_2$.

higher than the extravesicular concentrations (Fig. 5). Sodium uptake into the microsomes was inhibited markedly by relatively low concentrations of either Ca^{2+} or Mg^{2+} (compared to the concentrations of Na^+ or K^+ employed in this study). As little as 0.4 mM Ca^{2+} was capable of inhibiting Na^+ transport by 46%. The presence of intravesicular Ca^{2+} or Mg^{2+} did not alter the ability of either ion to inhibit Na^+ uptake from the extravesicular side of the membrane (Fig. 5).

When reconstituted proteoliposomes were preincubated with either Ca^{2+} or Mg^{2+}, and then diluted into solutions nominally free of divalent cations, no stimulation of Na^+ transport into the proteoliposomes was observed (Table III). Once again both extravesicular Ca^{2+} and Mg^{2+} inhibited Na^+ transport markedly, even at low concentrations (55–60% inhibition exerted by concentrations of 0.48 mM). The presence of high intravesicular levels of either cation appeared to enhance the observed inhibition of Na^+ uptake (to 86% in the case of Ca^{2+}) (Table III).

When reconstituted proteoliposomes were formed by dialysis against either Ca^{2+} or Mg^{2+}-containing buffer, the intravesicular divalent cation concentration would be expected to equal that of the dialysis medium. When proteoliposomes formed with intravesicular Ca^{2+} or Mg^{2+} concentrations of 2.5 mM were diluted 12-fold into

TABLE IV

EFFECT OF Ca^{2+} ON AMILORIDE-INHIBITED Na^+ UPTAKE INTO RECONSTITUTED PROTEOLIPOSOMES

(A) Reconstituted proteoliposomes were formed as described [3] and $^{22}Na^+$ uptake into the proteoliposomes was measured as described in the Legend of Table IIIA both with and without $CaCl_2$ and with and without amiloride (0.6 mM).

Compound added during incubation (incubation concentration)	Na^+ uptake (nmol/min per mg protein) (mean ± S.D. ($n = 2$)		
	− Amiloride	+ Amiloride	Difference
None	21.6 ± 0.4	6.5 ± 0.9	15.1
$CaCl_2$ (0.48 mM)	8.4 ± 1.4	5.6 ± 0.1	2.8

(B) The proteoliposomes were preincubated with and without $CaCl_2$ and then $^{22}Na^+$ uptake was measured after dilution with and without amiloride (0.6 mM) as described in the legend of Table IIIB.

Compound added during preincubation (preincubation concentration)	Na^+ uptake (nmol/min per mg protein) (mean ± S.D. ($n = 2$))		
	− Amiloride	+ Amiloride	Difference
None	20.3 ± 1.2	7.8 ± 0.2	12.5
$CaCl_2$ (2.4 mM)	3.0 ± 0.03	3.1 ± 0.2	− 0.1

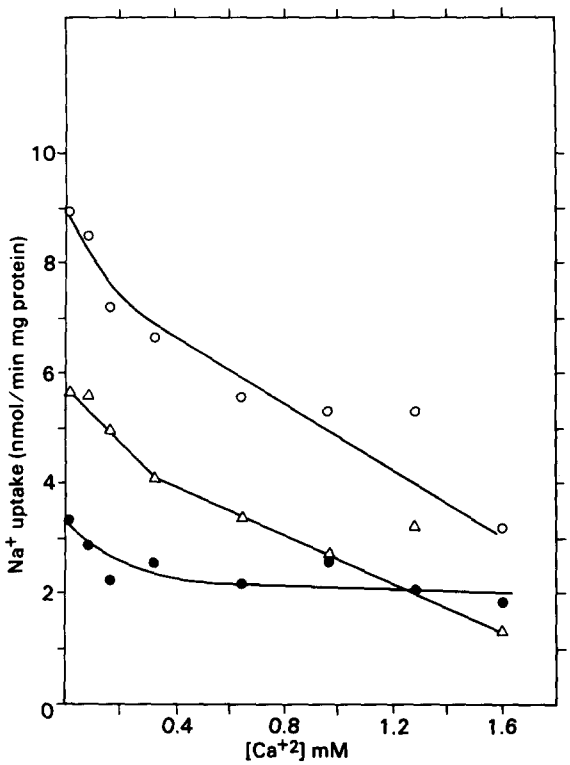

Fig. 6. Effect of external Ca^{2+} on amiloride-inhibited Na^+ uptake into kidney medulla microsomes. Aliquots (20 μl, 0.08 mg protein) of kidney medulla microsomes were incubated with ^{22}Na-Hepps (2 μCi, 4.8 mM, pH 8.2), sucrose (0.25 M) and increasing concentrations of $CaCl_2$ both with (●) and without amiloride (2 mM) (○) for 2 min at 20°C in a total volume of 0.25 ml and then applied to Dowex columns as described [2]. The difference between the data obtained in the presence and the absence of amiloride (amiloride-sensitive transport) is also shown (△).

solutions containing $^{22}Na^+$, the rate of Na^+ uptake into the vesicles was inhibited by 66% (data not shown). Once again significantly more inhibition of Na^+ uptake was observed in the presence of both intravesicular and extravesicular Ca^{2+} than was observed in the presence of extravesicular Ca^{2+} alone. These data indicate that Na^+ transport into either microsomes or proteoliposomes does not occur via Na^+-Ca^{2+} or Na^+-Mg^{2+} exchange. Such exchange processes might be expected to show enhanced Na^+ uptake into vesicles in the presence of high levels of intravesicular divalent cations.

Amiloride-inhibited Na^+ transport into microsomes was more sensitive to Ca^{2+} (76% inhibition) than was the amiloride-insensitive transport (45% inhibition) (Fig. 6). When the effects of extravesicular Ca^{2+} on amiloride-sensitive Na^+ uptake into reconstituted proteoliposomes was examined, the amiloride-sensitive portion of the transport was shown to be inhibited by 82%, while the amiloride-insensitive transport was not significantly inhibited (Table IV). When the proteoliposomes were preincubated with Ca^{2+} and then diluted with Ca^{2+}-free solution in order to create a gradient of high intravesicular Ca^{2+}, amiloride-sensitive Na^+ uptake was lowered by 100% while amiloride-insensitive transport was only decreased by 60% (Table IV). Control lipid vesicle preparations formed in the absence of kidney tissue by dialysis after octylglucoside treatment failed to take up significant amounts of $^{22}Na^+$ either in the presence or absence of Ca^{2+} (data not shown).

The apparent ability of high intravesicular levels of Ca^{2+} to inhibit Na^+ uptake into reconstituted proteoliposomes was unexpected. The possibility existed that this finding may have been a secondary result of some nonspecific apparent leak produced by the 60 min preincubation of the proteoliposomes with the concentration of Ca^{2+} used (2 mM). Such an apparent leak might have permitted the Na^+ accumulated within the proteoliposomes to leave the vesicles either before or during elution from the Dowex column (the procedure used to separate extravesicular $^{22}Na^+$ from intravesicular $^{22}Na^+$ during the transport assay).

When either kidney medulla microsomes or reconstituted proteoliposomes were preincubated with $^{22}Na^+$ to load them with the isotope and then exposed to increasing amounts of either K^+, Ca^{2+}, or Mg^{2+}, under certain circumstances, these cations were shown to produce apparent leaks in the vesicles and to permit Na^+ efflux. However, no such apparent leaks were produced unless the vesicles were exposed to relatively high concentrations of K^+ (> 10 mM) for 2 min, or unless they were exposed to K^+, Ca^{2+}, Mg^{2+} for relatively long periods of time (60 min) (data not shown). Therefore, the data shown in Tables III and IV and Figs. 4–6, indicate that extravesicular K^+, Ca^2, and Mg^{2+} could inhibit $^{22}Na^+$ transport into microsomes during short incubation periods (2 min) and that these effects did not reflect the

induction of apparent leaks by the cations. However, the apparent ability of intravesicular Ca^{2+} and Mg^{2+} to inhibit Na^+ uptake into reconstituted proteoliposomes probably does reflect the artifactual inducation of such leaks. These apparent leaks were completely reversible. When microsomes were pre-loaded with $^{22}Na^+$ andexposed to Ca^{2+} (2 mM) for 60 min, most of the $^{22}Na^+$ was shown to leak out of the microsomes. When microsomes were exposed to Ca^{2+} (2 mM) for 60 min and then diluted into a solution of $^{22}Na^+$ in order to lower the Ca^{2+} concentration to 0.1 mM, the rate of $^{22}Na^+$ uptake into the microsomes was restored to within 13% of the rate of $^{22}Na^+$ uptake observed in the absence of Ca^{2+} (data not shown). The rate of Na^+ uptake into microsomes was normally inhibited by about 10% in the presence of 0.1 mM Ca^{2+} (Fig. 5A), and so this experiment indicated that the Ca^{2+}-induced apparent leak could be reversed by simply lowering the Ca^{2+} concentration in the solution.

Sensitivity of Na^+ transport to inhibition by cations

Since K^+, Ca^{2+} and Mg^{2+} inhibited Na^+ transport into medulla microsomes, it seemed possible that these cations might interact with the Na^+-binding site on the transport protein. Other experiments indicated that such organic cations as Tris and *N*-methylglucamine also inhibited Na^+ transport into microsomes (data not shown). Of course the concentrations of monovalent cations required to inhibit Na^+ transport were at least an order of magnitude higher than the concentration of amiloride required to inhibit, which indicated that amiloride did not inhibit by the same mechanism as that employed by other cations. Divalent cations such as Ca^{2+} and Mg^{2+} also inhibited Na^+ transport into microsomes at concentrations about an order of magnitude lower than the concentrations required for inhibition by monovalent ions (Figs. 4 and 5). The trivalent ion La^{3+} inhibited Na^+ transport at concentrations about 25% as high as the concentrations of divalent ions required for inhibition (Fig. 7). Microsomes preloaded with $^{22}Na^+$ and exposed to $LaCl_3$ at a concentration sufficient to inhibit influx totally did not lose any of the accumulated $^{22}Na^+$, which indicated that La^{3+} did not induce apparent leaks in the vesicles at such concentrations (data not shown). The sensitivity of the transport process to monovalent, divalent, and trivalent cations strongly suggests that the ability of each ion to block Na^+ transport reflects its ability to interact with specific charged sites on the transport protein. When Na^+ transport into microsomes or reconstituted vesicles was measured in the presence of increasing Na^+ concentrations, with or without either K^+ or Ca^{2+} the inhibitory effects of each cation were shown to be nearly competitive. When the (rate of Na^+ transport)$^{-1}$ was plotted as a function of the (Na^+ concentrations)$^{-1}$, both in the presence and absence of Ca^{+2} or K^+, straight lines were obtained that intersected very close to the ordinate. This finding was also consistent with the suggestion that these ions inhibited Na^+ transport by interacting with the Na^+-binding site on the transport protein.

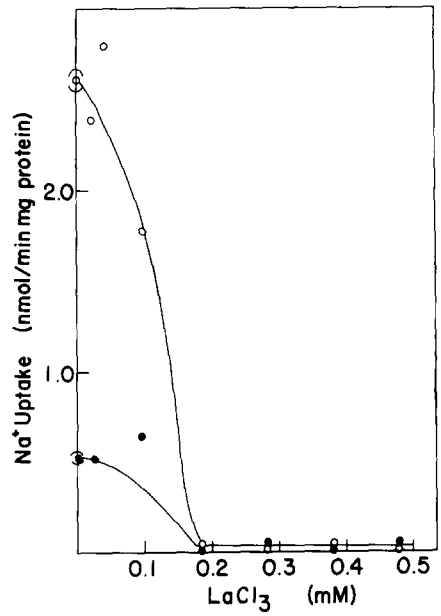

Fig. 7. Effect of La^{+3} on Na^+ uptake into microsomes. Aliquots (0.06 mg protein) of kidney medulla microsomes were incubated with ^{22}Na-Hepps (2.5 μCi, 6.2 mM, pH 8.2), sucrose (0.25 M) and increasing concentrations of $LaCl_3$ both with (●) and without (○) amiloride (2 mM) for 2 min at 20°C in a total volume of 0.25 ml and then applied to Dowex columns as described in LaBelle and Lee [2].

Discussion

Since Na^+ transport into kidney medulla microsomes and into reconstituted proteoliposomes

formed from these microsomes was sensitive to relatively high amiloride concentrations (10^{-3} M), and since this transport was insensitive to valinomycin-induced alterations in membrane potential, it seemed likely that this transport represented Na^+-H^+ exchange [2,3].

Warnock and Yee [28] have shown that valinomycin could stimulate $^{22}Na^+$ uptake into renal brush border membrane vesicles, and they claimed that this was evidence for an electrogenic amiloride-sensitive channel in these vesicles. The data in Fig. 1 show a similar effect, using medulla microsomes. However, in high enough concentrations valinomycin can carry $^{22}Na^+$ itself into vesicles. The small but finite ability of valinomycin to carry Na^+ across membranes has been described by Eisenman et al. [29]. Valinomycin may exert non-specific detergent-like effects at high concentrations. The valinomycin concentration required for $^{22}Na^+$ movement across lipid vesicles and medulla microsomes was 20 μg/ml, well below the concentration used by Warnock and Yee [28] (100 μg/ml). It will be important to examine Na^+ transport into brush border membrane vesicles using lower concentrations of valinomycin that exert unambiguous K^+-specific effects. Na^+-H^+ exchange was first demonstrated in renal brush border membrane vesicles by Murer et al. [9] and was shown to be amiloride-sensitive by Kinsella and Aronson [10]. These investigators were able to preincubate vesicles at low pH in order to load them with protons and such proton-loaded vesicles demonstrated enhanced levels of Na^+ transport. Vesicles incubated with high levels of external protons exhibited inhibited rates of Na^+ transport, due to the operation of the exchange mechanism in reverse (favoring Na^+ efflux). Since medulla microsomes also are sensitive to pH gradients, it seems likely that Na^+ transport into the microsomes represents Na^+-H^+ exchange. We previously reported that amiloride-sensitive Na^+ transport into medulla microsomes was not sensitive to the presence of a pH gradient [2]. However, these earlier studies were performed in the presence of rather low buffer concentrations, since Na^+ transport into membrane vesicles can be inhibited by high ionic strength (Dubinsky, W.P., personal communication). When sufficiently high buffer concentrations were used (the concentrations employed by Kinsella and Aronson [10], Na^+-H^+ exchange was observed in the medulla microsomes.

Relatively high (50 mM) buffer concentrations were required to induce pH gradients in the microsomes due to the intrinsic buffer capacity of the microsomes produced by the histidine solution in which the microsomes were stored and produced by the membrane protein itself. Sodium transport into the microsomes was insensitive to added HCO_3^- (Fig. 3) even though an indirect stimulation of Na^+-H^+ exchange by HCO_3^- might be expected if the CO_2 present with the added HCO_3^- diffused into the microsomes and acidified the microsomal interior. However, such an indirect effect of HCO_3^- did not occur since the amount of intramicrosomal H_2CO_3 generated could be calculated to be insufficient to lower the intra microsomal pH significantly due to the buffer capacity of the microsomes as indicated by the amount of extravesicular buffer required to alter the intravesicular pH [30]. Although the medulla microsomes are capable of Na^+-H^+ exchange, this does not indicate that this process is merely a reflection of brush border membrane vesicle contamination of our microsomes. We showed that the specific activities of brush-border membrane vesicle marker enzyme alkaline phosphatase, maltase, and trehalase were very low in medulla microsomes, while the specific activity of Na^+ transport in the medulla microsomes is comparable to the activity in brush border membrane vesicles [2].

The accumulation of the dye acridine orange has been used by many investigators to demonstrate proton accumulation by vesicles [11,31–36]. These investigators have measured acridine orange accumulation by vesicles by either direct spectrophotometric or spectrophotofluorometric procedures, but they did not separate extravesicular from intravesicular dye. Since acridine orange at neutral pH is 99.9% positively charged, we were able to use Dowex resin to bind extravesicular acridine orange, which permitted us to measure the intravesicular dye directly. Since medulla microsomes were able to exclude acridine orange in the presence of external Na^+, this provided strong evidence in support of Na^+-H^+ exchange by the vesicles. Since proteoliposomes preincubated with either pH 6 or pH 8 buffer accumulated the same

amount of dye, this provided direct evidence that proteoliposomes, unlike microsomes, could not maintain pH gradients. Nichols and Deamer [37] have shown that proton fluxes across pure phospholipid membranes are nearly as rapid as H_2O fluxes. Since our proteoliposomes were formed with 7-times as much added lipid as protein, we expected that such vesicles would be unable to maintain an imposed pH gradient and that reconstituted proteoliposomes treated with the different buffer solutions used in Table I would transport Na^+ at the same rate irregardless of buffer pH.

While we wish to draw no conclusions concerning the stiochiometry of the Na^+-H^+ exchange process based on the data shown in Table I, we were encouraged to observe that the number of nmoles of Na^+ taken up by the microsomes in response to the proton gradient was so similar to the number of nmoles of acridine orange excluded in response to the Na^+ gradient (0.24 nmol/min per mg protein).

Dubinsky and Frizzell [38] have shown that amiloride is capable of directly releasing proton gradients inside ileal brush-border membrane vesicles or inside liposomes, because of its properties as a weak base. Therefore, amiloride may be capable of inhibiting Na^+-H^+ exchange across membranes by a non-specific process under certain circumstances. Proton gradients can be eliminated by a weak base such as NH_3, since the uncharged form of the base diffuses freely across the membrane, becomes protonated and incapable of re-crossing the membrane. Thus the base can be accumulated inside vesicles at the expense of internal protons. This phenomenon has been reported by Boron and DeWeer [39], and by LaBelle and Racker [40]. However, the data in Table II show that Na^+ transport into kidney medulla microsomes was totally insensitive to the well-known uncoupler 1799 [40], either with or without valinomycin, and that NH_4^+ inhibited this process less than did amiloride itself. Since NH_3 can presumably penetrate membranes far more easily than the neutral form of amiloride, it seems logical that NH_4^+ would inhibit more effectively than amiloride if both were inhibiting by virtue of their ability to release a proton gradient. Most likely NH_4^+ inhibits Na^+ uptake into the microsomes by increasing the ionic strength of the solution (as does K^+). The inability of 1799 to lower Na^+ transport rates into the microsomes proves that amiloride does not inhibit Na^+ transport by merely removing a proton gradient from the microsomes. Amiloride most likely inhibits Na^+ transport by direct interaction with the transporter protein.

The data of Saier indicate that amiloride-inhibited, electroneutral Na^+-H^+ exchange across the plasma membranes of cultured kidney cells (MDCK) is inhibited by extracellular Ca^{2+} and and Mg^{2+} [12,13,24]. Villereal [23] has also observed that amiloride-inhibited serumdependent Na^+-H^+ exchange across the plasma membranes of human fibroblasts is also blocked by extracellular Ca^{2+}. Amiloride-inhibited Na^+ transport into brush-border membrane vesicles from rabbit ileum also appears to be sensitive to Ca^{2+} (Dubinsky, W.P., personal communication). The ability of K^+, Ca^{2+}, Mg^{2+}, and La^{3+} to inhibit Na^+ transport into kidney medulla microsomes indicated that these ions interact with the Na^+-binding site on the transport protein. The binding of Na^+ to a site on the membrane surface portion of the transport protein may be required before the Na^+ can be transported into the vesicles and this Na^+ binding may be inhibited by cations. Such cation effects were observed by Frankenhaeuser and Hodgkin [41] who demonstrated an increase in the membrane surface potential of the squid axon by Ca^{2+} and by McLaughlin et al. [42] who observed similar effects of divalent cations on the surface potential of pure bilayers. One would expect that La^{3+} would be three times as effective as Ca^{2+} in exerting such effects and that Ca^{2+} would be ten times as effective as K^+ and this has been shown to be the case (Figs. 4, 5, and 7). Likewise, the ability of increasing Na^+ concentrations to diminish the effects of K^+ and Ca^{2+} on Na^+ transport can be considered as more evidence that K^+ and Ca^{2+} interact with the Na^+ binding site.

The ability of K^+, Ca^{2+}, and Mg^{2+} to cause apparent leaks in the vesicles after long incubation periods may reflect the ability of these ions to displace $^{22}Na^+$ from intravesicular binding sites. Such sites have been observed inside intestinal brush-border vesicles by Liedtke and Hopfer [43]. Whatever the mechanism of the apparent leak generated by the cations, it was shown to be clearly reversible, and the reversibility of the ap-

parent leak ruled out any vesicle 'destruction' brought on by treatment with cations.

LaBelle and Lee [3] showed that the amiloride-inhibited Na^+ transporter from medulla microsomes could be incorporated into reconstituted proteoliposomes. We have demonstrated above that this transporter is responsible for Na^+-H^+ exchange, and that the transporter is sensitive to inhibition by cations both in the microsomes and in the reconstituted proteoliposomes. This information concerning the sensitivity of Na^+-H^+ exchange to such cationic inhibition has not been reported previously, nor has the existence of such a Na^+-H^+ exchange protein been reported in kidney medulla. Medullary structures such as the papillary collecting duct and perhaps the loop of Henle play important roles in the regulation of urinary pH, and the Na^+-H^+ exchange protein in the medulla microsomes may be responsible for this regulation. The absence of brush-border membrane marker enzymes from the medulla microsomes [2] indicates that the microsomal transport activity is not merely a reflection of contamination by brush-border membranes from proximal tubules. We have obtained much information concerning the behavior of the Na^+-H^+ exchanger in the native microsomes, and the similarity of function between these microsomes and the reconstituted proteoliposomes provides additional evidence that the protein responsible for Na^+-H^+ exchange has been incorporated intact into the proteoliposomes. The fact that this protein has been reconstituted into proteoliposomes may permit its eventual isolation. Of course Na^+-H^+ exchange proteins have been reported in a wide variety of cell types, and so one cannot be sure if the protein in the medulla microsomes plays a specific role in renal tubule function, or merely plays the same role in kidney cells that it plays in all mammalian cells.

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