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INHIBITION BY AMILORIDE OF SODIUM TRANSPORT INTO RABBIT KIDNEY MEDULLA MICROSOMES

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Sodium transport into rabbit kidney medulla microsomes was 50% inhibited by amiloride. This Na^+ uptake was shown to represent transport when the uptake process was reversed by the ionophore nigericin. The transport was complete within 60 min and proportional to the microsomal protein concentration. The effect of amiloride on transport was specific since the similar compound sulfaguanidine failed to affect microsomal Na^+ transport. Amiloride-sensitive Na^+ transport into microsomes was inhibited 70% by decreasing the pH (from 7.0 to 5.9), but was unaffected by the presence of a pH gradient. The kinetics of Na^+ transport could be explained by a simple model, assuming that amiloride lowered the rate of Na^+ entrance into the vesicles but had no effect on the rate of efflux. The failure of amiloride to effect efflux from the vesicles was also demonstrated directly.

Introduction

Epithelial cells lining the distal tubules of the mammalian kidney are responsible for the active transport of sodium out of the urine into the blood [1]. This sodium enters the epithelial cells across the apical membrane by means of a transporter which can be specifically inhibited by amiloride [2–5]. Sodium ions are driven out of the cells across the basal-lateral membrane by the ouabain-inhibited (Na^+-K^+)-pump [6,7]. Similar active Na^+ transport occurs across the epithelia of both frog skin and tropical toad urinary bladder [1,8,9]. The mineralocorticoid aldosterone has been shown to stimulate Na^+ transport across the above epithelia, perhaps by stimulating the synthesis of the amiloride-inhibited Na^+ transporter [1,10–14].

Recently a fraction was isolated from mammalian kidney that was enriched in distal tubule segments, aldosterone receptors, and vasopressin-stimulated adenylate cyclase [15,16]. Vasopressin has been shown to stimulate H_2O transport by the distal tubule via a cyclic AMP mediated process [17]. Membrane fractions rich in vasopressin-stimulated adenylate cyclase have been obtained from mammalian kidney medulla [18–20], and both aldosterone-induced protein synthesis and aldosterone-induced citrate synthetase activity can be most easily identified in kidney medulla [21,22]. This evidence suggested that an amiloride-inhibited Na^+ transporter, perhaps under aldosterone control, known to be present in the distal tubule of the mammalian kidney, could be obtained from kidney medulla. Recently, we prepared a membrane vesicle fraction from the urinary bladder of the tropical toad which was capable of amiloride-inhibited Na^+ transport [23]. Since the toad urinary bladder is often used as a model for the distal tubule of the mammalian kidney, we also

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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tried to obtain a microsomal membrane fraction from rabbit kidney medulla that was capable of amiloride inhibited Na^+ transport. Such a mammalian system would further justify the validity of the toad bladder model. It is also considerably easier to purify plasma membrane from rabbit kidney than from toad bladder [18–20,24,25] and the possibility existed that rabbit kidney would serve as a much richer source of the amiloride-inhibited Na^+ channel than would toad bladder. Therefore, in order to begin the purification of this transporter from rabbit kidney medulla, we have obtained a microsomal membrane fraction from this tissue that is capable of amiloride-inhibited Na^+ transport.

Materials

New Zealand white rabbits were obtained from Nichols Rabbitry, Lumberton,Texas. Amiloride hydrochloride (*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. Nigericin was generously provided by Dr. R.J. Hosley of Eli Lilly, Indianapolis, IN. Ouabain, Hepes, Mes, ATP, sulfaguanidine and Dowex 50X8-100 (H^+ form) were obtained from Sigma Chemical Co., St. Louis, MO. $^{22}Na^+$, D-[^{3}H] glucose, L-[^{3}H] glucose and Aquasol-2 were obtained from New England Nuclear, Boston, MA.

Methods

Rabbit kidney medulla subcellular fractions. Subcellular fractions were isolated from rabbit kidney medulla by a slight modification of the procedure of Barnes et al [18]. The published procedure was altered by substituting rabbit kidney for bovine kidney and scissors for razor blades during tissue mincing [18]. The fractions isolated included nuclei, basal-lateral plasma membrane, mitochondria, and microsomes. The microsomes were washed once after isolation by centrifugation for 30 min at 200,000 $\times$ *g* and resuspended in 0.25 M sucrose/6 mM histidine buffer (pH 7.2) solution at a final protein concentration of 5–6 mg/ml. They were then stored at -20°C in aliquots of 0.25 ml. Protein concentrations were determined by the method of Lowry et al. [26]. The succinate dehydrogenase activity of each fraction was determined by the method of Pennington [27], and the ($Na^+ + K^+$)-ATPase activity measured by the method of Jorgenson [28].

Na^+ transport measurements. Na^+ transport into subcellular fractions of rabbit kidney medulla was measured by the method of LaBelle and Valentine [23]. Aliquots of the tissue were incubated at 22°C with 0.25 M sucrose, 0.1 mM ouabain, 1 μCi $^{22}Na^+$, and either 0.6 mM NaCl, plus 1.28 mM sodium phosphate (pH 7.4), or 0.6 mM amiloride-HCl plus 1.6 mM sodium phosphate (pH 7.4), in a total volume of 0.25 ml. Each incubation mixture was placed on a 1-ml Dowex 50X8 (Tris) 100 mesh column and eluted with sucrose as described [23]. The eluted vesicles were dissolved in 6.5 ml Aquasol-2-scintillation fluid and radioactivity was determined with a Beckman LS-100-C Liquid Scintillation Spectrometer.

Glucose transport measurements. [^{3}H]Glucose transport into membrane vesicles was measured by a modification of the Sephadex-centrifugation method of Penefsky [29]. Membrane vesicles were incubated at 20°C with 0.15 M NaCl, 2 mM $MgSO_4$, 10 mM Tris-HCl (pH 7.2) and either 0.12 mM D-[^{3}H]glucose (4 μCi) or 0.12 mM L-[^{3}H]glucose (4 μCi) in a total volume of 0.15 ml. The incubation was terminated by first applying 0.1 ml aliquots of the incubation mixtures to syringe barrels (1 ml tuberculin) previously filled with Sephadex G-50 (40–80 mesh) and dehydrated by 2 min, 250 $\times g$ centrifugation and then centrifuging the syringe barrels for 2 min at 250 $\times g$. The membrane vesicles passed through the Sephadex into the centrifuge tube containing the syringe barrel together with accumulated [^{3}H]glucose. The free extravesicular [^{3}H]glucose was retained by the Sephadex.

Brush border marker enzymes. The activity of alkaline phosphatase was measured by the method of Bessey et al. [30]. The activities of maltase and trehalase were measured by the method of Dahlquist [31].

Electron microscopy. Pelleted microsomes were fixed in a freshly made 1:1 (v/v) solution of 2.5% glutaraldehyde in 0.2 M sodium phosphate buffer (pH 7.4) and 2% aqueous OsO_4 for 30 min at 4°C [32]. The pellets were washed twice with the sodium

phosphate buffer and distilled water, dehydrated in graded ethanol and embedded in Epon 812. Thin sections (600–800 Å, grey-silver) were cut with a diamond knife (Diatome) on an LKB-2 ultramicrotome and mounted on 100 mesh, formvar coated grids. The sections were stained for 20 min with 2% uranyl acetate and Reynolds lead citrate before examining with a Phillips 201 electron microscope at 80 kV [33].

Results

Subcellular fractionation of rabbit kidney medulla

Subcellular fractions of kidney medulla were examined for amiloride-inhibited Na^+ transport. The medulla was used because vasopressin, aldosterone, and amiloride have been shown to exert their primary effects on the distal tubules of the mammalian kidney [2–5,12–14,17] and vasopressin-sensitive adenylate cyclase activity, together with aldosterone induced protein synthesis have been clearly demonstrated in the kidney medulla [18–21]. These subcellular fractions consisted of nuclei, basal-lateral plasma membranes, mitochondria and microsomes. The basal-lateral plasma membrane fraction produced by centrifugation at low speed, has been shown by several investigators to be enriched in basal-lateral membrane enzymes such as the $(Na^+ + K^+)$-ATPase and vasopressin-stimulated adenylate cyclase [18,20]. The basal-lateral fraction, prepared in our laboratory, also was highly enriched in $(Na^+ + K^+)$-ATPase activity (Fig. 1). Iyengar, et al. [19] have indicated that a fraction obtained from kidney medulla by high

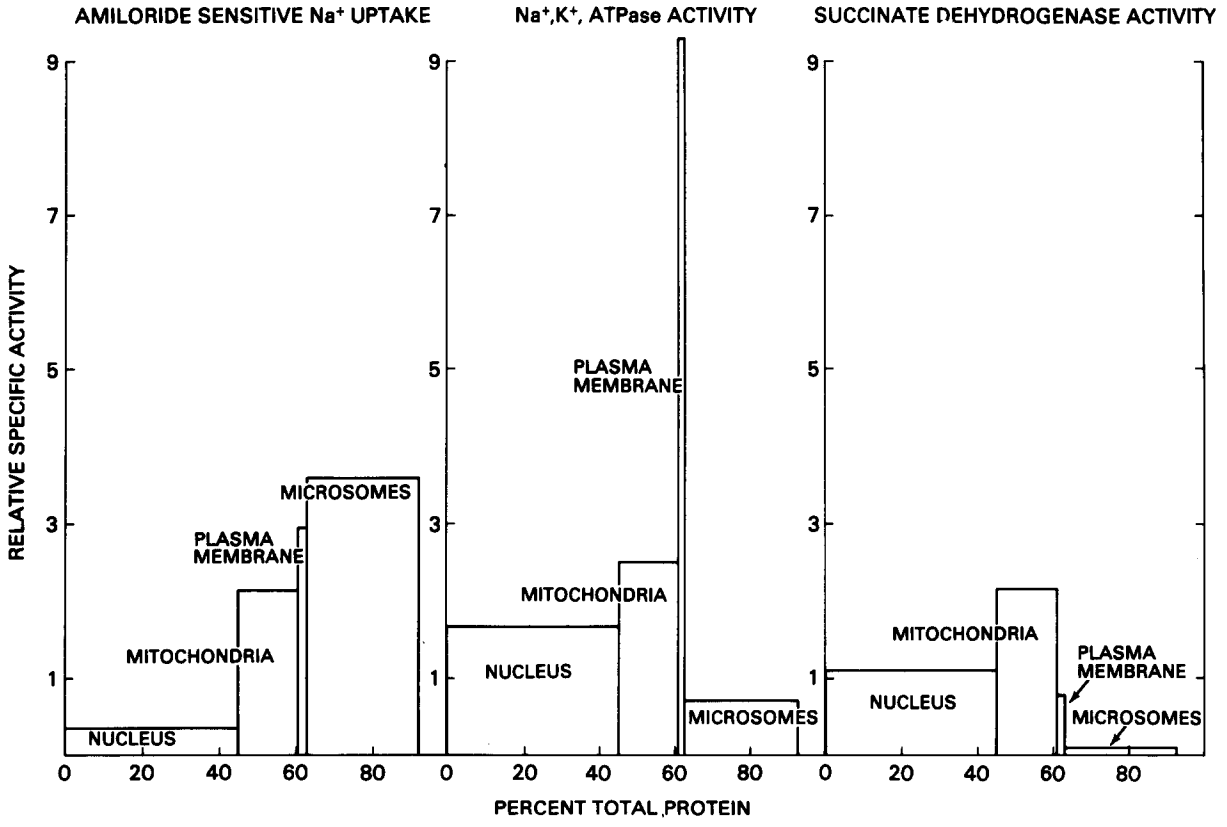

Fig. 1. Subcellular fractionation of rabbit kidney medulla. The homogenate of rabbit kidney medulla was divided by the procedure of Barnes et al. [18] into subcellular fractions: Nucleus, basal-lateral plasma membrane, mitochondria and microsomes. The specific activities of amiloride-sensitive $^{22}Na^+$ transport, succinate dehydrogenase, and the $(Na^+ + K^+)$-ATPase were determined for each fraction. These specific activities were measured relative to the specific activities of the initial homogenate and plotted as a function of the percent of total membrane protein occupied by each fraction.

speed centrifugation consisted of apical plasma membranes. The fraction that demonstrated the highest specific activity of amiloride-inhibited Na^+ transport was the microsomal fraction (Fig. 1) (obtained by high-speed centrifugation). About 67% of the total amiloride-inhibited Na^+ transport activity was present in the microsomal fraction. These data also show that this fraction was almost totally lacking in mitochondrial contamination (due to the absence of the mitochondrial marker enzyme, succinate dehydrogenase).

Characterization of amiloride-inhibited Na^+ transport into microsomes

Sodium accumulation by rabbit kidney medulla microsomes was 50% inhibited by amiloride. This amiloride-inhibited Na^+ uptake activity was extremely stable and was undiminished even after 6 weeks at −20°C. The microsomes were normally stored in the presence of an isotonic sucrose-histidine buffer solution, but microsomes could be stored for at least 3 weeks in the absence of histidine without losing activity. The presence of the amino acid buffer had no influence on the transport activity. Sodium uptake by the microsomes was normally measured in the presence of phosphate anion, but identical results were obtained when phosphate was replaced entirely by Hepes.

Sodium accumulation by the microsomes was extremely rapid. The process was half-complete within 5 min and reached equilibrium within 60 min (Fig. 2A). The kinetics of Na^+ accumulation are consistent with a simple reaction scheme represented by the equation:

$$Na_o^+ \underset{l}{\overset{k}{\rightleftharpoons}} Na_i^+ \qquad (1)$$

wherein Na_o^+ represents the Na^+ outside the vesicles, Na_i^+ represents Na^+ inside the vesicles and k and l represent rate constants for the forward and reverse reactions (influx into and efflux from vesicles). Under conditions obtained when the initial internal Na^+ concentration is zero and the external Na^+ concentration is essentially constant, the relationship expected between internal Na^+ concentration and time can be expressed by:

$$[Na^+]_i^t = [Na^+]_o K_{Na}(1 - e^{-lt}) \qquad (2)$$

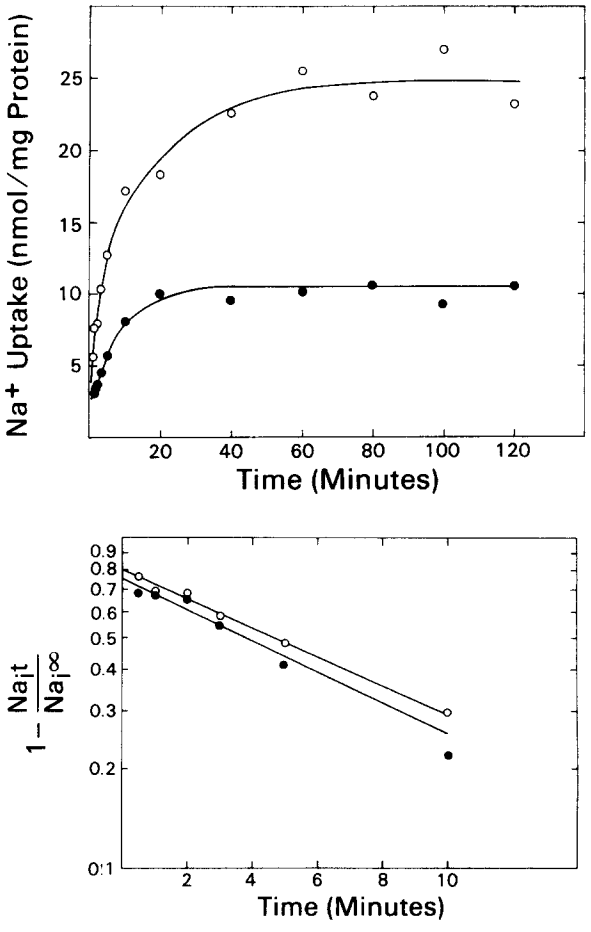

Fig. 2. Effect of time on $^{22}Na^+$ accumulation by rabbit kidney medulla microsomes. (A) Aliquots of rabbit kidney medulla microsomes (0.11 mg protein) were incubated for the times indicated at 22°C with sucrose (0.25 M), ouabain (0.1 mM), $^{22}Na^+$ (0.5 μCi, 2.8 mM), Cl^- (0.6 mM), and sufficient phosphate (pH 7.4) to serve as a counterion for Na^+ both with (●——●), and without (○——○) amiloride (0.6 mM), in a total volume of 0.25 ml. The incubations were terminated by the application of the incubation mixtures to Dowex columns (1 ml). The columns were eluted with 1 ml sucrose (0.25 M), and the radioactivity in the eluants determined. (B) The data in (A) (first 10 min) are plotted as $\ln(1 - ((Na)_i^t/(Na)_i^\infty))$ vs. time.

wherein $[Na]_i^t$ represents the internal sodium concentration at time t, $[Na^+]_o$ represents the initial external Na^+ concentration and K_{Na} represents the equilibrium constant of the transport reaction (the ratio of the forward rate constant to the reverse rate constant). One of the consequences of this model is the fact that a plot of $\ln(1 -$

$((Na)_i^t/(Na)_i^\infty))$ vs. time should be a straight line with the reverse reaction rate constant l as its slope. $(Na)_i^t$ represents the amount of Na^+ inside the vesicles at time t and $(Na)_i^\infty$ represents the amount of Na^+ inside under equilibrium conditions. When the relationship between $\ln(1 - ((Na)_i^t/(Na)_i^\infty))$ and time was plotted for the data shown in Fig. 2A, two straight lines were obtained (Fig. 2B). From the slope of each line the reverse rate constant l was calculated to be 0.044 min^{-1}, both with and without amiloride. When the efflux of $^{22}Na^+$ from preloaded microsomes was measured directly, no effect of amiloride was observed on this process (Fig. 3).

Another expected consequence of the rate equation would be a linear relationship between sodium accumulation at a constant time t and the external sodium concentration, and such a relationship was not observed (Fig. 4). Since the vesicles were formed with very few internal cations and transport was measured in the presence of relatively membrane-impermeant anions (phosphate or Hepes), Na^+ uptake by the vesicles would be expected to produce a high membrane potential (inside positive) that would block Na^+ movement at higher Na^+ concentrations. This would result in decreased ion movement at the higher concentrations as shown in Fig. 4.

When Na^+ uptake into medulla microsomes was determined, both in the presence and absence of amiloride, as a function of increasing protein concentration, the relationship observed was strictly linear (data not shown). The Na^+ accumulation could be extrapolated to zero at zero protein concentration, and the rate of uptake was 7.37 nmol/min mg in the absence of amiloride and 3.77 nmol/min mg in the presence of amiloride. The concentration of amiloride required for the half-maximal inhibition of Na^+ transport into the microsomes was 60 μM, and a significant inhibitory effect was observed when 24 μM amiloride was used in the incubation (Fig. 5). Preincubation of the microsomes with amiloride before the Na^+ uptake assay did not increase the effect of the inhibitor on the process (data not shown).

Controls that identify Na^+ accumulation as transport

The following controls indicated that the amiloride sensitive Na^+ accumulation by the microsomes represented transport and not binding. First, the extent of Na^+ accumulation by the microsomes was decreased as the osmotic pressure of the solution was increased by the addition of sucrose to the solution (Fig. 6). Therefore the vesicles apparently behaved as osmometers, decreasing their internal volume as H_2O left in response to the gradient across the vesicle membrane produced by the membrane impermeable sucrose. Due to their decreased volume, less Na^+ could be taken into the vesicles. Sodium uptake into the

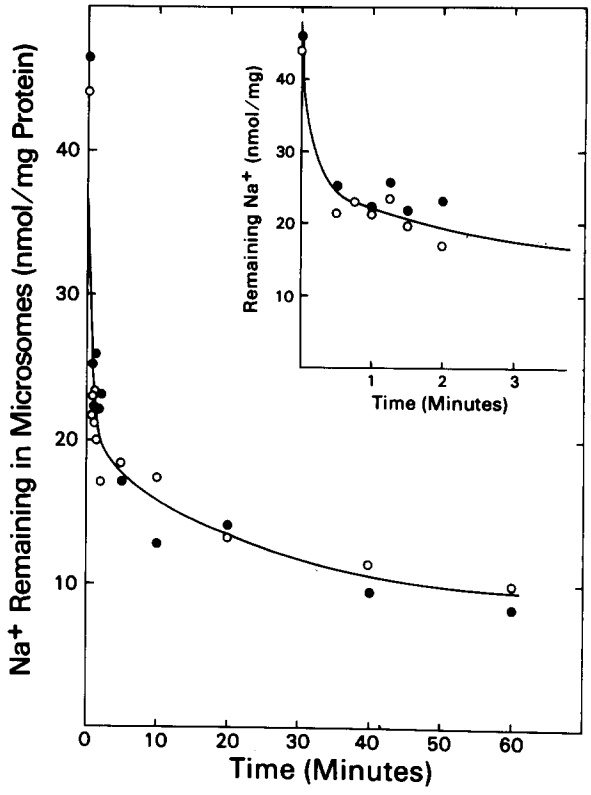

Fig. 3. Effect of time on $^{22}Na^+$ efflux from rabbit kidney medulla microsomes. Rabbit kidney medulla microsomes (0.66 mg protein) were incubated for 30 min at 22°C with sucrose (0.25 M), ouabain (0.5 mM, and [^{22}Na]sodium phosphate (1 μCi, 10 mM, pH 7.4) in a total volume of 0.3 ml. Aliquots (40 μl) of the incubation mixture were either applied directly to Dowex columns as described in Fig. 2 or diluted 1:6 with sucrose (0.25 M) and sodium phosphate (15 mM, pH 7.4) either with (●——●) or without (○——○) amiloride (0.5 mM) for the times indicated and then applied to Dowex columns. Data obtained after short periods of dilution (0.5–2 min) are shown in the inset.

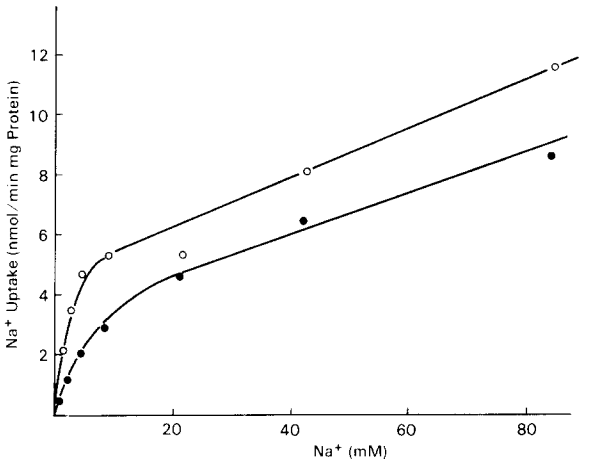

Fig. 4. Effects of Na^+ concentration on $^{22}Na^+$ accumulation by microsomes. Rabbit kidney medulla microsomes (120 μg protein) were incubated for 2.0 min at 22°C with ouabain (0.1 mM), Cl^- (0.6 mM), increasing amounts of $^{22}NaP_i$ (1.2 μCi/μmol Na^+), and sufficient sucrose to keep the osmolarity constant, both with (●——●) and without (○——○) amiloride (0.6 mM), in a total volume of 0.25 ml. The incubation mixtures were applied to Dowex columns as described in the legend to Fig. 2.

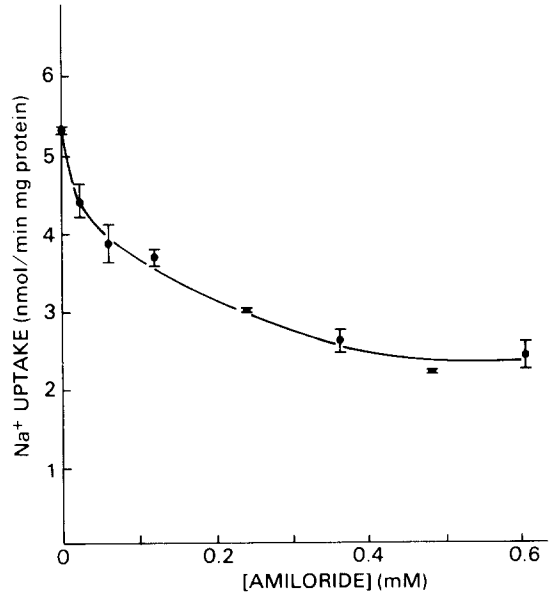

Fig. 5. Effect of the increasing concentration of amiloride on $^{22}Na^+$ accumulation by microsomes. The accumulation of $^{22}Na^+$ by rabbit kidney medulla microsomes during a 2-min incubation in the presence of increasing amounts of amiloride was determined as described in the legend to Fig. 2.

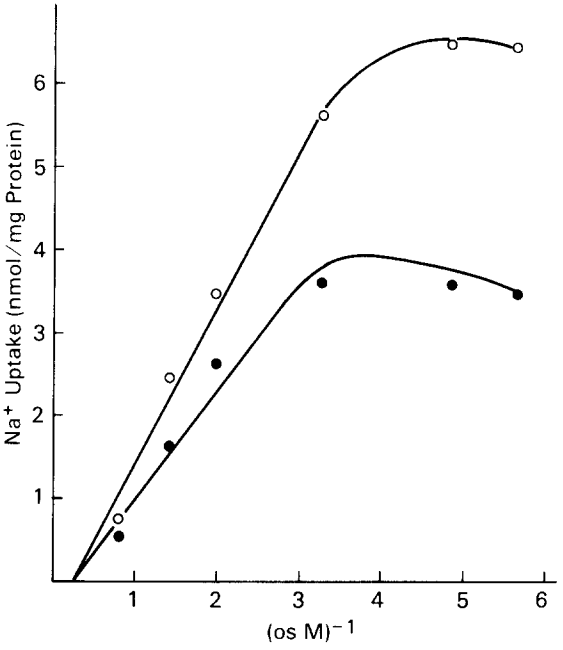

Fig. 6. Effect of increasing osmolarity on sodium accumulation by medulla microsomes. Sodium uptake into rabbit kidney medulla microsomes was measured as described in the legend to Fig. 2, except that the osmolarity was altered by changing the sucrose concentration and the Dowex columns were equilibrated and eluted with sucrose solutions that were isotonic to the incubation mixtures. The time of incubation was 20 min.

vesicles both in the presence and absence of amiloride could be extrapolated to near zero at infinite osmolarity (1/osM = 0) (Fig. 6), indicating that nearly all of the Na^+ taken up by the vesicles represented ions transported into an osmotically active space. Na^+ uptake was not a strictly linear function of the inverse of osmolarity, no doubt because of the inability of the extremely small vesicles to expand in solutions of low osmolarity (less than 0.25 osM sucrose). Second, the Na^+ accumulated by the microsomes could be removed by the monovalent ionophore nigericin. When microsomes were preloaded with $^{22}Na^+$, both in the presence and absence of amiloride, and then diluted 6-fold into a non-radioactive sucrose solution, very little $^{22}Na^+$ was removed from the vesicles (Table I). When the pre-loaded microsomes were diluted into a sucrose solution containing the ionophore nigericin, roughly two-thirds of the accumulated Na^+ was removed from the microsomes (Table I). If the nigericin was able to

TABLE I

NIGERICIN SENSITIVITY OF Na^+ ACCUMULATION BY MICROSOMES

Rabbit kidney medulla microsomes (0.15 mg protein) were incubated for 2 min at 22°C with sucrose (0.25 M), ouabain (0.1 mM), and [^{22}Na]sodium phosphate (5 μCi, 8.7 mM, pH 7.4), both with and without amiloride (0.6 mM) as indicated in a total volume of 0.27 ml. Aliquots (40 μl) of the incubation mixtures were either applied directly to Dowex columns as described in Fig. 2 or diluted 1:6 with sucrose (0.25 M) with and without nigericin, incubated 30 s further and then applied to Dowex columns. The values represent means of duplicate determination ± S.D. (n = 2).

	Na^+ accumulated (nmol/mg protein)	
	−Amiloride	+Amiloride (0.6 mM)
Before dilution	18.4 ± 1.1	9.7 ± 0.6
After dilution − Nigericin	19.1 ± 0.3	8.9 ± 0.2
After dilution + Nigericin (33 μg/ml)	6.8 ± 1.2	3.2 ± 0.4

permit the complete equilibration of the $^{22}Na^+$ after the 1:6 dilution of the vesicles, the vesicles should have retained about 16% of their initial $^{22}Na^+$ in the presence of the ionophore. Since the vesicles retained from 33 to 37% of their $^{22}Na^+$ in the presence of the ionophore, the possibility existed that from 17 to 21% of the accumulated isotope might have been irreversibly bound to proteins on the vesicle interior.

Specificity of Na^+ transport into microsomes

The ability of amiloride to inhibit Na^+ uptake into rabbit kidney medulla microsomes was not shared by the structurally related compound sulfaguanidine. During a representative experiment, kidney medulla microsomes took up 5.40 ± 0.47 nmol Na^+/min per mg in the absence of inhibitor, while in the presence of amiloride (0.6 mM) the rate of Na^+ accumulation was 3.20 ± 0.08 nmol/min per mg and in the presence of sulfaguanidine (0.6 mM) the rate was 5.22 ± 0.27 nmol/min per mg (the same as control accumulation). This indicated that amiloride was most likely exerting an effect on a specific biological transport system rather than merely blocking charge movement across the microsomal membrane by virtue of its hydrophobic character and positive charge (qualities shared by sulfaguanidine).

Amiloride was not capable of decreasing vesicle size, as shown by electron microscopy (Fig. 7). The mean diameter of untreated vesicles was determined to be 1730 ± 730 Å ($n = 106$) while the mean diameter of amiloride-treated vesicles was determined to be 1790 ± 770 Å ($n = 104$). Amiloride was also totally incapable of inhibiting either the transport of D-[^{3}H]glucose or L-[^{3}H]glucose into the vesicles (Fig. 8). This indicated that amiloride was not merely increasing membrane permeability non-specifically.

Sensitivity of Na^+ transport to pH

Kinsella and Aronson [34] have demonstrated the existence of an (Na^+-H^+)-antiporter in brush-border membrane vesicles obtained from the proximal tubules of the kidney cortex. This antiporter was highly sensitive to the presence of a pH gradient across the membrane and also inhibited by amiloride [34]. In order to determine the sensitivity of rabbit kidney medulla microsomes to a pH gradient, aliquots of medulla microsomes were preincubated at either pH 5.9 or pH 7.0 for 30 min and then Na^+ uptake into the microsomes was measured at either pH. The pH of the incubation mixture was established by using the same buffer mixtures used by Kinsella and Aronson [34] containing Tris, Hepes and Mes (Table II).

The amiloride-sensitive transport of Na^+ into kidney microsomes was more than 70% inhibited by a decreased extravesicular pH (from pH 7.0 to pH 5.9) (Table II). Preincubation of the vesicles at pH 5.9 for 30 min did not affect amiloride-sensitive Na^+ uptake, provided that Na^+ uptake was measured at pH 7.0. This suggested that the decreased pH did not destroy transport activity irreversibly but merely inhibited it. Likewise, amiloride-sensitive Na^+ transport was not significantly altered by the presence of a pH gradient across the microsomal membrane (Table II). However, the amiloride-insensitive Na^+ transport did appear to be sensitive to such a gradient, showing 36% stimulation when the internal pH was less than the external pH and 34% inhibition when the reverse was true. Neither the uptake of D-glucose nor the uptake of L-glucose into kidney medulla micro-

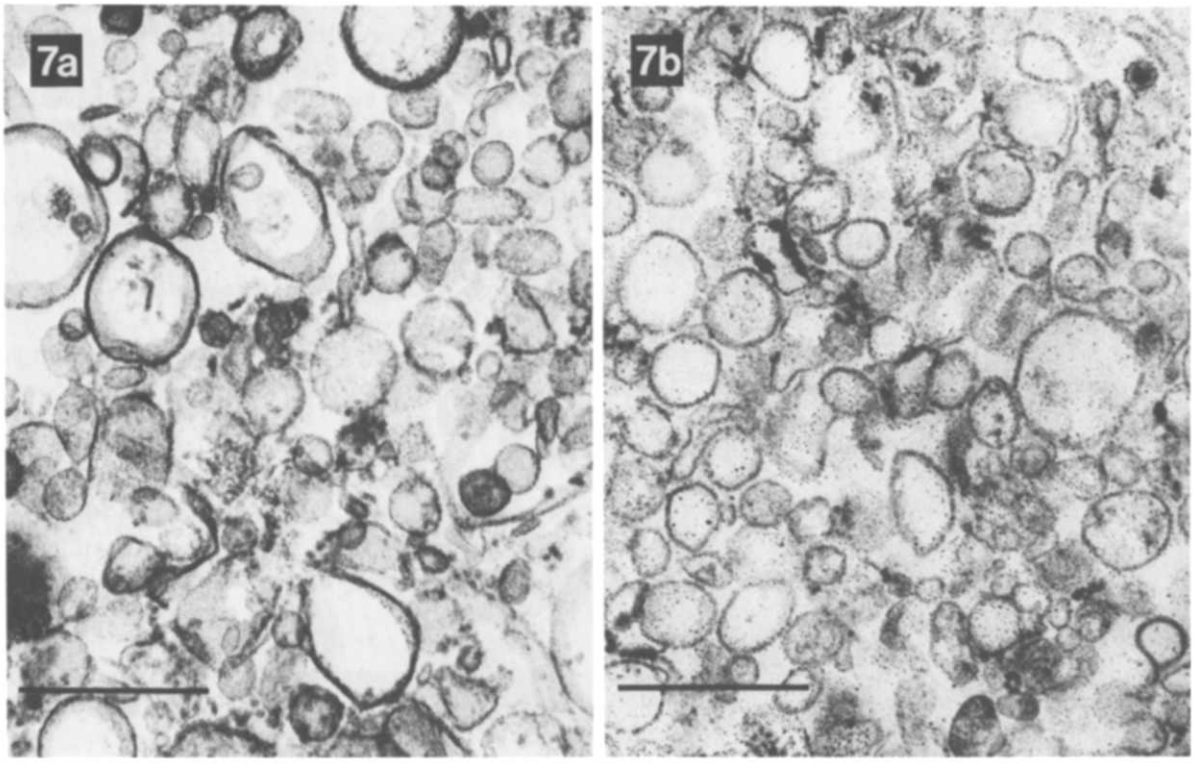

Fig. 7. Electron micrographs of kidney medulla microsomes. (A) Untreated microsomes. (B) Amiloride-treated microsomes. Scale bars = 0.5 μm. Micrographs by John S. Bergmann.

somes were sensitive to the pH gradients used in the experiment shown in Table II (data not shown).

Substantial contamination of the rabbit kidney medulla microsomes by brush-border vesicles from the proximal tubules was ruled out by the measurement of brush-border marker enzyme activi-

TABLE II

SENSITIVITY OF Na^+ UPTAKE BY MEDULLA MICROSOMES TO pH EFFECTS

Rabbit kidney medulla microsomes (0.3 mg protein) were preincubated for 30 min at 22°C with either a pH 5.9 buffer solution containing Tris (2.9 mM), Hepes (1.4 mM), and Mes (9.1 mM) or a pH 7.0 buffer solution containing Tris (4.7 mM), Hepes (7.9 mM), and Mes (1.8 mM), in a total volume of 0.1 ml. $^{22}Na^+$ uptake into aliquots (20 μl) of each preincubation mixture was measured for 2 min as described in the legend of Fig. 1, except that each $^{22}Na^+$ incubation mixture was either buffered with the above pH 5.9 buffer solution or with the above pH 7.0 buffer solution. $^{22}Na^+$ uptake was determined either without or with (0.6 mM) amiloride, and the differences between these values (amiloride-sensitive uptake) were calculated.

Internal pH (preincubation pH)	External pH (incubation pH)	Na^+ uptake (nmol/min per mg) ± S.D. ($n=2$)		
		−Amiloride	+Amiloride	Amiloride-sensitive uptake
5.9	5.9	1.22 ± 0.04	1.15 ± 0.02	0.07 ± 0.04
5.9	7.0	2.99 ± 0.08	1.66 ± 0.12	1.33 ± 0.12
7.0	7.0	2.30 ± 0.1	1.22 ± 0.05	1.10 ± 0.1
7.0	5.9	1.09 ± 0.09	0.81 ± 0.01	0.28 ± 0.09

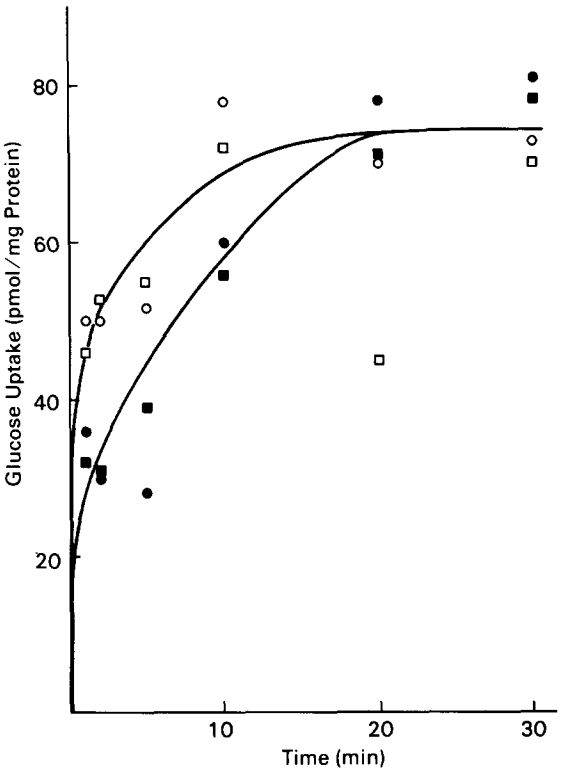

Fig. 8. Uptake of [^{3}H]glucose by rabbit kidney medulla microsomes. The uptake of either D-[^{3}H]glucose (□, ○), or L-[^{3}H]glucose (■, ●) by medulla microsomes was measured as described in Methods, both in the presence (○, ●) and absence (□, ■) of amiloride (0.6 mM).

ties. The activity of the brush-border marker enzyme maltase was determined to be 36.5 nmol/min per mg in crude kidney cortex homogenate. This was almost identical to the value found for maltase activity in kidney cortex homogenate by George and Kenny (42 nmol/min per mg) [35]. The maltase activity of the kidney medulla microsomes was found to be 40 nmol/min per mg in comparison with the maltase activity of the brush border fraction of George and Kenny [35], which was 944 nmol/min per mg. Therefore, one could infer that brush border vesicle protein comprised only about 4% of the total protein content of the medulla microsomes. Likewise the specific activities of alkaline phosphatase and trehalase (other marker enzymes for brush border membranes) in the medulla microsomes were 180 and 20 nmol/min per mg, respectively, while the activities of these enzymes in purified brush border membranes were determined by George and Kenny [35] to be 2755 and 1170 nmol/min per mg, respectively. These values suggested that brush border vesicle protein comprised from 2 to 6% of the total protein in the medulla microsomes.

The kidney medulla microsomes were capable of stereospecific D-glucose transport (D-glucose uptake in excess of L-glucose diffusion) (Fig. 8). However, this D-glucose transport was totally independent of sodium since the same results were obtained when all of the NaCl in the incubation medium was replaced by KCl (data not shown).

Discussion

Since the diuretic amiloride has been shown to block Na^+ movement out of the urine and into the epithelial cells lining the kidney distal tubules [2–5], whereas the $(Na^+ + K^+)$-ATPase has been shown to pump Na^+ out of these cells into the blood [6,7], it seemed reasonable that subcellular fractionation of the kidney might yield two plasma membrane fractions. One fraction might possess a highly active amiloride-inhibited Na^+ transport system while the other would be highly enriched in $(Na^+ + K^+)$-ATPase activity. The data in fig. 1 suggest that these two plasma membrane fractions have been partially separated by differential centrifugation. A similar separation has been obtained by Iyengar et al. [19], although they used a bicarbonate-stimulated ATPase activity as a marker for apical membranes rather than $^{22}Na^+$ transport activity. Of course, $(Na^+ + K^+)$-ATPase activity was found in all subcellular fractions (Fig. 1). Since cell surface receptors and enzymes are made in the endoplasmic reticulum and some surface receptors (e.g., the asialoglycoprotein receptor in the liver cell) actually exist inside the cell in much higher quantities than on the surface [36], the presence of $(Na^+ + K^+)$-ATPase activity and also amiloride-inhibited Na^+ transport activity in all fractions may represent either contamination of all fractions with plasma membrane or with endoplasmic reticulum containing newly made Na^+ transporter.

The exponential kinetics of the Na^+ accumulation process were consistent with a simple transport model. Amiloride lowered the rate of Na^+

entrance into the vesicles, thereby decreasing k, and did not inhibit either the calculated reverse rate constant l nor the observed rate of Na^+ efflux. Therefore, under equilibrium conditions defined by the equation

$$K_{Na} = \frac{k}{l} = \frac{(Na)_i^{\infty}}{(Na)_o} \quad (3)$$

The amount of Na^+ expected in the vesicles at infinite time $(Na)_i^{\infty}$ would be less in the presence of amiloride than in its absence. This was indeed observed in the experiment shown in Fig. 2A. From the above equation, it can be calculated that amiloride lowers k by about 50%. The fact that Na^+ accumulation was directly proportional to the protein concentration of the microsomes was consistent with transport and identical with what other investigators have observed with similar systems [23,37,38].

The amiloride-inhibited accumulation of Na^+ by the rabbit kidney microsomes was proven to represent membrane transport rather than binding. Sodium accumulation by the microsomes was sensitive to changes in the osmotic strength of the incubation medium, and could be almost totally reversed by the ionophore nigericin (Table I). Similar effects of nigericin on membrane transport have been reported by other investigators of ion transport [23,39,40].

Amiloride inhibition of Na^+ uptake into kidney medulla microsomes was observed at concentrations as low as 24 μM. This concentration was higher than the concentration observed to block Na^+ transport across the epithelia of isolated perfused kidney tubules [4,41]. This may have resulted from the presence of non-specific amiloride-absorbing sites present in the kidney medulla microsomes that lowered the effective concentration of amiloride observed by the Na^+ transporter. These sites would not be available to the amiloride in an isolated tubule preparation.

Bentley [8] has shown that sulfaguanidine did not block Na^+ transport across the isolated toad bladder [8], (an organ that is extremely sensitive to amiloride) in spite of the fact that the structure of sulfaguanidine is nearly identical to that of amiloride [23]. Likewise LaBelle and Valentine did not observe an effect of sulfaguanidine on Na^+ transport into toad bladder microsomes [23] nor was there any effect of sulfaguanidine on Na^+ uptake into kidney medulla microsomes. This provided strong evidence that amiloride was interacting with a specific biological transport system rather than simply slowing nonspecific charge movement. Amiloride was also shown by LaBelle and Valentine [23] to be incapable of either inhibiting gramicidin-mediated movement of Na^+ into phospholipid vesicles or of affecting the slow leakage of Na^+ into phospholipid vesicles that can be observed in the absence of gramicidin. This also indicated that the inhibitory effects of amiloride on Na^+ transport represented the inhibition of a specific process. The possibility that amiloride might have decreased vesicle size was ruled out by electron microscopy (Fig. 7), and the possibility that amiloride might have nonspecifically enhanced vesicle permeability was ruled out when amiloride was shown to have no effect on either D- or L-glucose transport into the vesicles (Fig. 8).

Since Kinsella and Aronson [34] observed amiloride-inhibited Na^+ transport into brush border vesicles from rabbit kidney cortex, the possibility existed that the transporter they studied was the same as the transporter investigated in our laboratory. Preliminary experiments showed that amiloride-sensitive Na^+ transport into kidney medulla microsomes was insensitive to the presence of a pH gradient which suggested that this transporter was different from the amiloride-sensitive (Na^+-H^+)-antiporter [34] in kidney brush border vesicles. Contamination of medulla microsomes by brush border vesicles from proximal tubules (primarily found in the cortex), was shown to be minimal when very low levels of brush border marker enzymes (maltase, trehalase, and alkaline phosphatase) were detected in the medulla microsomes. Since the Na^+ transport activity of the medulla microsomes was actually higher than the Na^+ transport activity of brush border vesicles [34,42], it seemed extremely unlikely that the transport activity found in the microsomes resulted solely from brush border vesicle contamination. Further evidence that the medulla microsomes were not contaminated substantially by brush border vesicles was provided by the electron micrograph (Fig. 7), which did not show any evidence of the microvilli characteristic of brush

border membranes [43]. Brush border vesicles are capable of sodium-dependent D-glucose transport, and the medulla microsomes were capable only of sodium-independent D-glucose transport, once again suggesting minimal contamination of medulla microsomes by brush border vesicles. This does not imply that the apical membrane of the distal tubule is capable of stereospecific D-glucose transport, but that the medulla microsomes are no doubt contaminated by either basal-lateral membrane from the distal tubule or by endoplasmic reticulum containing newly synthesized D-glucose transporter. The amiloride-inhibited Na^+ transport system contained within the medulla microsomes may be related to the amiloride-sensitive system in the brush border vesicles, but the medulla microsomes are conclusively different from the brush border vesicles as befits their distal tubular origin.

Studies in our laboratory are currently in progress to reconstitute the amiloride-sensitive Na^+ channel from rabbit kidney medulla into phospholipid vesicles and to use these reconstituted vesicles as an assay during the purification of the amiloride-sensitive channel. The development of amiloride-inhibited microsomes from kidney medulla represents an important first step in the isolation of the channel.

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