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INHIBITION BY AMILORIDE OF $^{22}Na^+$ TRANSPORT INTO TOAD BLADDER MICROSOMES

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Summary

The diuretic, amiloride, inhibited $^{22}Na^+$ accumulation by microsomes from toad (*Bufo marinus*) bladder by between 40 and 70%. Accumulated $^{22}Na^+$ was separated from free isotope by ion-exchange chromatography. The amiloride-sensitive process was directly proportional to the protein concentration and a hyperbolic function of the Na^+ concentration. The inhibition was competitive. At least 9 times more activity was found in the microsomal fraction than the mitochondrial fraction, and none at all was found in the cytosol. Amiloride inhibition could not be reproduced by similar pyrazine and guanidine compounds, such as sulfaguanidine. Amiloride-sensitive Na^+ accumulation was totally reversed by nigericin. The intravesicular volume of the bladder microsomes as determined by the $[^3H]H_2O/[^{14}C]$inulin method was shown to be nearly equal to the volume occupied by the amiloride-sensitive portion of the $^{22}Na^+$ accumulation. The amiloride-sensitive Na^+ accumulation most likely represents transport into vesicles. Since amiloride does not affect gramicidin-mediated $^{22}Na^+$ uptake into phospholipid vesicles, the amiloride inhibition seen with bladder microsomes probably represents a specific channel blockade and not simply interference with cation movement that might be produced by any cationic hydrophobic molecule.

Introduction

The study of ion transport across the isolated toad urinary bladder has provided much useful information about the function of the distal mammalian nephron [1]. Both the toad bladder and the distal nephron are lined with

epithelial cells that actively transport sodium from the apical or mucosal side of the cells to the basolateral or serosal side, a process that is stimulated by the mineralocorticoid, aldosterone, and inhibited by the diuretic, amiloride (*N*-amidino-3,5-diamino-6-chloropyrazine carboxamide) [1—4]. This epithelial sodium transport has been explained by a two-barriers-in-series model [5], wherein sodium enters the epithelial cell on the apical side via facilitated diffusion and leaves the cell on the basolateral side via active transport by the $(Na^+ + K^+)$-ATPase [6]. Since amiloride blocks epithelial sodium transport when applied to the apical side of the tissue and this process is readily reversible, it appears that amiloride prevents sodium entrance into the cells via a sodium channel or carrier in the apical plasma membrane [3]. There is much evidence that, when aldosterone stimulates sodium transport across epithelial tissue, the synthesis of this amiloride-sensitive sodium transporter in the apical membrane is simultaneously increased [6]. Direct evidence in support of this could be obtained if the sodium transporter from the apical membrane were purified.

Purification of the sodium transporter would require an assay. Such an assay could involve the incorporation of the transporter into phospholipid vesicles. A similar reconstitution assay has been essential to the purification of the glucose transporter from the human erythrocyte [7,8]. Before the Na^+ transporter could be incorporated into phospholipid vesicles, it would be highly desirable to determine whether or not the activity of the transporter could be detected in a subcellular fraction of toad bladder. Characterization of such an activity would greatly increase the probability of a successful reconstitution. For these reasons the amiloride-sensitive accumulation of $^{22}Na^+$ by a toad bladder membrane fraction has been examined.

The apical membrane of the toad bladder epithelium has been partially purified by Rodriguez and Edelman [9,10] from the post-nuclear supernatant obtained after bladder homogenization. Such a purification has not been attempted in the current study because it seemed that the amiloride-sensitive Na^+ transport process would be more active in a post-nuclear supernatant fraction that had been subjected to a minimum of manipulation. Cell surface receptors and enzymes are made in the endoplasmic reticulum and in some cases surface proteins (e.g., the asialoglycoprotein receptor of rat liver [11]) exist inside the cell in much higher amounts than on the cell surface. Since the purified plasma membranes might only contain a small fraction of the total cellular sodium transporter, the study of the amiloride-sensitive Na^+ transporter began with a microsomal fraction from toad bladder.

Materials and Methods

Materials

Tropical toads (*Bufo marinus*) of Mexican origin were obtained from Rand McNally, Somerset, WI. 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 the generous gift of Dr. R.J. Hosley of Eli Lilly, Indianapolis, IN. Ouabain, guanidine hydrochloride, Hepes, gramicidin, sulfaguanidine, 2-pyrazine carbox-

amide and Dowex 50X8-100 (H^+ form) were obtained from Sigma Chemical Co. St. Louis, MO. Pyrazine, 2-pyrazine carboxylic acid and 3-amino-2-pyrazine carboxylic acid were obtained from Aldrich Chemical Co., Milwaukee, WI. ^{22}Na, [^{3}H]sucrose, [^{3}H]H_2O, [^{14}C]methoxyinulin, and Aquasol-2 were obtained from New England Nuclear, Boston, MA. Soybean phospholipid (asolectin) came from Associated Concentrates, Woodside, NY.

Methods

Toad bladder membrane vesicles. Toad urinary bladders were removed by dissection and immersed at once in homogenization medium containing sucrose (0.25 M), histidine (30 mM, pH 7.0), Na_2EDTA (1 mM) at 0°C. The bladders were suspended in 9 vols. of homogenization medium, minced with dissection scissors, and then homogenized at 0°C for two, 20 s bursts with a Polytron tissue grinder (PT-10) (setting of 6). The homogenate was centrifuged at 800 × *g* for 10 min to remove connective tissue, nuclei, and undisrupted cells, and the supernatant centrifuged for 10 min at 8000 × *g* to pellet the mitochondria. The post-mitochondrial supernatant was then centrifuged for 30 min at 210 000 × *g* to pellet the microsomes. Both mitochondria and microsomes were resuspended in homogenization medium and washed once by centrifugation. The final mitochondrial and microsomal pellets were suspended in homogenization medium at a protein concentration between 3 and 6 mg/ml. Tissue could be stored 1 week at 0°C without any loss of activity and could also be frozen for longer periods. Protein concentrations were determined by using the method of Lowry et al. [12]. Succinate dehydrogenase activity was measured by using the method of Pennington [13] and cytochrome oxidase activity was measured by using the method of Wharton and Tzagoloff [14]. Phospholipids were extracted from toad bladder microsomes by using the procedure of Bligh and Dyer [19] and the lipid phosphorus was measured by using the method of Ames and Dubin [20].

Measurement of ion transport. Tissue samples were incubated with $^{22}Na^+$, sucrose (0.25 M), P_i (2.0 mM), histidine (2.4 mM, pH 7.0), and various compounds in a total volume of 0.25 ml. Ouabain (0.1 mM) was included to minimize interference by the ($Na^+ + K^+$)-ATPase from the basolateral membrane. Amiloride was added to the incubation mixture as the hydrochloride salt, and NaCl was added to the control incubation mixture (without amiloride) to keep the Cl^- concentration constant. Each incubated sample was placed on a Dowex-50X8 (Tris), 100 mesh column with a void volume of 0.5 ml following the procedure of Gasko et al. [15] and LaBelle and Racker [16]. The vesicles were allowed to enter the column and then eluted with a 0.25 ml aliquot followed by a 0.8 ml aliquot of sucrose (0.25 M). The eluted vesicles were dissolved in 6.5 ml Aquasol-2 scintillation fluid and their radioactivity was determined with a Beckman LS-100 C liquid scintillation spectrometer.

Measurement of intravesicular volume. The intravesicular volume of the toad bladder microsomes was determined by a modification of the procedure of Stock et al. [21]. The microsomes were suspended in homogenization medium containing [^{3}H]H_2O and [^{14}C]methoxyinulin. They were pelleted by centrifugation at 210 000 × *g* for 30 min and the amounts of ^{3}H and ^{14}C in the pellet and supernatant determined. Since inulin has a molecular weight of 5000 it can

be assumed that it cannot penetrate membrane vesicles, while all membranes are freely permeable to H_2O. Therefore, the inulin can be used as a marker for the extravesicular volume of the pellet, while the [^{3}H]H_2O can be used as a marker for the total volume, and the difference between these volumes would be the intravesicular volume. The volume occupied by either isotope in the microsomal pellet was calculated by dividing the amount of each isotope in the pellet by the isotope concentration in the supernatant.

Radioactively labeled sucrose was used in another method to determine the intravesicular volume of the microsomes. The toad bladder was homogenized in the presence of [^{3}H]sucrose (10 μCi), the homogenate centrifuged at 8000 $\times g$ for 10 min, the supernatant diluted with 4 vols. of unlabeled homogenization medium and centrifuged twice at 210 000 $\times g$ for 30 min. The amount of [^{3}H]-sucrose trapped in the microsomal pellet was measured.

Phospholipid vesicles. Phospholipid vesicles were prepared from soybean phospholipid (asolectin) that had been partially purified by extraction with acetone [17]. The lipid was exposed to sonication using a bath-type sonicator from Laboratory Supplies Co., Hicksville, NY (Model GI-225 P1), following the procedure of Racker [18].

Results

Sodium accumulation by a microsomal fraction from toad bladder was inhibited from 40 to 70% by amiloride depending upon the preparation. The rate of Na^+ accumulation decreased markedly during the first 10 min of incubation, but the amiloride-sensitive Na^+ uptake process did not reach completion during 10 min (Fig. 1). Since the amount of Na^+ accumulated by the microsomes sometimes declined if the incubation continued beyond 10 min, Na^+ uptake was not measured after periods substantially longer than 10 min. The amiloride-sensitive Na^+ uptake was directly proportional to the microsomal protein concentration (Fig. 2). The Na^+ accumulation was shown to be a hyperbolic function of the Na^+ concentration by a Lineweaver-Burk plot of the data (Fig. 3). The apparent K_m value for total Na^+ uptake was about 2 mM, while the V value varied from 5 to 15 nmol/min per mg depending on the preparation. Amiloride was shown to be a competitive inhibitor of Na^+ uptake by Lineweaver-Burk plot. In order to measure Na^+ accumulation as a function of increasing Na^+ concentration, it was necessary to increase the concentrations of both Na^+ and P_i simultaneously. When the membrane impermeant anion, Hepes, was used to replace P_i in this experiment, data identical to the data in Fig. 3 were obtained indicating that the increased Na^+ accumulation was a result of the increased Na^+ concentration and not a result of the altered P_i concentration. The effect of increasing the amiloride concentration on Na^+ uptake is shown in Fig. 4. A significant effect was observed at a concentration of 0.12 mM. Cl^- also lowered Na^+ uptake slightly. Since amiloride was added as the hydrochloride salt, the effect of Cl^- itself on the system was carefully measured. The same level of Cl^- was used in the presence of amiloride as in its absence in order to ensure that only the effect of added amiloride was being observed (Figs. 1—3).

It was necessary to determine whether Na^+ accumulation by the microsomes

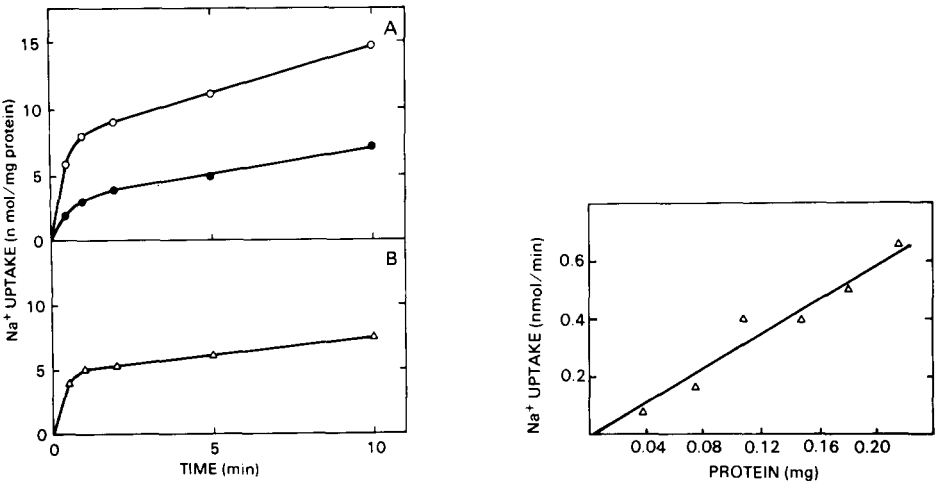


Fig. 1. Effect of time on $^{22}Na^+$ accumulation by toad bladder microsomes. (A) Aliquots of toad bladder microsomes (67 μg protein) were incubated for 10 min at 22°C with sucrose (0.25 M), ouabain (0.1 mM), Na^+ (3.6 mM), Cl^- (0.6 mM) and sufficient P_i (pH 7.4) to serve as a counterion for Na^+ both with (●———●) and without (○———○) amiloride (0.6 mM) in a total volume of 0.25 ml. After the addition of $^{22}Na^+$ (1 μCi) the incubations were continued for the times indicated and 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 difference between the two curves in A (△———△) which is the amiloride-sensitive Na^+ accumulation.

Fig. 2. Effect of protein concentration on $^{22}Na^+$ accumulation by microsomes. Increasing amounts of toad bladder microsomes were incubated for 2 min at 22°C with $^{22}Na^+$ and the compounds indicated in Fig. 1 both with and without amiloride (0.6 mM) and the incubations were terminated as described in the legend of Fig. 1. The difference between Na^+ uptake in the absence of amiloride and Na^+ uptake in its presence is presented above.

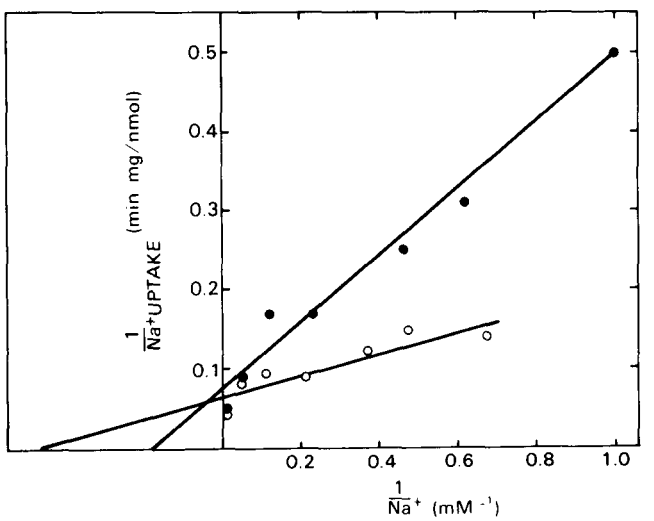


Fig. 3. Effects of Na^+ concentration on $^{22}Na^+$ accumulation by microsomes. Toad bladder microsomes (67 μg protein) were incubated for 2 min at 22°C with ouabain (0.1 mM), Cl^- (0.6 mM), increasing amounts of $^{22}NaP_i$ (1.9 μCi/μmol Na^+), and sufficient sucrose to keep the osmolarity constant, 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 of Fig. 1. A double-reciprocal plot of the data is shown.

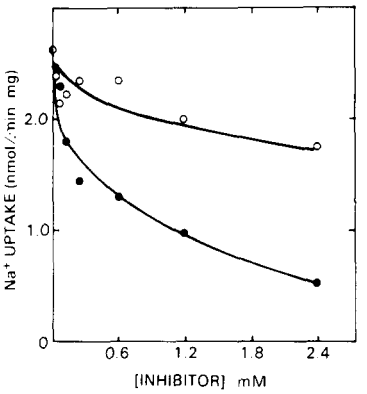


Fig. 4. Effect of increasing concentrations of Cl^- and amiloride hydrochloride on $^{22}Na^+$ accumulation by microsomes. Toad bladder microsomes (60 μg protein) were incubated for 2 min at 22°C with sucrose (0.25 M), ouabain (0.1 mM), $^{22}Na^+$ (1 μCi, 3.1 mM), and increasing amounts of either Cl^- (○———○), or amiloride hydrochloride (●———●) in a total volume of 0.25 ml. The counterion for Na^+ was either P_i (pH 7.4) or Cl^-, and the incubation mixtures were applied to Dowex columns as described in the legend of Fig. 1.

resulted from a binding or a transport process. When bovine serum albumin, boiled toad bladder microsomes, or dialyzed toad bladder cytosol were incubated with $^{22}Na^+$ under conditions identical to conditions employed with the intact microsomes, no $^{22}Na^+$ accumulation was observed. Likewise, soy phospholipid vesicles prepared by sonication could take up only 2% as much $^{22}Na^+$ as could the microsomes and the uptake into lipid vesicles was totally insensitive to amiloride. These data provided indirect evidence that the intact microsomes were indeed responsible for something other than simple nonspecific binding. More direct evidence in support of the theory that the microsomes were actually transporting Na^+ was provided by experiments with the ionophore, nigericin. Toad bladder microsomes preincubated with $^{22}Na^+$, with and without amiloride, and then diluted 6-fold with sodium-free sucrose, with and without nigericin, were applied to Dowex columns. The amiloride-sensitive portion of the sodium accumulation was totally removed from the microsomes by nigericin, while the amiloride-insensitive sodium accumulation was largely insensitive to the ionophore (Table I). Likewise, the nigericin-insensitive Na^+ accumulation was unaffected by amiloride.

The intravesicular volume of the microsomal fraction as determined by the [^{14}C]methoxyinulin/[^{3}H]H_2O method was about 3.0 μl/mg protein (Table II). This volume was shown to be unaffected by either added amiloride or by low levels (0.5—2 mM) of either Cl^- or P_i. This indicated that the slight inhibitory effect of Cl^- on Na^+ transport (Fig. 4) did not stem from the exchange of a non-permeable anion (P_i) by a permeable one (Cl^-). The intravesicular volume occupied by the [^{3}H]sucrose was found to be 1.5 μl/mg protein. Only a negligible amount of [^{3}H]sucrose was shown to bind to the microsomes when the isotope was added after the tissue had been homogenized. If the amiloride-sensitive $^{22}Na^+$ accumulation is assumed to represent transport into vesicles, the intravesicular volume occupied by the transported Na^+ ions can be calculated

TABLE I

NIGERICIN SENSITIVITY OF Na^+ ACCUMULATION BY MICROSOMES

Toad bladder microsomes (97 μg protein) were incubated for 2 min at 22°C with sucrose (0.25 M), ouabain (0.1 mM), $^{22}Na^+$ (0.6 μCi, 3.0 mM), Cl^- (0.6 mM), and sufficient P_i (pH 7.4) to serve as a counter-ion for Na^+, both with and without amiloride as indicated in a total volume of 0.12 ml. Aliquots of the incubation mixtures were either applied directly to Dowex columns as described in Fig. 1, or diluted 1 : 6 with sucrose (0.25 M) with and without nigericin, incubated 2 min further, and then applied to Dowex columns.

	Na^+ accumulated (nmol/mg protein)	
	—amiloride	+amiloride (0.6 mM)
Before dilution	6.10	3.33
After dilution — nigericin	5.26	2.96
After dilution + nigericin (33 μg/ml)	2.28	2.06

from the equation:

$$V_{Na} = \frac{CPM_a \cdot V_T}{CPM_T \cdot P_T}$$

where V_{Na} is the intravesicular volume in μl/mg protein, CPM_a is the amount of amiloride-sensitive $^{22}Na^+$ accumulated by the microsomes, CPM_T the total amount of $^{22}Na^+$ incubated with the microsomes, P_T the amount of microsomal protein in the incubation mixture, and V_T the total volume of the incubation mixture. When this equation was applied to the results of the $^{22}Na^+$ accumulation experiments, V_{Na} was calculated to be about 2.0 μl/mg protein. The phospholipid content of the microsomes was determined to equal about 0.52 mg

TABLE II

DETERMINATION OF INTRAVESICULAR VOLUME OF TOAD BLADDER MICROSOMES

Toad bladder microsomes (0.56 mg protein) were incubated for 5 min at 20°C with or without amiloride (0.(mM) in a total volume of 0.25 ml. They were diluted to 6 ml with homogenization medium containing [^{3}H] H_2O (3.75 μCi) and [^{14}C]methoxyinulin (1.25 μCi). They were centrifuged for 30 min at 210 000 × *g* and th radioactivity in both the supernatant and the pellet was determined. Values indicate the mean of quadruplicat determinations ±S.D. About 41% of the ^{14}C was detected in the ^3H channel and had to be subtracted from th total radioactivity in the ^3H channel to permit the determination of the ^3H present. No radioactivity from th ^3H was detected in the ^{14}C channel. Pellet volumes occupied by the [^{3}H]H_2O and the [^{14}C]methoxyinuli were calculated as described in Materials and Methods. The intravesicular volume was equal to the differenc between the pellet volumes occupied by the two labeled compounds.

	[^{3}H]H_2O		[^{14}C]Methoxyinulin		Intravesicular volume (μl/mg pellet protein)	
	—amiloride	+amiloride	—amiloride	+amiloride	—amiloride	+amiloride
Supernatant (cpm/μl)	487 ± 13	483 ± 10	218 ± 5	216 ± 1		
Pellet						
Total cpm	11 000 ± 160	10 500 ± 350	4 715 ± 185	4 460 ± 230		
Total μl	22.6	21.7	21.6	20.61		
μl/mg pellet protein	67.8	65.1	64.8	61.8	3.0	3.3

TABLE III

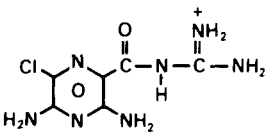
EFFECT OF CERTAIN PYRAZINE AND GUANIDINE DERIVATIVES ON $^{22}Na^+$ ACCUMULATION BY MICROSOMES

Toad bladder microsomes (50 μg protein) were incubated for 2 min at 22°C with sucrose (0.25 M), ouabain (0.1 mM), $^{22}Na^+$ (1 μCi, 4.8 mM), Cl^- (1.4 mM), sufficient P_i (pH 7.4) to serve as a counterion for Na^+ and the compounds below (each 0.6 mM), in a total volume of 0.25 ml. The mixtures were applied to Dowex columns as described in Fig. 1. Results are expressed as nmol/min per mg protein, ±S.D. (n = 3).

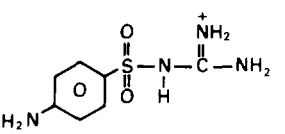
Derivative added	$^{22}Na^+$ accumulation
None	9.22 ± 0.41
Amiloride	4.98 ± 0.27
Sulfaguanidine	9.10 ± 0.53
Guanidine	7.63 ± 0.45
2-Pyrazine carboxamide	7.58 ± 0.18
Pyrazine	8.64 ± 0.39
2-Pyrazine carboxylate	8.45 ± 1.43
3-Amino-2-pyrazine carboxylate	7.40 ± 0.96

phospholipid per mg protein so that the intravesicular volumes could be calculated in terms of μl/mg lipid if necessary. Such volumes would be twice the volumes calculated in μl/mg protein.

The effect of amiloride on Na^+ transport into the microsomes was shown to be specific for the precise structure of a guanidinopyrazine diuretic. The effect of several derivatives of pyrazine and guanidine other than amiloride on Na^+ uptake into microsomes was shown to be very slight (Table III, Fig. 5). Amiloride

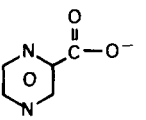


amiloride

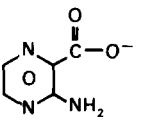


sulfaguanidine

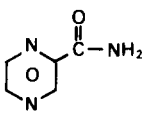
pyrazine



2 pyrazine carboxylic acid



3 amino 2 pyrazine carboxylic acid



2 pyrazine carboxamide

Fig. 5. Structures of pyrazine and guanidine derivatives used in Table III.

TABLE IV

SUBCELLULAR FRACTIONATION OF THE AMILORIDE-SENSITIVE Na^+ TRANSPORTER

Toad bladders were separated into mitochondrial and microsomal fractions and the specific activities of amiloride-sensitive Na^+ transport, succinate dehydrogenase, and cytochrome oxidase were measured as described in Materials and Methods. All values are in nmol/min per mg protein

Subcellular fraction	Amiloride-sensitive Na^+ uptake	Succinate dehydrogenase	Cytochrome oxidase
Mitochondria	2.34	20.6	150
Microsomes	5.91	10.1	75

itself inhibited uptake by 46%, guanidine and 2-pyrazine carboxamide inhibited uptake by about 18% and the other compounds failed to exert a statistically significant effect on Na^+ accumulation.

Since amiloride is positively charged at neutral pH, it was important to determine whether or not it could diminish all cation movements across membranes nonspecifically. When gramicidin was added to soy phospholipid vesicles formed by sonication, $^{22}Na^+$ entry into the vesicles increased 5- to 7-fold and this increased rate of sodium uptake was insensitive to amiloride (0.6 mM).

The total amiloride-sensitive Na^+ transport activity was more than 9 times greater in the microsomal fraction than in the mitochondrial fraction. The specific activity of the transport was nearly 3 times higher in the microsomal fraction while the specific activities of the mitochondrial marker enzymes, succinate dehydrogenase and cytochrome oxidase, were twice as high in the mitochondrial fraction as in the microsomal fraction (Table IV). Amiloride-sensitive Na^+ transport activity could not be measured in either the initial homogenate (before centrifugation) or the nuclear pellet because their high viscosity made it impossible to elute them from the Dowex columns.

Discussion

The rate of amiloride-sensitive Na^+ accumulation by the toad bladder microsomes declined within minutes as the tissue became saturated with the ion. Such a kinetic relationship is consistent with membrane transport. The direct proportionality between Na^+ accumulation and tissue concentration and the hyperbolic relationship between Na^+ accumulation and Na^+ concentration are also consistent with transport. Since the amiloride-sensitive portion of microsomal Na^+ accumulation was totally removed from the microsomes by the ionophore, nigericin, it could be concluded that this accumulation process represented transport into vesicles. The amiloride-insensitive Na^+ accumulation may represent a combination of binding and transport processes.

The presence of vesicles in the bladder microsome preparation was demonstrated in several ways. First, the presence of vesicles was indirectly confirmed by decreasing the osmotic strength of the incubation medium. The extent of $^{22}Na^+$ uptake was stimulated by decreasing the sucrose concentration of the incubation medium. Second, the presence of a vesicular fraction in the bladder microsomes was demonstrated directly by the use of $[^3H]H_2O$ and [^{14}C]methoxyinulin. The intravesicular volume calculated from the difference between

the volumes occupied by the two labeled compounds was 3.0 μl/mg protein. This agrees fairly well with the calculated intravesicular volume occupied by the transported Na^+ (V_{Na}) (2.0 μl/mg protein). Likewise, the volume occupied by [^{3}H]sucrose was about 75% of V_{Na}, which would be expected since complete equilibration of the [^{3}H]sucrose during tissue homogenization is highly unlikely (some membrane fragments enclose homogenization medium, some enclose cytosol, and some do both). Since the agreement between the measured volume of $^{22}Na^+$ and [^{3}H]sucrose was extremely close, it appeared that most of the intact vesicles in the system were capable of Na^+ transport.

Sodium transport into microsomes was largely insensitive to a number of compounds that were structurally similar to amiloride (Table III, Fig. 5). These compounds included sulfaguanidine, pyrazine, guanidine, 2-pyrazine carboxylate, 2-pyrazine carboxamide and 3-amino-2-pyrazine carboxylate. Bentley [3] has shown that pyrazine and sulfaguanidine did not exert any effects on sodium transport in the intact toad bladder, and that guanidine only exerted an effect at a concentration above the effective concentration of amiloride in his system. Sulfaguanidine and amiloride are both positively charged at neutral pH due to the guanidino moieties and highly hydrophobic due to the aromatic rings. Such compounds might be capable of binding to negatively charged membranes and blocking the movement of positive ions nonspecifically. Yet sulfaguanidine had no significant inhibitory effect on Na^+ transport into toad bladder microsomes, indicating that the effect of amiloride most likely reflects the blockade of a specific transport system. This view is strengthened by the fact that amiloride did not block the channel produced by gramicidin in phospholipid vesicles, nor did it block the diffusion of Na^+ into such vesicles in the absence of gramicidin.

Sodium transport across the isolated intact toad bladder is inhibited more than 50% by 1 μM amiloride [3], but an amiloride effect on toad bladder microsomes required at least 120 μM amiloride. This apparent discrepancy might be explained by the fact that the total volume of the Ussing chamber used in the intact bladder studies is 160 times larger than the volume used in the microsomal studies above (0.25 ml) [3,22]. Therefore, the total amount of amiloride used in either study is comparable. Perhaps amiloride is accumulated at the membrane surface so that the total amount of amiloride employed is more important than the concentration. Furthermore, the ratio of transporter present at the cell surface to total transporter present in the cell is unknown. The hypothetical accumulation of amiloride at the membrane surface could not take the form of irreversible binding since amiloride exerted a plainly competitive effect on Na^+ uptake into the microsomes (Fig. 3). Such accumulation might result from a transient attraction between the negatively charged membrane surface and the positively charged amiloride. It is possible that such accumulation does not occur and that the disparity between the K_i value for amiloride in the intact toad bladder and the K_i value demonstrated with microsomal vesicles results from some as yet unexplained phenomenon.

When toad bladder homogenates were separated into two subcellular fractions, the specific activity of the amiloride-sensitive Na^+ transporter was 3 times higher in the microsomal fraction than in the mitochondrial fraction. This distribution was similar to the distribution obtained by Jorgensen [23] during

subcellular fractionation of the kidney ($Na^+ + K^+$)-ATPase. Based on the distribution of succinate dehydrogenase and cytochrome oxidase activity between the mitochondrial and microsomal fractions of the bladder, it appears that the microsomal fraction was contaminated somewhat by mitochondrial fragments, but it seems likely that at least 80% of the amiloride-sensitive Na^+ transport activity was limited to microsomal rather than to mitochondrial vesicles. The discovery of amiloride-sensitive Na^+ transport into toad bladder microsomes provides the first direct assay for this transporter in a subcellular fraction.

Studies are currently in progress to measure $^{22}Na^+$ uptake into fractions of purified toad bladder apical membranes [9,10]. Studies are also in progress to incorporate the transporter into phospholipid vesicles and to use this reconstitution procedure as an assay that will permit purification of the sodium transporter.

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