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Amiloride Blockable Sodium Fluxes in Toad Bladder Membrane Vesicles

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Summary. Recently we reported a simple manual assay for the measurements of isotope fluxes through channels in heterogenous vesicle populations (Garty et al., *J. Biol. Chem.* **258:**13094–13099 (1983)). The present paper describes the application of this method to the assessment of amiloride blockable fluxes in toad bladder microsomes. When $^{22}Na^+$ uptake was monitored in the presence of an opposing Na^+ gradient, a relatively large and transient amiloride-sensitive flux was observed. Such an amiloride-blockable flux could also be induced by a KCl + valinomycin diffusion potential. The effects of the intra- and extravesicular ionic composition on the rate of $^{22}Na^+$ uptake were examined. It was shown that the amiloride-blockable fluxes occur in particles permeable to Na^+ and Li^+ but relatively impermeable to K^+, $Tris^+$ and Cl^-. Analysis of the amiloride dose-response relations revealed a complex "non Michaelis-Menten" behavior. The data could be accounted for by assuming either a strong negative cooperativity in the amiloride-membrane interaction, or two amiloride-sensitive Na^+ conducting pathways with K_i values of 0.06 and 6.4 μM. Both pathways appear to be electrogenic and therefore the possibility of an electroneutral amiloride-blockable Na/H exchange was excluded. Calcium ions could block the amiloride-sensitive flux from the inner but not from the outer phase of the membrane. It is suggested that although a substantial part of the $^{22}Na^+$ flux is inhibited only by a relatively high concentration of amiloride, this uptake represents transport through the apical Na-specific channels. The data also define the optimal experimental conditions for the study of amiloride-sensitive fluxes in toad bladder microsomes.

Key Words amiloride · epithelial transport · Na^+ channels · toad bladder · vesicles

Introduction

Sodium transport across the toad urinary bladder is a two-step process as originally suggested for frog skin [19]. According to this scheme, Na ions diffuse from the lumen into the epithelial cell through Na-specific, amiloride-blockable channels and are later being pumped to the interstitial space by the (Na + K)-ATPase. In recent years it became apparent that the apical Na channels are subject to a number of regulatory processes which control the transepithelial flow of Na^+. For instance, hormones that stimulate Na^+ transport, such as aldosterone and vasopressin, appear to act by increasing the density of electrically detectable channels in the apical membrane [25, 32]. In addition, a number of intracellular regulatory mechanisms were proposed. These mechanisms are: direct feedback inhibition of Na channels by the cellular Na^+ activity [11, 23], inhibition by the cellular Ca^{2+} [3, 8, 14, 38], and Na^+-independent metabolic control of the Na^+ permeability [13].

Study of the molecular events involved in the regulation of the apical Na^+ permeability depends on the ability to measure fluxes through the Na^+ channels under simplified, well defined, and controlled conditions, e.g., in membrane vesicles. However, measurements of fluxes through the Na^+ channels in vesicle preparations are subject to two major difficulties which, in fact, are common to almost any channel containing vesicle preparation. First, the flow rate through a single channel will be in the order of 10^6 ions/sec [26]. Accordingly, the equilibration of a tracer added to a suspension of vesicles will occur within 1 sec [8]. Second, the abundance of the Na^+ channels in the apical membrane is usually less than 1 channel/μm^2 [25, 32]. In addition, any apical membrane preparation will invariably be contaminated by basolateral and intracellular membranes. Thus, only a small fraction of the vesicles is expected to contain Na channels. Indeed, previous attempts to measure amiloride blockable Na^+ fluxes in vesicles derived from toad bladder either failed to demonstrate high sensitivity to amiloride [20, 22] or required a stop-flow apparatus [8].

Recently we described a simple and highly sensitive transport assay, which overcomes the above difficulties [15]. In this assay, uptake of a tracer of the ion of interest is measured against a large chemical gradient of the same ion. As a result of the imposed gradient, a transient electrical diffusion potential is set up across the membrane of those

vesicles that are highly permeable to the ion of interest and are impermeable to the other ions present in the suspension. The isotope tends to equilibrate with this potential and is therefore concentrated selectively and transiently in the vesicles of interest. When measured in this way, the tracer uptake is relatively large, it takes place preferentially in the channel-containing vesicles, and occurs over minutes rather than sub-seconds time range. The efficacy of this assay was demonstrated in a number of systems, among which was a crude microsomal preparation from toad bladder epithelium.

In this paper the characteristics of the $^{22}Na^+$ uptake induced by Na^+ diffusion potential are further explored. It is shown that the amiloride-sensitive flux is an electrogenic process that occurs in particles permeable to Na^+ and Li^+ but impermeable to K^+, $Tris^+$ and Cl^-. Externally added $CaCl_2$ (1 mM) does not affect this uptake but can abolish it if the membrane is made permeable to Ca^{2+} with A23187. It is claimed that although $^{22}Na^+$ uptake by microsomes is less sensitive to amiloride than Na^+ transport in the intact epithelium, this process represents transport through the apical Na^+ specific channels.

Materials and Methods

Animals

Toads (*Bufo marinus*, Mexican origin) were obtained from Lemberger, WI, and kept in tanks with an access to tap water.

Vesicle Preparation

Plasma membrane from toad bladder epithelium was isolated by the method of Palmer and Edelman [7]. Usually two toads were used for each preparation. The animals were double pithed and perfused through the ventricles with 500 ml Ringer's solution. The urinary bladders were removed and immersed in an ice-cold solution, which usually contained (in mM): 87.5, sucrose; 55, NaCl; 10, imidazole or PIPES buffers (pH = 7); and 5, EDTA. This composition is quite similar to the homogenization media used previously [7, 35], except that part of the sucrose was substituted by NaCl in order to trap Na^+ ions inside the vesicles. The epithelial cells were scraped from the connective tissue using a microscope slide, and harvested by centrifugation at 1,000 $\times$ *g* for 5 min. The harvested cells were washed twice by suspension in 30 ml of the above homogenization solution and recentrifugation. Washed cells were suspended in minimal volume (1–3 ml) of the medium and homogenized. The homogenization was performed with a Dounce-type tissue grinder (0.025–0.075 mm clearance) using 20–40 strokes. Unbroken cells and nuclei were sedimented at 1000 $\times$ *g* for 10 min. The pellet was resuspended in 1–3 ml homogenizing medium and the above homogenization and centrifugation were repeated. The supernatants of the two spins were combined and centrifuged at 10,000 $\times$ *g* for 5 min to remove intact mitochondria. The pellet obtained had most of the cytochrome oxidaze activity (a mitochondrial marker) but also contained some plasma membrane, assayed as (Na + K)-ATPase activity and apical membrane bound ^{125}I (H. Garty, *unpublished data*). The loose plasma membrane layer could be separated from the tight mitochondrial pellet by gentle shaking. It was combined with the supernatant and centrifuged for 1 hr at 27,000 $\times$ *g*. Membranes (~0.5 mg protein per hemibladder) were suspended in the homogenization medium to a final concentration of 1 mg/ml, stored at 0°C, and used within 24 hr. In experiments that assessed the effects of Ca^{2+} on Na^+ transport, the epithelial cells were first washed under the usual conditions, then washed again, and processed in EDTA-free medium. In the ion substitution experiments, homogenizing media in which NaCl was replaced by either LiCl or KCl were used. In order to obtain matched NaCl and LiCl or KCl loaded preparations, one hemibladder from each toad was processed in the usual homogenizing medium and the other in the modified medium. Special care was taken that the homogenization and separation steps would be identical in the two preparations to be compared.

In some preparations the plasma membrane was fractionated on a sucrose density gradient. The solutions used contained 20, 40 or 60% sucrose (wt/vol) plus 55 mM NaCl, 10 mM imidazole (pH = 7) and 5 mM EDTA. A step density gradient was formed in 14 ml cellulose nitrate tubes using 3.3 ml of each of the above solutions. One ml of vesicles (~1 mg) was layered on top of the sucrose solution and the tubes were centrifuged at 65,000 $\times$ *g* for 2 hr (Beckman L5-50 ultracentrifuge, rotor SW-41). Two vesicle fractions were collected from the 20–40% and 40–60% interfaces. They were diluted with homogenization medium, centrifuged at 100,000 $\times$ *g* for 1 hr, and resuspended in a minimal volume.

Transport Measurements

$^{22}Na^+$ uptake by the cell membrane vesicles was monitored in the presence of Na^+ diffusion potential as described previously [15]. Dowex 50WX8 (50–100 mesh, Tris form) columns were used both for substituting the external cations by Tris (prior to the transport assay) and for separating intra- and extravesicular $^{22}Na^+$ [16]. These columns were poured in Pasteur pipettes plugged with glass wool. The columns used for establishing the Na^+ gradient were prewashed with 175 mM sucrose (1.5 ml) and maintained at room temperature. The columns used to assay $^{22}Na^+$ uptake were prewashed with 1.5 ml of 175 mM sucrose + 10 mg/ml BSA (pH = 7) and kept at 0°C.

The experimental protocol used for the flux assays was as follows: 100 μl vesicles suspended in homogenization medium, were applied to a Dowex column and eluted with 1 ml sucrose solution (175 mM). This step exchanged all external cations (and in particular Na^+) by Tris, and diluted the vesicles by an isotonic sucrose solution. A small volume of the eluted vesicles was saved for protein assay, and the rest was immediately mixed with a reaction mixture whose composition depended on the experimental design. This reaction mixture contained $^{22}NaCl$ (1–2 μCi, carrier free) plus additional reagents such as: amiloride, Ca^{2+}, A23187, KCl, NaCl, etc. Its osmolarity was adjusted to ~190 mosm/kg with a concentrated sucrose solution. The time course of tracer uptake was measured by removing 100–200 μl aliquots from the suspension, applying them to Dowex columns and eluting them into counting vails with 1.5 ml of an ice-cold sucrose solution (175 mM). The background radioactivity, assayed by sampling a vesicle-free solution, was not higher than 0.01% of

the total radioactivity. In most experiments only the initial rate of $^{22}Na^+$ uptake was measured, i.e., 3–4 aliquots were sampled during the first 3 min after mixing of vesicles with $^{22}Na^+$. The fluxes were calculated by fitting the data points to a straight line. The rates were expressed as picomoles of $^{22}Na^+$ taken up per minute per mg protein (pmol · mg^{-1} · min^{-1}).

Statistics

Data are expressed as mean ± SEM.

Materials

BSA (albumin bovine fraction V), gramicidin D, valinomycin, and PIPES buffer were obtained from Sigma Chemical Co., A23187 from Calbiochem and Dowex 50Wx8 (50–100 mesh) from Fluka. Amiloride.HCl was a gift from Merck Sharp and Dohm Co., and $^{22}NaCl$ (200 μCi/ml, carrier free) was purchased from Amersham Radiochemicals. All conventional chemicals were of analytical grade.

Abbreviations and Symbols

PIPES—piperazine-N,N′-bis[2-ethane-sulfonic acid].
P_X—The membrane permeability to ion X.
X_{in}, X_{out}—Internal and external concentrations of ion X, respectively.

Results

The Time Course of the Amiloride-Sensitive $^{22}Na^+$ Flux

Figure 1A illustrates the time course of $^{22}Na^+$ uptake by toad bladder microsomes in the presence of Na^+ gradient (Na^+_{in} = 55 mM, Na^+_{out} < 10 μM). As described previously [15], the externally added $^{22}Na^+$ is first taken up, and after the vesicles' radioactivity reaches a maximal value at $t \sim 20$ min it decreases slowly. The initial uptake represents accumulation of $^{22}Na^+$ in the vesicles driven by the gradient imposed across their membrane. The later tracer efflux is due to the slow dissipation of the Na gradient (NaCl efflux and Na/Tris exchange). Amiloride (200 μM), added to the suspension at $t = 0$ blocks about $\frac{2}{3}$ of the $^{22}Na^+$ influx and shifts the point of maximal internal radioactivity to longer times. The mean initial uptake rates measured in 12 vesicle preparations were 4.3 ± 0.4 pmol $^{22}Na^+$ · mg^{-1} · min^{-1} and 1.8 ± 0.2 pmol $^{22}Na^+$ · mg^{-1} · min^{-1} in the absence and presence of amiloride, respectively,[1] i.e., 58% of the total rate could be blocked by amiloride. The amiloride-insensitive uptake represents Na^+ transport through other pathways, possibly in a different population of vesicles. Part of it may also come from 'inside-out' vesicles in which the amiloride binding site is not readily accessable to the externally added diuretic (*see* below). In three additional preparations $^{22}Na^+$ fluxes were measured in vesicles loaded with only 10 mM NaCl. In these measurements the mean initial rate of $^{22}Na^+$ uptake was 2.0 ± 0.35 pmol · mg^{-1} · min^{-1} and 74.2 ± 2.4% of this flux could be inhibited by amiloride (200 μM), i.e., lowering the internal Na activity from 55 to 10 mM reduced the $^{22}Na^+$ uptake to less than half of the usual value; however, the amiloride-insensitive flux dropped even further.

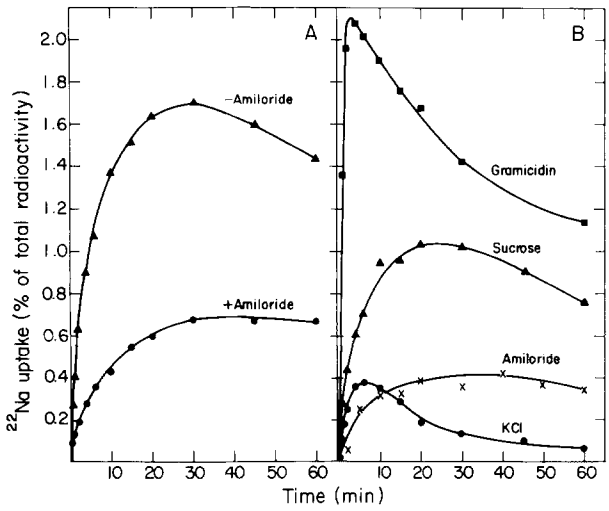

Fig. 1. The time course of $^{22}Na^+$ uptake by toad bladder vesicles. (A): The time course of $^{22}Na^+$ uptake was measured as described under Materials and Methods in the presence (●—●) and absence (▲—▲) of amiloride. Amiloride was added to the reaction mixture to a final concentration of 200 μM from an aqueous 5 mM solution adjusted to pH = 7 by Tris. The other reaction mixture received an equal volume of diluent. (B): $^{22}Na^+$ uptake was measured in the presence of various reagents indicated in the figure, all added to the reaction mixture (i.e., interacted with the vesicles at $t = 0$). The tested reagents were: (■—■) 10^{-8} M gramicidin D added from a stock solution of 10^{-5} M in ethanol, (●—●) 25 mM KCl added from isotonic aqueous solution, (×—×) 20 μM amiloride added from aqueous 500 μM solution adjusted to pH = 7, and (▲—▲) diluent. In each case (except for gramicidin D) the measurement was performed twice, in the presence and absence of 200 μM amiloride. The data presented are the amiloride-blockable fluxes, i.e., the differences between the values measured in the presence and absence of amiloride.

Both the initial rate and the peak value of the $^{22}Na^+$ uptake are determined by the permeabilities and concentrations of all the ions present [15]. The effects of KCl, gramicidin D, and submaximal concentration of amiloride on these parameters are demonstrated in Fig. 1*B*. The curves of this figure (except for gramidicin D) are the amiloride-sensi-

[1] These appear to be minute fluxes. However, it should be noted that the initial rate of Na^+ uptake is proportional to the external Na activity which in these experiments is about 1 μM.

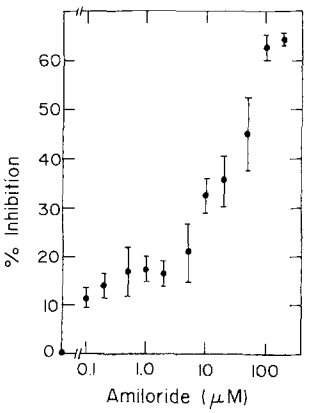

Fig. 2. The amiloride dose-response relations. The initial rate of $^{22}Na^+$ uptake was measured as described under Materials and Methods in the presence of different amiloride concentrations. Amiloride was applied from a 100-fold concentrated stock solution adjusted to pH = 7 with Tris. The data is expressed as % of the flux inhibited as a function of the diuretic concentrations. Mean ± SEM of measurements in six membrane preparations are presented

tive fractions, i.e., the differences between tracer fluxes obtained in the presence and absence of 200 μM amiloride. Substituting part of the sucrose in the suspending medium by KCl (25 mM) evoked a substantial decrease in the rate of $^{22}Na^+$ uptake, accompanied by a shift of the peak internal radioactivity to shorter times (i.e., from 20 to 6 min). On the other hand, similar decrease of the initial rate, induced by 20 μM amiloride, was accompanied by a shift of the peak radioactivity to longer times (i.e., from 20 to 35 min). The differences in the effects of KCl and amiloride on the time course are accounted for by the different effects of these reagents on the parameters determining the kinetic behavior. K^+ ions serve as counter ions to Na^+; the addition of KCl to the suspension will facilitate the rate at which the Na^+ gradient dissipates and will therefore shift the peak radioactivity to shorter times (*cf.* Fig. 5*B* in [15]). The effect of amiloride, on the other hand, can be described either as a decrease of the Na^+ permeability of each vesicle or a decrease of the total volume included in the Na^+ permeable vesicles.[2] Both modifications will decrease the initial rate and shift the peak radioactivity to longer times (*cf.* Fig. 5*A*,*C* in [15]). These data also illustrate the fact that the transient tracer accumulation measured by this procedure can be used to distinguish between effects of an unknown reagent on the driving force and permeability of the ion of interest.

The effect of increasing permeability to Na^+ (or increasing the fraction of Na^+-conducting vesicles) was demonstrated by measuring the time course of $^{22}Na^+$ uptake in the presence of the cationic ionophore gramicidin D. The addition of gramicidin (10^{-8} M) induced more than a fourfold increase of the initial rate and a proportionate decrease of the time at which the internal radioactivity reaches maximal value, as expected from our previous calculations [15]. The data with gramicidin D, a well characterized channel, also confirm the fact that under the experimental conditions employed in this study, the equilibration time of $^{22}Na^+$ through ion channels is slowed down to the minute time range. This, of course, is one of the major advantages of this transport assay.

The Amiloride Dose-Response Relations

At high concentrations amiloride is known to block Na/H exchangers [2, 4] and might also have additional, nonspecific, effects [10]. Therefore, an important requirement for the identification of the observed amiloride-sensitive fluxes as a transport mediated by the apical Na^+ channels is to demonstrate inhibition by sufficiently low concentrations of this diuretic. The concentration dependence of the amiloride-induced inhibition was evaluated by measuring the initial rate of $^{22}Na^+$ uptake in the presence of different amounts of amiloride (0.1–200 μM) added to the suspension at time zero. Figure 2 summarizes the dose-response relations obtained in six membrane preparations. It appears that although certain inhibition is achieved for less than 0.1 μM amiloride, the main effect requires much higher concentrations, and unlike in the intact epithelium [9, 25] the data cannot be fitted to a simple Michaelis-Menten kinetics. Figure 3 shows an Eadie-Hofstee analysis of this data. It was found that all the points corresponding to less than 20% inhibition could be fitted to a straight line with a slope of $-16.7\ \mu M^{-1}$ (i.e., $K_i = 0.06\ \mu M$). The rest of the data strongly deviated from this line, but could be fitted to another straight line with a slope of $-0.156\ \mu M^{-1}$ (i.e., $K_i = 6.4\ \mu M$). This analysis indicates either the presence of two independent amiloride-blockable Na^+ conducting sites or the existence of a strong negative cooperativity in the interaction of this diuretic with the Na^+-transporting protein [41]. One possibility that immediately comes to mind is that the Na uptake with higher affinity to amiloride is mediated by the apical channels while the second phase indicates the operation

[2] From the measured density of Na channels in the apical membrane and the average diameter of the membrane vesicles, a ratio of less than one channel per vesicle was calculated [15]. Accordingly, partial blocking by amiloride can also be described as a decrease in the fractional volume of the Na^+-conducting vesicles.

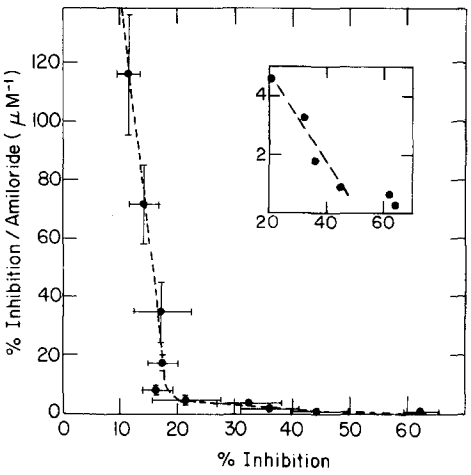

Fig. 3. Analysis of the inhibition by amiloride. An Eadie-Hofstee analysis of the data of Fig. 2 is plotted. The insert shows part of the data points on an enlarged scale

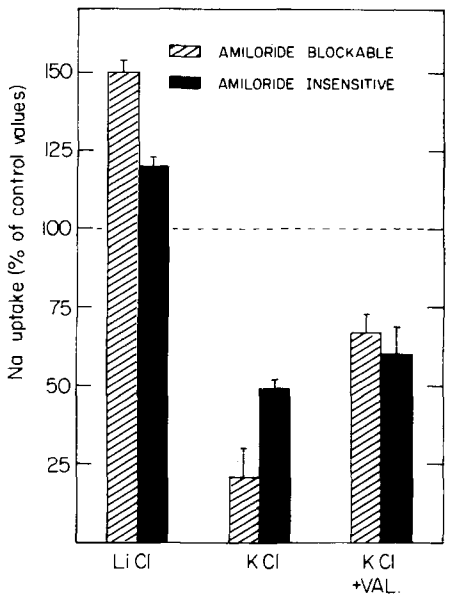

Fig. 4. The effects of internal ion composition on $^{22}Na^+$ uptake. Matched preparations of NaCl and either KCl or LiCl loaded vesicles were obtained as described under Materials and Methods. The initial rate of $^{22}Na^+$ uptake was measured in the presence and absence of amiloride (200 μM), and the data were expressed as % of the values obtained in the corresponding NaCl-loaded preparation (denoted "control values"). Valinomycin was added to a final concentration of 1 μM at time zero. Mean ± SEM of 3 pairs of vesicle preparations are presented

of an Na/H exchanger. Such exchangers are typically characterized by K_i values of 7–30 μM [2]. However, in the experiments summarized below it was shown that both phases of the amiloride dose-response curve shown in Fig. 3 represent an electrogenic $^{22}Na^+$ uptake. Therefore contributions of an electroneutral $^{22}Na^+/H^+$ or $^{22}Na^+/Na^+$ exchanges to the observed flux are unlikely. Other possible explanations to the curved Eadie-Hofstee plot obtained are discussed in the last section.

The Dependence of the $^{22}Na^+$ Fluxes on the Ionic Composition

To evaluate the permeability characteristics of the Na-transporting vesicles and to further explore the nature of the two amiloride-sensitive fractions obtained, the influence of the intra- and extravesicular ionic composition on the $^{22}Na^+$ fluxes was studied. These measurements were taken in the presence and absence of amiloride, and the uptake that could not be inhibited by 200 μM amiloride was defined as the amiloride-insensitive fraction.

In the first set of experiments the ability of LiCl and KCl to substitute for NaCl was tested. Accordingly, vesicles were prepared to contain 55 mM of either LiCl or KCl, and the $^{22}Na^+$ uptake by them was measured and compared to the fluxes in a matched NaCl-loaded preparation. It was found that internal Li^+ can successfully substitute for Na^+ in driving $^{22}Na^+$ uptake, and the observed rates in the LiCl-loaded particles were in fact even higher (Fig. 4). On the other hand, substituting NaCl by KCl abolished most of the amiloride-sensitive uptake, and only a residual flux of 21% of the control value was measured. Interestingly enough, at least part of the amiloride-blockable flux could be recovered if valinomycin (1 μM) was added to the reaction mixture. The amiloride-insensitive portions of the uptake seem to be much less sensitive to the substitution of Na^+ by K^+ or the addition of valinomycin.

The data of Fig. 4 is compatable with the possibility that the observed amiloride blockable $^{22}Na^+$ uptake is an electrogenic process taking place in particles that are permeable to Na^+ and Li^+ but not to K^+. As discussed before [15], the formation of an ion gradient in the initial phase of the measurement imposes an electrical potential difference that will drive the $^{22}Na^+$ uptake. The magnitude of this potential depends on P_X/P_{Cl} (X being the cation trapped in the particles) and will be relatively large for Na^+ and Li^+ but not for K^+. If, however, P_K is increased by the addition of valinomycin, a large enough K^+ diffusion potential can be established as well. The fact that NaCl- and LiCl-loaded vesicles exhibit much larger amiloride blockable fluxes than KCl-loaded particles can also be accounted for by assuming electroneutral $^{22}Na^+/Na^+$ and $^{22}Na^+/Li^+$ exchange processes. However, in this case valinomycin, a specific K^+ carrier, should not influ-

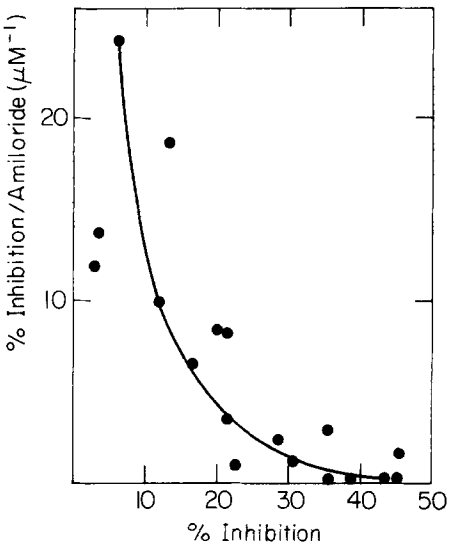

Fig. 5. The amiloride dose-response relations in KCl-loaded vesicles. The initial rate of $^{22}Na^+$ uptake was measured in KCl-loaded vesicles in the presence of valinomycin (1 μM) and different concentrations of amiloride. Data from 3 different membrane preparations are presented

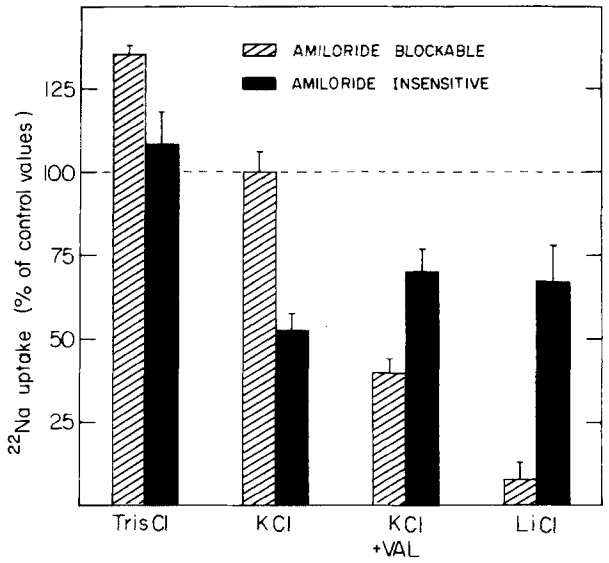

Fig. 6. The effects of the external ionic composition on $^{22}Na^+$ uptake. The initial rate of $^{22}Na^+$ uptake in NaCl-loaded vesicles was measured as described under Materials and Methods in the presence and absence of amiloride and the salts indicated in the figure. All salts were added at time zero to a final concentration of 10 mM. The control sample received an equal volume of isotonic sucrose solution. Uptake rates are presented as % of the control values, and are the mean ± SEM of 4 experiments

ence the tracer uptake. The ability of valinomycin to induce an amiloride-blockable $^{22}Na^+$ uptake is therefore a strong indication of the electrogenic nature of this process.

In light of the above conclusion, it was of interest to examine more closely the sensitivity of the ionophore-dependent flux to amiloride. An amiloride dose-response curve of the valinomycin-induced $^{22}Na^+$ uptake was measured, and the results obtained are given in Fig. 5. It was found that, similarly to the NaCl loaded particles, the data points could not be fitted to a straight line and a strongly curved Eadie-Hofstee plot was observed. The apparent K_i values estimated from the upper and lower parts of this curve are 0.5 and 3.6 μM, respectively. Thus, the substitution of NaCl by KCl + valinomycin did not abolish the flux with lower affinity to amiloride, and its relative contribution to the total amiloride-blockable uptake was quite similar to the one observed in Fig. 3. Therefore this, as yet unexplainable, fraction does not seem to represent an electroneutral $^{22}Na^+/Na^+$ or $^{22}Na^+/H^+$ exchange process.

In the above experiments the extravesicular medium was always composed of about 5 mM Tris · Cl and an isotonic sucrose solution (175 mM). The following experiments, summarized in Fig. 6, examined the effect of externally added K^+, Li^+ and $Tris^+$ on the uptake rates. The application of 10 mM Tris · Cl caused an increase of about 35% in the amiloride blockable flux and had no significant effect on the amiloride-insensitive fraction. KCl (10 mM), on the other hand, had no effect on the initial rate of the amiloride-sensitive $^{22}Na^+$ uptake but substantially reduced the amiloride-insensitive portion. At higher KCl concentration a reduction in the amiloride-blockable flux was observed, too (*cf.* Fig. 1*B*). If valinomycin (1 μM) was added together with KCl, about 60% of the amiloride-blockable uptake was inhibited, but the amiloride-insensitive flux was not significantly affected. LiCl or NaCl (not shown) both induced nearly complete abolishment of the amiloride-blockable uptake but affected the amiloride-insensitive flux to a much lesser degree. The above data can all be explained by the possible effects of these ions on the Na^+ diffusion potential that drives the $^{22}Na^+$ uptake. If P_{Na}/P_{Cl} is about 100, the addition of 10 mM NaCl (or Li) to vesicles that contain 55 mM NaCl and are suspended in sucrose, should lower the membrane potential from −115.6 to −41.6 mV (*cf.* Eq. (1) in [15]). K^+, on the other hand, will not depolarize the membrane if its permeability is much lower than that of Na^+. Such depolarization can be induced by the addition of valinomycin together with KCl. The fact that KCl + valinomycin is less effective than LiCl in inhibiting the amiloride-sensitive $^{22}Na^+$ uptake, may indicate that the relevant vesicles are more permeable to Na^+ (and Li^+) than to K^+ even in the presence of the ionophore. Since the estimated single Na^+ channel current is at least 10^3 times higher than the turn-

over rate of valinomycin, this is not an unlikely possibility. The increased $^{22}Na^+$ fluxes observed upon addition of Tris · Cl could at least partly be due to the decrease in the Cl^- gradient across the membrane. Such a gradient will tend to limit the Na^+ diffusion potential.

In other experiments it was observed that the amiloride-blockable fluxes are very sensitive to the external Na^+ activity, and introducing as little as 0.5 mM NaCl to the external medium was sufficient to lower the uptake rate by about 50%. This extremely high sensitivity to the external Na^+ may signal that P_{Na}/P_{Cl} is larger than 100 (e.g., for $P_{Na}/P_{Cl} = 1000$ the addition of 0.5 mM NaCl to the external solution will reduce the membrane potential from −170 to −116 mV). However, it can also be accounted for by assuming the existence of an essential binding site on the outer surface, with a very high affinity to Na^+.

In another set of experiments the ability of the delayed addition of unlabeled Na^+ to elute $^{22}Na^+$ from the vesicles was examined. Accordingly, vesicles were first incubated for about 20 min with $^{22}Na^+$ under the usual conditions (i.e., $Na^+_{in} \gg Na^+_{out}$). When the rate of $^{22}Na^+$ uptake decreased to nearly zero the suspension was mixed with a small volume of a reaction mixture that contained NaCl (final concentration 5 mM) with or without amiloride (200 μM). The fluxes induced by these additions were monitored during 3 min, and the results obtained are summarized in Table 1. As seen from this table, the addition of NaCl to the vesicle suspension induced a substantial $^{22}Na^+$ efflux that was mostly inhibited by amiloride. On the other hand, the application of amiloride alone caused only a slight increase in the $^{22}Na^+$ efflux above the control value. In a limited number of experiments the NaCl-induced efflux was measured in the presence of different concentrations of amiloride. It was found that under these conditions, too, part of the flux was inhibited by less than micromolar concentrations of the diuretic while the rest was inhibited by a much higher concentration only. The fact that 200 μM amiloride substantially inhibited the tracer efflux rules out the possibility that the decrease in $^{22}Na^+$ uptake induced by the relatively high diuretic concentration results from effects other than reducing P_{Na}, e.g., depolarization of the membrane [10], displacement of adsorbed $^{22}Na^+$, or "detergent-like" effects. In all three cases enhancement of the efflux by amiloride is expected.

Table 1. $^{22}Na^+$ efflux in NaCl loaded vesicles[a]

Additions	Efflux rate (pmol $^{22}Na^+ \cdot mg^{-1} \cdot min^{-1}$)
Diluent	0.47 ± 0.06
Amiloride (200 μM)	0.70 ± 0.10
NaCl (5 mM)	4.8 ± 1.0
NaCl + amiloride	1.55 ± 0.25

[a] NaCl-loaded particles suspended in Na^+-free sucrose solution were incubated with $^{22}Na^+$ for 20 min. At the end of this period the vesicles were mixed with a small volume (<10%) of a reaction mixture whose composition is given in the table. $^{22}Na^+$ effluxes induced by these additions were monitored for 3 min. Mean ± SEM of 3 measurements are presented.

The Orientation of the Channel-Containing Vesicles

The design of many experiments depends on knowing the sideness of the membrane vesicles with respect to the cellular orientation, i.e., are they "right-side-out" or "inside-out" oriented. The fact that most of the initial $^{22}Na^+$ uptake was blocked by externally added amiloride seems to indicate that at least ⅔ of the vesicles are right-side-out oriented. However, it is possible that amiloride can also block Na channels from the cytoplasmic side or diffuse fast enough into the particles. Thus the sensitivity to amiloride itself is not sufficient for the determination of the vesicle orientation. Additional information could, however, be obtained by measuring the effect of Ca ions on the $^{22}Na^+$ uptake.

Many findings suggest that cellular Ca can block Na channels in tight epithelia [3, 7, 14, 27, 38, 40]. Direct inhibition of Na channels by physiological intracellular Ca activities had been recently demonstrated in apical enriched membrane vesicles derived from toad bladder [8]. Since Na^+ transport across the intact bladder is not sensitive to the mucosal Ca activity [39], the interaction between channels and Ca must be restricted to the cytoplasmic phase of the channel. Ca^{2+} is, of course, a highly impermeable ion, and the sideness of its effects in vesicles may be used to determine their orientation. Such effects were evaluated by measuring the initial rate of $^{22}Na^+$ uptake ± amiloride in a solution that contained either 1 mM Ca^{2+} or Ca^{2+} plus the Ca^{2+} ionophore A23187, or the ionophore alone. The data was compared to a control measurement performed in the absence of Ca and A23187.

The presence of 1 mM Ca^{2+} in the vesicle-suspending medium had no significant effect on the amiloride-sensitive Na uptake, but reduced the amiloride insensitive fraction to 65.9 ± 5.2% of the original value (Fig. 7). If 15 μM A23187 were added at time zero, together with the 1 mM $CaCl_2$, the amiloride blockable uptake was almost completely abolished (11.9 ± 4.1%) and the amiloride insensitive uptake was further reduced (to 41.2 ± 3.8%). A23187 alone had no effect on the $^{22}Na^+$ uptake,

Table 2. $^{22}Na^+$ uptake in different membrane fractions[a]

Fraction	$^{22}Na^+$ uptake (pmol $^{22}Na^+ \cdot mg^{-1} \cdot min^{-1}$)	
	Amiloride blockable	Amiloride insensitive
20–40% interface	3.00 ± 0.38	2.2 ± 0.35
40–60% interface	1.90 ± 0.35	1.75 ± 0.28
Mitochondrial fraction (10,000 × *g* pellet)	0.67 ± 0.21	1.60 ± 0.45

[a] The initial rate of $^{22}Na^+$ uptake was measured as described under Materials and Methods. Mean ± SEM of the values obtained in 4 preparations are given.

and the amiloride-blockable and amiloride-insensitive fluxes were 99.1 ± 16.4% and 102.3 ± 8.4% of the control values, respectively. The internal Ca^{2+} activity in this experiment is not defined and will probably be higher than physiological intracellular Ca^{2+} activities. It should, however, be noted that, in spite of the membrane potential imposed, Ca^{2+} will not accumulate in the particles since A23187 supports an electroneutral $Ca^{2+}/2H^+$ exchange [34]. The presence of 10 mM buffer in the vesicles will probably prevent variation of the internal pH as a result of the Ca^{2+}/H^+ exchange.

The data in Fig. 7 shows that the amiloride-sensitive uptake occurs in normally oriented vesicles in which the Ca^{2+} binding site is facing in. About one third of the amiloride-insensitive flux could, however, result from "inside-out" particles which can be blocked by externally added Ca but not by amiloride. This possibility is supported by the finding that trapping amiloride inside the particles somewhat decreases the amiloride-insensitive $^{22}Na^+$ uptake. In these experiments amiloride (200 μM) was included in the homogenization medium and therefore trapped inside the vesicles. Passing them through Dowex column, prior to the addition of $^{22}Na^+$, removed the external, but presumably not the internal, amiloride and therefore its effect on the $^{22}Na^+$ uptake could be assessed.[3] The fraction of $^{22}Na^+$ uptake which could not be blocked by 200 μM amiloride added to these preparations was 31 ± 2.5%, as compared to 43.6 ± 2.1% measured in the absence of internal amiloride. Thus, the data could signal the presence of a small population of "inside

[3] At pH = 7 practically all the amiloride will be in the charged form [4, 6], and therefore it will be exchanged for Tris on the Dowex column. Since the membrane permeability to this diuretic is not extremely high (e.g., [5, 36]), leakage of the internal amiloride prior to the uptake measurement may not be very large. Moreover, the membrane potential established should help to retain it in the vesicles.

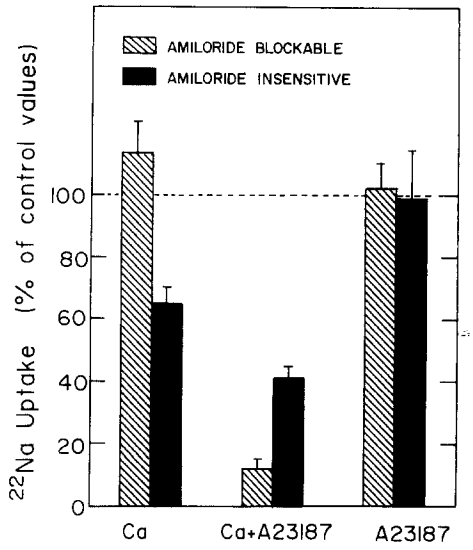

Fig. 7. The influence of Ca^{2+} and A23187 on $^{22}Na^+$ uptake. Uptake rates in NaCl-loaded vesicles were measured as described under Materials and Methods and the legends to the previous figures. $CaCl_2$ and A23187 were added at time zero to a final concentration of 1 mM and 15 μM, respectively. Mean ± SEM of 5 experiments are presented

out" apical vesicle. More important is the observation that trapping amiloride inside the vesicles did not abolish the sensitivity of the $^{22}Na^+$ uptake to externally added amiloride. This finding provides the first indication that the Na channels are not symmetrical with respect to amiloride and cannot be blocked from the cytoplasmic side by this diuretic. This conclusion, however, is based on the assumption that the internally trapped amiloride does not leak out during the first 3 min (*see* footnote 3). Direct measurements with radioactively labeled amiloride are required in order to establish this.

Fractionation of the Toad Bladder Vesicles

The above experiments were all performed using the crude microsomal preparation which contains in addition to the apical membrane nearly all the basolateral membrane. Partial separation between apical and basolateral membranes was achieved by fractionating the vesicles on a sucrose step-density gradient [7, 8]. In this separation procedure two membrane fractions are collected at the 20–40% and 40–60% interfaces. Both fractions were assayed for amiloride blockable $^{22}Na^+$ uptake, and in addition also measured tracer fluxes in the "mitochondria enriched" fraction precipitated at 10,000 × *g*. The mean values of the amiloride-sensitive and amiloride-insensitive fluxes measured in four experiments are given in Table 2. As seen from this table,

the largest amiloride-sensitive $^{22}Na^+$ uptake was measured in vesicles collected from the 20–40% interface. The values obtained for the vesicles collected from the 20–40% interface were lower by about 40%, and much smaller amiloride-sensitive uptake was detected in the mitochondria-enriched fraction. The density distribution of the Na-conducting vesicles in this case is opposite to the one reported previously [8]. Possible reasons for this discrepancy are discussed below.

The amiloride dose-response relations of the 20–40% and 40–60% membrane fractions were also compared. In both cases quite similar plots were obtained (*not shown*), and the above membrane fractionation failed to separate the two amiloride-sensitive processes.

Discussion

This paper describes measurements of $^{22}Na^+$ fluxes in toad bladder vesicles using a method recently reported by us [15]. The advantages of this flux assay over other methods for monitoring ion fluxes through channels in vesicles are its simplicity and high sensitivity. Its main disadvantage is the fact that $^{22}Na^+$ uptake is measured under a specific set of conditions, i.e., very low Na^+_{out}, very high Na^+_{in} and a relatively large membrane potential. These nonphysiological conditions may affect the properties of the studied channels (e.g., their interaction with amiloride). It proved possible, however, to monitor amiloride-blockable $^{22}Na^+$ fluxes under two other sets of conditions, namely K^+ + valinomycin-induced $^{22}Na^+$ uptake and Na^+_{out} driven $^{22}Na^+$ efflux. In the first case the measurement did not require high Na^+_{in}, and in the second case the membrane potential was probably very low and Na^+_{out} could be raised to its physiological activity. Thus, the combination of these three flux assays overcomes the problem of assaying the channel activity under specific nonphysiological conditions.

Ion substitution experiments showed that the amiloride-blockable $^{22}Na^+$ fluxes occur in particles highly permeable to Na^+ and Li^+ but relatively impermeable to K^+ and Cl^-. This selectivity order is typical for the apical, Na channel-containing, membrane, and opposite to the one expected for the basolateral membrane [18, 29]. A K^+ gradient could also drive an amiloride-sensitive $^{22}Na^+$ uptake if valinomycin was added to the suspension. The fact that this flux was not larger than the corresponding Na^+-driven $^{22}Na^+$ uptake argues against the possibility that a self inhibition of channels by Na^+_{in} plays a major role in the transport monitored in vesicles. The amiloride-sensitive $^{22}Na^+$ uptake was also found to be sensitive to the internal, but not external, Ca activity. From this finding it was concluded that the transport takes place in vesicles that are "right-side-out" with respect to the cellular orientation.

The density distribution of the amiloride inhibitable Na^+-transporting vesicles turned out to be opposite to the one reported by Chase and Al-Awqati [8]. This discrepancy can be accounted for in one of two ways:

1) In the previous studies cells were homogenized in a low ionic strength medium [7, 8]. In the present experiments 55 mM NaCl was contained in the homogenizing buffer. It was observed that the higher ionic strength considerably increased the cell resistance to mechanical rupture. Accordingly, more intense homogenization had to be performed in order to obtain high yields of isolated membrane. It is quite possible that these changes also modify the density distribution of the isolated vesicles. Recently we succeeded in incorporating amiloride-blockable Na^+-specific channels into lipid bilayers by fusing the above membrane preparations with a black lipid film (R. Reinhardt, H. Garty and B. Lindemann, *unpublished data*). In these experiments too, the highest frequency of successful incorporation was obtained with the 20–40% fraction.

2) The sedimentation on a sucrose density gradient exposes the vesicles to an osmotic shock, which can irreversibly increase their unspecific permeability. Such an effect will tend to lower the diffusion potential that drives the tracer uptake, and will in particular be large for the fraction that bands at a higher sucrose density (i.e., the 40–60% fraction). Accordingly, the transport rates measured in this vesicle population can be artificially low.

A puzzling observation in this study was the complex amiloride dose-response relations which are not normally found in the intact epithelia (*see*, however, [6]). This finding can be accounted for in one of two ways. (i) Part of the amiloride-sensitive $^{22}Na^+$ flux in vesicles occurs via a pathway that does not contribute to the short-circuit current in the intact bladder. (ii) The isolation of membrane vesicles and/or the assay conditions modify the interaction of amiloride with the Na channels. An amiloride inhibition constant of several micromolars is usually associated with the operation of a Na/H exchanger [2, 4]. Such an electroneutral Na/H exchange in toad bladder microsomes was recently described by LaBelle and Eaton [21]. However, two lines of evidence strongly argue against the possibility that the phase with a K_i of 6.4 μM represents a Na/H antiport. First, all Na/H antiports described so far appear to be electroneutral [2, 4, 21]. The amiloride-sensitive flux in this study is clearly elec-

trogenic. Second, the $^{22}Na^+$ fluxes measured by LaBelle and Valentine were not sensitive to less than 120 μM amiloride [22]. In the present study the inhibition was nearly complete at this concentration. Moreover, studies in intact bladders [1, 28] failed to detect Na/H exchange. It is unlikely that such a hypothetical process will become the major Na transporting pathway in vesicles. Consideration was also given to the possibility that amiloride blocks the electrogenic basolateral Na/Ca exchanger [7]. This possibility can easily be excluded since most of the experiments were performed in a Ca-free EDTA solution, and $^{22}Na^+/Na^+$ exchange by this transporter is, of course, an electroneutral event. In addition, the ion substitution experiments showed that the uptake occurs in apical and not basolateral membrane vesicles. Dubinsky and Frizzell [10] had recently demonstrated that amiloride, at high enough concentrations, can act as an uncoupler and collapse a pH gradient established across brush border membranes. If the charged form of this diuretic is permeable enough it could also depolarize the membrane potential imposed in the present measurements and thus inhibit $^{22}Na^+$ uptake without blocking a Na^+ conductive site. This possible artifact can be eliminated, too. An amiloride-induced depolarization will either enhance or not affect the Na^+_{out}-driven $^{22}Na^+$ efflux, quite opposite to the strong inhibition observed. In addition, as discussed with relation to Fig. 1*B*, the shift of the maximal radioactivity time induced by submaximal doses of amiloride is typical of the situation with decreased P_{Na} and opposite to the expected change in the case of increased permeability (or concentration) of counter ions. The inhibition of $^{22}Na^+$ efflux by amiloride also rules out other possible artifacts, e.g., displacement of $^{22}Na^+$ adsorbed to the particles by this diuretic, or a "detergent like" effect. Thus, considering the fact that the process characterized by a lower affinity to amiloride is electrogenic, takes place in the apical membrane and is inhibited by the cytoplasmic Ca^{2+}, it seems logical to postulate that it either represents a class of modified Na^+ channels or a modification in the amiloride-channel interaction, rather than a different, as yet unknown pathway.

Accumulating data suggest that the inhibition of Na^+ current involves the binding of amiloride to more than one site of the membrane [4, 33] and that its binding is voltage dependent [17, 31]. Such effects are expected to produce a negative cooperativity in the amiloride-channel interaction and might be responsible for the strongly curved Eadie-Hofstee plots in Figs. 3 and 5 [41]. Yet, since the deviation from the normal Michaelis-Menten kinetics and the voltage effects in the intact epithelium are very small, one would have to assume an amplification of these interactions in vesicles, e.g., some uncoupling between the amiloride receptor and the Na^+-conducting site during the isolation of the membrane vesicles.

Shum and Fanelli [37] developed a method for 'eliminating' the basolateral membrane in frog skin and measuring serosa-to-mucosa passive Na^+ fluxes. These fluxes, measured in the presence of high serosal (and cellular) Na^+ activity with no Na^+ in the mucosal compartment, were found to be much less sensitive to amiloride than the fluxes measured for the same skin in the opposite direction. The similarity in the conditions used by them and those employed in this study raises the possibility that the decreased sensitivity to amiloride stems from the very high Na^+_{in} and/or the nearly zero Na^+_{out}. The experiments with K^+ + valinomycin and the efflux measurements argue against the hypothesis that a combination of high Na^+_{in} and zero Na^+_{out} is required to reduce the sensitivity to amiloride. Yet, the possibility that one of these conditions is sufficient in order to increase the K_i value of amiloride cannot be excluded on the basis of the above data.

Another interesting hypothesis, which will have to be evaluated in the future, is that the fraction with lower sensitivity to amiloride represents a population of channels which in the intact bladder are either in cytoplasmic vesicles [24, 30], or in the apical membrane but in a nonconductive state [12]. Thus, although the reason for the reduced sensitivity of the $^{22}Na^+$ fluxes in vesicles to amiloride is not yet understood, this is a potentially interesting phenomenon which may reflect the presence of a population of modified channels involved in the regulation of the apical Na^+ permeability.

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