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Inhibition of Na^+-Ca^{2+} Exchange in Rat Brain by Amiloride

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SUMMARY

Amiloride (*N*-amidino-3,5-diamino-6-chloropyrazine carboxamide) reversibly inhibits Na^+-dependent $^{45}Ca^{2+}$ uptake (Na^+-Ca^{2+} exchange) by plasmalemma-enriched vesicles prepared from microsomes of rat cerebral cortex and by vesicles from osmotically shocked synaptosomes. The drug inhibits Na^+-dependent Ca^{2+} uptake in a competitive manner with a K_I of 0.25–0.34 mM. Na^+-dependent $^{45}Ca^{2+}$ efflux from vesicles is also inhibited by extravesicular amiloride. The drug does not appear to affect nonmitochondrial ATP-dependent Ca^{2+} transport in these vesicle preparations. Membranes containing Na^+-Ca^{2+} carrier can be solubilized in Na^+-cholate and reconstituted into phospholipid vesicles. Na^+-dependent Ca^{2+} uptake by the vesicles is inhibited by amiloride.

INTRODUCTION

Amiloride, a potassium-sparing diuretic, inhibits Na^+ transport across frog skin (1), toad bladder (2), and other Na^+-transporting epithelia (3, 4). In these tissues, Na^+ presumably first enters the cell at the mucosal surface via a facilitative diffusion Na^+ carrier or channel. The intracellular Na^+ is then pumped out the serosal side of the cell by the Na^+,K^+-ATPase. Amiloride inhibits transepithelial Na^+ transport only when present at the mucosal surface of the cell layer, and the drug has no effect when present at the serosal surface (1, 2). This evidence and other data (5, 6) indicate that the drug acts as a diuretic by binding to (7) and inhibiting the mucosal facilitative diffusion Na^+ transport system.

In addition to the diuretic activity of amiloride, the drug affects ion transport in red blood cells (8), fibroblasts (9), murine erythroleukemia cells (10), and cultured hepatocytes (11). Amiloride also may inhibit Na^+transport in cardiac tissue. For example, in perfused dog heart, Yamashita *et al.* (12) demonstrated that amiloride lengthened the refractory period and increased conduction time in the atrioventricular node, had a negative chronotropic effect on the sinoatrial node, and prolonged the refractory period and had a positive inotropic effect on Purkinje fibers (see also ref. 13). Amiloride also appears to delay the onset of arrhythmias and cardiac arrest induced by digitalis (14, 15). These findings may reflect an amiloride effect on Na^+ transport and consequently an effect on the Na^+ gradient across the heart cell membrane. Since Ca^{2+} efflux in heart is thought to be coupled to the inward-directed Na^+ gradient (Na^+-Ca^{2+} exchange transport), amiloride may indirectly alter intracellular Ca^{2+} levels and thus alter the Ca^{2+}-mediated contractility properties of the heart (13). Recently Villereal (9), working with human fibroblasts, demonstrated that intracellular Ca^{2+} stimulates an amiloride-sensitive Na^+ influx pathway. Smith *et al.* (10) have extended this observation in murine erythroleukemia cells and presented evidence that amiloride directly inhibits the Na^+-Ca^{2+} exchange transport system. The drug is thought to inhibit the exchange system at the inner surface of the cell membrane. Thus, in these cells and in other tissues (including heart), amiloride may directly alter intracellular Ca^{2+} levels by inhibiting the Na^+-Ca^{2+} exchange transport system rather than by exerting an indirect effect on other Na^+ transport mechanisms.

Cytoplasmic Ca^{2+} levels in excitable cells are regulated in part by two plasmalemma Ca^{2+} efflux transport systems: a Na^+-Ca^{2+} exchange system and an ATP-dependent transport system (16–18). In this paper, we examine the effects of amiloride on these two systems in rat cerebral cortex, using membrane vesicles prepared from microsomal fractions (18) and from lysed synaptosomes (19). The data demonstrate that amiloride inhibits the Na^+-dependent Ca^{2+} transport system but not the ATP-dependent mechanism.

EXPERIMENTAL PROCEDURES

Preparation of membranes. Microsomal membranes containing Na^+-Ca^{2+} exchange activity were prepared from the cerebral cortex of adult male rats. Crude microsomes obtained by differential centrifugation were purified by discontinuous sucrose density gradient centrifugation as previously described (18). When compared with the crude homogenate, the final preparation was enriched 10-fold in Na^+-Ca^{2+} exchange activity, 2-fold in (Na^+-K^+)-ATPase activity, 2.2-fold in 5′-nucleotidase activity, and 9.4-fold in antimycin A-resistant NADPH-dependent cytochrome *c* reductase activity (18). The resulting membranes were judged to be free of mitochondrial contamination by the absence of both succinate dehydrogenase and oligomycin-dinitrophenol-azide sensitive ATP-dependent Ca^{2+} uptake (18). Membranes in

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160 mM NaCl/20 mM Tris-HCl (pH 7.4) could be stored at −70° for several months without loss of activity.

Synaptosomes were prepared by a modification (20) of the procedure of Gray and Whittaker (21). Membrane vesicles from lysed synaptosomes were prepared as described by Gill *et al.* (19).

Preparation of lipid for reconstitution. Aliquots of the stock lipid solution (100 mg/ml in $CHCl_3$) were dried under a stream of N_2 at 25° followed by two cycles of resuspension in freshly distilled ether with subsequent drying under N_2. The lipid was resuspended in 1-ml aliquots (40 mg/ml) in 200 mM sodium oxalate/20 mM Tris-HCl (pH 7.4). The mixture was clarified by sonication under N_2 at 25°, using a bath type sonicator (Laboratory Supplies Company, Hicksville, N. Y.).

Solubilization and reconstitution. Microsomal membranes were solubilized, and the Na^+-Ca^{2+} exchange carrier was reconstituted into phospholipid vesicles as previously described (22) by a modification of the method of Miyamoto and Racker (23). The Na^+-Ca^{2+} carrier was solubilized for 1 hr (0°) in media containing membranes (2 mg/ml), sonicated soybean phospholipid (20 mg/ml), 20 mM Tris-HCl (pH 7.4), 100 mM sodium oxalate, 2.5% sodium cholate, and 0.5 M NaCl. Insoluble protein was removed by centrifugation at 4° for 1 hr at 140,000 × *g* (Type 50 rotor, Beckman). The resulting supernatant was diluted 7-fold in 200 mM sodium oxalate/20 mM Tris-HCl (pH 7.4) and incubated overnight at 4°. The reconstituted vesicles were collected by centrifugation at 140,000 × *g* for 2 hr at 4°.

Ca^{2+} uptake assays. The microsomal membrane vesicles and the reconstituted phospholipid vesicles were assayed for Na^+-dependent Ca^{2+} uptake essentially as previously described (18). The membranes were prepared for uptake experiments by overnight incubation at 4° in 160 mM NaCl/20 mM Tris-HCl (pH 7.4) to allow Na^+ to equilibrate across the microsomal membranes. Unless indicated otherwise, uptake assays routinely contained 20 mM Tris-HCl (pH 7.4), 160 mM KCl or NaCl, and the indicated concentrations of $^{45}Ca^{2+}$ (0.26 mCi/nmole) in a final volume of 150 μl. The reaction mixture and membrane vesicles were incubated separately at the final assay temperature (23°) for 5 min prior to initiation of the assay. Ca^{2+} uptake was started by dilution of the membranes 30-fold (≃20 μg of protein per assay) into the reaction media. Uptake was terminated by the addition of 5 mM EDTA followed by rapid filtration through a nitrocellulose filter (Schleicher and Schuell, 0.45 μM). The filter was washed 3 times with 3-ml aliquots of 160 mM KCl/20 mM Tris-HCl (pH 7.4).

ATP-dependent Ca^{2+} uptake was assayed in 160 mM KCl/20 mM Tris-HCl (pH 7.4), 2.5 mM $MgCl_2$, 0.01 mM $^{45}Ca^{2+}$, 0.1 mM ouabain, 0.2 mM dinitrophenol, 0.2 mM NaN_3, oligomycin (0.15 μg/ml), and either 4 mM Tris·ATP or 4 mM Tris·ADP, or no added nucleotide. The reaction was initiated by the addition of vesicles and terminated as described above. The $^{45}Ca^{2+}$ trapped by the filters was determined by standard scintillation counting techniques, as previously described (18).

The effect of membrane vesicles, reconstituted vesicles, Mg^{2+}·ATP, and amiloride on the ionized Ca^{2+} concentration under the transport assay conditions described above was checked by using a Ca^{2+}-selective electrode coupled to a pH meter (Corning 130). The electrode was calibrated in both NaCl and KCl reaction media using solutions of known Ca^{2+} concentration. Under the assay conditions used, Mg^{2+}·ATP and amiloride did not significantly alter free Ca^{2+} levels. The concentration of vesicles used in uptake assays (0.05–0.10 mg/ml) resulted in less than a 5% reduction in free Ca^{2+} due to binding to the external surface of the membranes, binding to other components of the assay media, and uptake of Ca^{2+} into the lumen of the vesicles. Endogenous Ca^{2+} contributed less than 0.5 μM to the final Ca^{2+} concentration in these assays.

Protein determination. Protein was determined by the method of Lowry *et al.* (24) as modified by others. Bovine serum albumin was used as a standard.

Materials. Asolectin was purchased from Associated Concentrates (Woodside, N. Y.). Stock solutions of the lipid (100 mg/ml in redistilled $CHCl_3$) were stored at −70° under N_2 and used within 1–2 months. Amiloride was the gift of Merck Sharp & Dohme (Rahway, N. J.). The amiloride was checked for contaminating Ca^{2+} by atomic absorption spectroscopy using a Perkin-Elmer Model 290 spectrophotometer. The drug did not contain detectable levels of Ca^{2+} (<0.5 μM Ca^{2+} in a 3 mM solution). The Ca^{2+}-selective electrode was from W. Simon.

RESULTS

Amiloride inhibition of Na^+-dependent Ca^{2+} uptake. Figures 1 and 2 illustrate the time course of Na^+-dependent Ca^{2+} uptake by microsomal vesicles (Fig. 1) and resealed vesicles from lysed synaptosomes (Fig. 2). The microsomal vesicles are enriched in Na^+-Ca^{2+} exchange activity, Na^+,K^+-ATPase, and 5′-nucleotidase activity and contain a non-mitochondrial ATP-dependent Ca^{2+} uptake system. These membranes are free of contamination by mitochondria and mitochondrial fragments (18). The vesicles derived from lysed synaptosomes are thought to be resealed fragments from the synaptic plasma membrane (see Discussion, ref. 19). This vesicle preparation contains some mitochondrial contamination. Both the microsomal vesicles (18) and the membranes from lysed synaptosomes (25) contain endoplasmic reticulum fragments.

When both types of membrane vesicles are loaded with Na^+ by preincubation and diluted into Na^+-free media (an outwardly directed Na^+ gradient), Ca^{2+} is rapidly taken up by a time-dependent process which plateaus after 10–20 min (Fig. 1A–C and Fig. 2). The same vesicles diluted into Na^+-containing media (no Na^+ gradient) do not accumulate Ca^{2+} (Figs. 1D and 2). Previous work from our laboratory (18) and by Gill *et al.* (19) has shown that the Ca^{2+} accumulated by both types of vesicles can be released by the Ca^{2+} ionophore, A23187, but not by EGTA[1] (see also Fig. 3). Thus the Ca^{2+} associated with the membranes is transported into the lumen of the vesicles. Furthermore, uptake is time- and temperature-dependent, and is saturable with respect to the external Ca^{2+} concentration (18, 19).

Amiloride inhibits Na^+-dependent Ca^{2+} uptake (Figs. 1A–C and 2) without affecting the Ca^{2+} associated with the vesicles in the absence of a Na^+ gradient (Figs. 1D and 2). The drug inhibits both the initial rate of uptake (measured after 10 sec) and the amount of Ca^{2+} associated with the vesicles after apparent equilibrium has been reached (Fig. 1). Na^+-dependent Ca^{2+} uptake by the microsomal vesicles is not dependent on the extravesicular cation present; uptake occurs in media containing K^+, choline$^+$, or Li^+. Likewise, amiloride inhibition does not depend on which cation is present. As previously described (18, 19), Ca^{2+} uptake is reduced when Li^+ is the predominant extravesicular cation.

The data presented in Figs. 1 and 2 suggest that amiloride inhibits Na^+-Ca^{2+} exchange directly, possibly by interacting with the carrier. However, several other interpretations of these experiments are possible. First, amiloride could complex with extravesicular Ca^{2+}, thereby reducing the concentration of the cation available to the carrier, and cause a reduction in Ca^{2+} uptake. This explanation does not seem possible since amiloride (pK_a = 8.80) is predominantly positively charged at neutral pH (26). However, the effect of amiloride on ionized Ca^{2+} was examined using a Ca^{2+}-specific electrode. The

[1] The abbreviation used is: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′, N′*-tetraacetic acid.

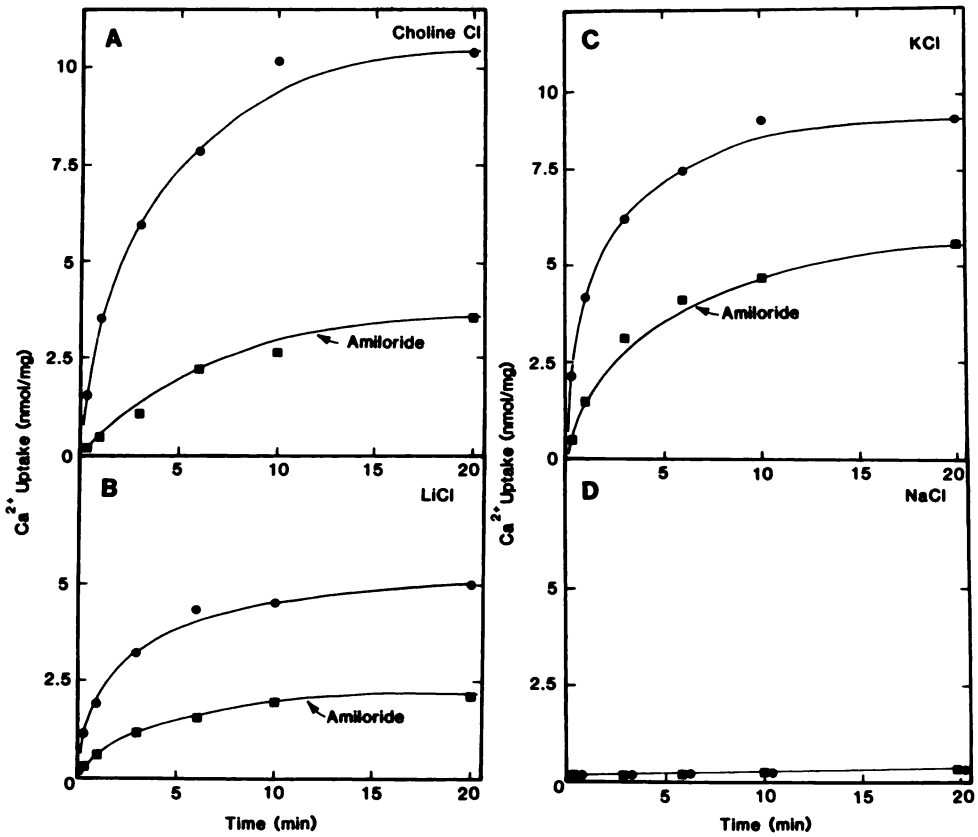

FIG. 1. *Time course of Na^+-dependent Ca^{2+} uptake by microsomal membrane vesicles*

Ca^{2+} uptake assays were initiated by diluting Na^+-loaded microsomes into medium containing 10 μM $^{45}Ca^{2+}$, 20 mM Tris-HCl (pH 7.4), and either 160 mM choline chloride (A), 160 mM LiCl (B), 160 mM KCl (C) or 160 mM NaCl (D). Uptake was terminated by the addition of EGTA (5 mM final concentration), and vesicular $^{45}Ca^{2+}$ was determined as described under Experimental Procedures. Uptake was measured in the presence (■) or absence (●) of 2 mM amiloride. The data points shown are the averages of three determinations. The standard deviation for triplicate determinations varied from ±1.2% to ±14%, and the average for the above points was ±4.6%.

drug, at a concentration of 3 mM, did not alter the ionized Ca^{2+} concentration when Ca^{2+} was varied between 10 and 100 μM. Identical results were obtained in Na^+-Ca^{2+} exchange assay media containing either 160 mM NaCl or 160 mM KCl. A second possible explanation of the effect of amiloride on Na^+-Ca^{2+} exchange is that the amiloride contains trace amounts of $^{40}Ca^{2+}$ which dilute out the specific activity of the $^{45}Ca^{2+}$ present in the uptake assay. However, as determined by atomic absorption spectroscopy, the amiloride preparation used did not contain measurable amounts of Ca^{2+}(or Na^+), and a 2.5 mM solution could only result in $^{40}Ca^{2+}$ levels of 0.5 μM or less. Finally, amiloride conceivably could have no effect on Na^+-dependent Ca^{2+} uptake but rather increase the leakiness of the membranes to Ca^{2+}. If this were the case, Ca^{2+} accumulated by Na^+-dependent uptake would leak out of the vesicles down its chemical gradient faster in the presence of amiloride than in the absence of the drug. This explanation does not appear likely, since the greatest effect of amiloride occurred when uptake was assayed over a very short period of time (initial rates). Under initial rate conditions, the amount of Ca^{2+} accumulated would be minimal, and the effect of an increase in Ca^{2+} efflux would also be small. Furthermore, the effect of amiloride on the permeability of membrane vesicles was examined directly (Fig. 3). Vesicles were allowed to accumulate Ca^{2+} by Na^+-dependent Ca^{2+} uptake for 20 min, until apparent equilibrium was reached. EGTA was added to stop uptake, and the effect of amiloride on accumulated Ca^{2+} was determined over 10 min. As seen in Fig. 3, amiloride did not result in an appreciable increase in Ca^{2+} efflux and did not affect the noncarrier Ca^{2+} permeability of the membrane. The above data demonstrate that amiloride directly inhibits the Na^+-dependent Ca^{2+} transport mechanism present in these membranes.

ATP-dependent Ca^{2+} uptake. In both the microsomal membrane preparation and in vesicles prepared from lysed synaptosomes, the Na^+-Ca^{2+} exchange system and an ATP-dependent Ca^{2+} transport system occupy the same membrane vesicles (18, 19). The effect of amiloride on ATP-dependent uptake was examined using lysed synaptosomal vesicles. In the absence of a Na^+ gradient, Ca^{2+} is accumulated in the presence of Mg^{2+}·ATP. Little uptake occurs in the absence of added nucleotide (Fig. 4) or in the presence of Mg^{2+}·ADP (data not shown). The mitochondrial poisons dinitrophenol, azide, and oligomycin were included in these assays to prevent ATP-driven Ca^{2+} uptake by contaminating mitochondria or mitochondrial fragments. Amiloride (2 mM) did not affect ATP-dependent uptake. In these experiments, even though the vesicle preparations are most likely a mixed population, only inside-out vesicles have the ATP-hydrolyzing site accessible to ATP, and only the ATP-depend-

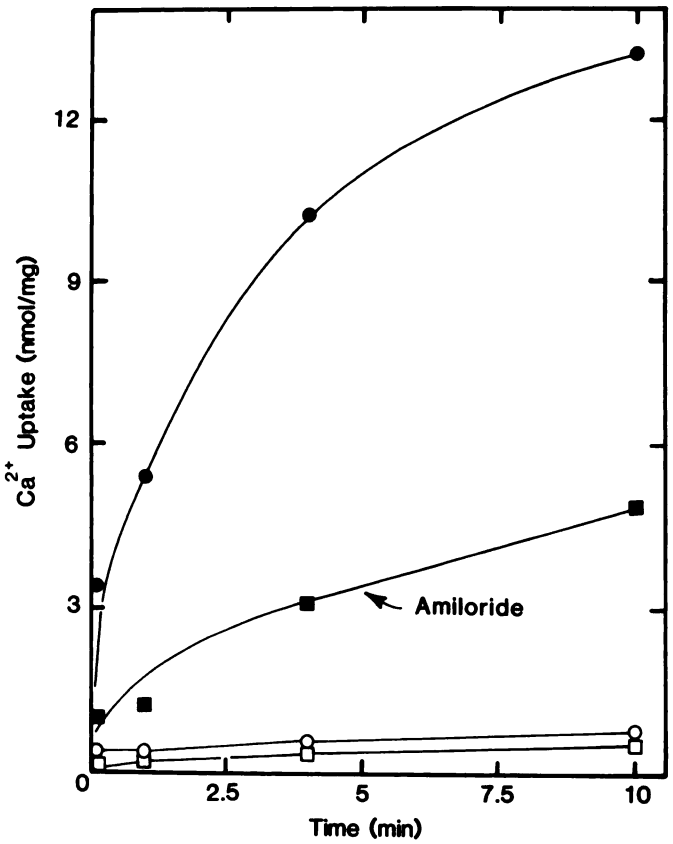

FIG. 2. *Amiloride inhibition of Ca^{2+} uptake by membrane vesicles derived from lysed synaptosomes*

Assays were performed as described in Fig. 1. The uptake medium contained 10 μM $^{45}Ca^{2+}$, 20 mM Tris-HCl (pH 7.4), and either 160 mM KCl (●), 160 mM KCl and 2.5 mM amiloride (■), 160 mM NaCl (○), or 160 mM NaCl and 2.5 mM amiloride (□). The standard deviation for triplicate determinations varied from ±2.0% to ±17%, and the average for the above points was ±7.6%.

ent transport complexes present in inside-out membranes function in Ca^{2+} pumping. At present it is not known whether amiloride penetrates the vesicles over the time period of this experiment (10 min). If the drug does not penetrate the vesicles, the data in Fig. 4 demonstrate that amiloride does not affect ATP-dependent Ca^{2+} uptake at the intracellular membrane surface, leaving the possibility that the drug could affect this ATP-dependent system at the extracellular surface. If amiloride does rapidly equilibrate across the membrane, then amiloride has no effect on ATP-dependent uptake at either surface of the membrane.

Amiloride inhibition of Na^+-dependent Ca^{2+} efflux. The Na^+-Ca^{2+} transport system is capable of transporting Ca^{2+} across a membrane in either direction, depending on the respective Na^+ and Ca^{2+} gradients present (17, 18). As a result of this property, when vesicles containing this transport system are loaded with Ca^{2+} by Na^+-dependent uptake and the outwardly directed Na^+ gradient is reduced by the addition of extravesicular Na^+, a portion of the accumulated Ca^{2+} is released (Na^+-dependent Ca^{2+} efflux). The effect of amiloride on this efflux process was examined in vesicles from lysed synaptosomes and microsomal vesicles (Fig. 3A and B). The membranes were loaded with Ca^{2+} by Na^+-Ca^{2+} exchange until apparent equilibrium was reached (20 min). Uptake was terminated by the addition of EGTA, which reduces the extravesicular Ca^{2+} effectively to zero. Efflux was initiated by the addition of Na^+ (36 mM). As shown in Fig. 3, Na^+ induced a rapid release of accumulated Ca^{2+}, whereas Ca^{2+} efflux did not occur when only EGTA was added. Amiloride inhibited the initial rate of Na^+-dependent Ca^{2+} efflux and increased the amount of Ca^{2+} retained by the vesicles after the new apparent equilibrium was reached.

In the above experiments (Figs. 1–3), the vesicles used are most likely a mixed population of inside-out and right side-out vesicles, and both types of membranes probably contribute to the observed Ca^{2+} efflux. Na^+-dependent Ca^{2+} efflux was also examined under conditions where only inside-out vesicles were loaded with Ca^{2+}. Synaptosomal vesicles were loaded with $^{45}Ca^{2+}$ by ATP-dependent uptake and allowed to accumulate $^{45}Ca^{2+}$ for 20 min in the presence of Mg^{2+}·ATP and in the absence of Na^+. Under these conditions, only inside-out vesicles were loaded with Ca^{2+}. Ca^{2+} uptake was terminated by the addition of EGTA, and Ca^{2+} efflux was induced by the addition of extravesicular Na^+ (Fig. 5). In the absence of the drug, Na^+ induced a rapid efflux of Ca^{2+}, whereas in the presence of amiloride, efflux was inhibited.

Concentration dependence of amiloride inhibition of Na^+-dependent Ca^{2+} uptake. Figure 6 illustrates the inhibitory effect of various concentrations of amiloride on the initial rate of Na^+-dependent Ca^{2+} uptake for both types of vesicles. Inhibition was examined at 10 μM and 50 μM extravesicular Ca^{2+}. The $K_{m_{Ca^{2+}}}$ values for Na^+-dependent Ca^{2+} uptake by microsomes and synaptosomal vesicles are 23 μM (18) and 40 μM (19), respectively. Maximal inhibition required amiloride concentrations of 1 mM or greater. At the higher Ca^{2+} concentration examined, the effectiveness of amiloride as an inhibitor was reduced. When the data were analyzed by the method of Dixon (27), plots characteristic of competitive inhibition resulted (Fig. 6). K_I values of 0.34 mM and 0.25 mM were obtained with microsomal vesicles and synaptosomal vesicles, respectively. The linearity of the Dixon transformations suggests that amiloride inhibits Ca^{2+} transport at a single class of sites. At 10 μM $^{45}Ca^{2+}$ and 2.5 mM amiloride, Na^+-dependent Ca^{2+} uptake was inhibited by 82% and 86% in microsomal vesicles and lysed synaptosomal vesicles, respectively.

Reversibility of amiloride inhibition. In toad bladder preparations (3), amiloride inhibits Na^+ transport in a reversible manner. When tissue incubated with amiloride is washed free of the drug, Na^+ transport is restored. The reversibility of amiloride inhibition of Na^+-Ca^{2+} exchange was examined using microsomal (Table 1) and syaptosomally derived vesicles (data not shown). Membranes were incubated in the presence of 2 mM amiloride or in the absence of the drug (control samples) for 10 min at 23°. Samples were removed from the preincubation media by centrifugation, washed, and assayed for amiloride-sensitive Na^+-dependent Ca^{2+} uptake. Vesicles preincubated in amiloride retained amiloride-sensitive Ca^{2+} uptake and the specific activities of the pretreated membranes were comparable to the activities of the control samples. Thus, amiloride acts reversibly.

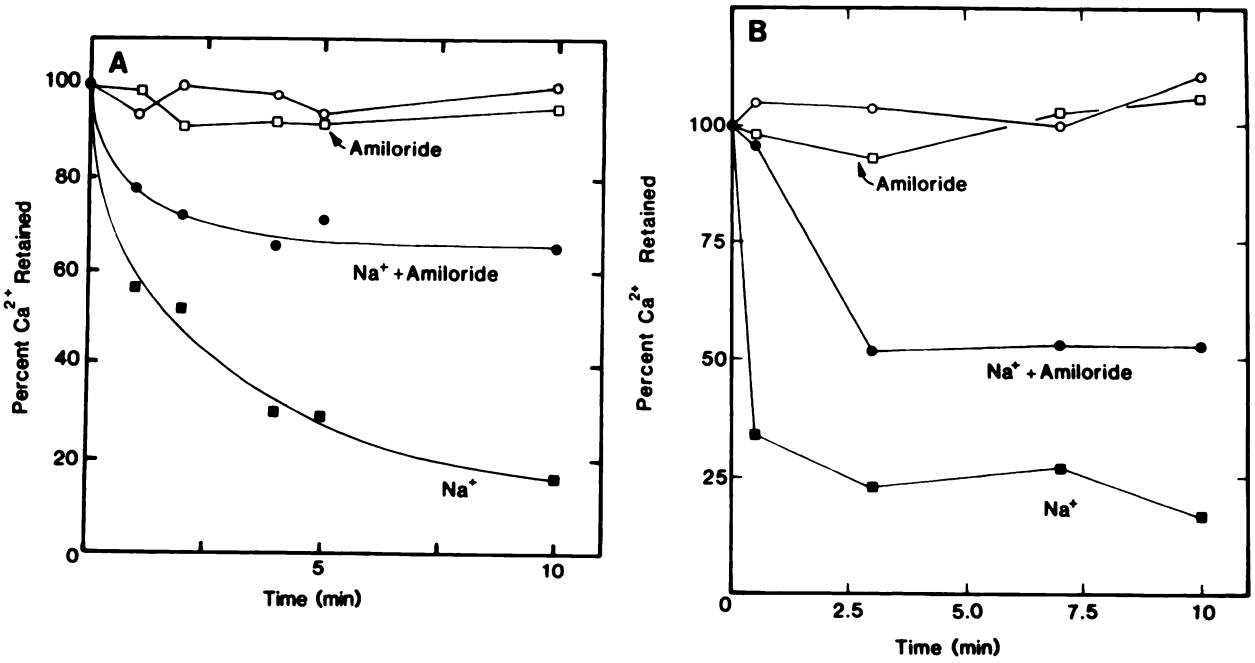

FIG. 3. *Effect of amiloride on vesicular Ca^{2+} permeability and on Na^+-dependent Ca^{2+} efflux*

Sodium-loaded microsomes (A) or membrane vesicles from lysed synaptosomes (B) were diluted 30-fold into Na^+-free medium (160 mM KCl/20 mM Tris-HCl, pH 7.4) containing 2 μM $^{45}Ca^{2+}$ and allowed to accumulate $^{45}Ca^{2+}$ for 20 min. Uptake was terminated by the addition of EGTA. $^{45}Ca^{2+}$ efflux was initiated (time zero) by the addition of Na^+ to some samples in the presence or absence of amiloride. Additions at time zero were none (□), 2.5 mM amiloride (○), 36 mM NaCl (■), and 36 mM NaCl/2.5 mM amiloride (●). Vesicular $^{45}Ca^{2+}$ was determined by filtration over the next 10 min as described under Experimental Procedures. The data points shown are the averages of three determinations. The Ca^{2+} associated with Na^+-loaded vesicles diluted into NaCl medium rather than KCl medium under similar conditions was taken as background. The standard deviation for triplicate determinations varied from ±3.6% to ±17%, and the average for the above points was ±6.9%.

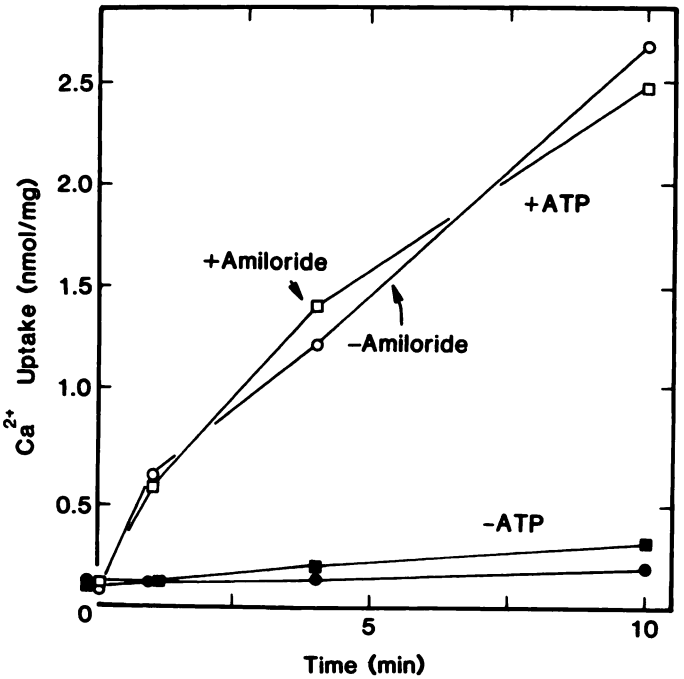

FIG. 4. *Effect of amiloride on ATP-dependent Ca^{2+} uptake by membrane vesicles from lysed synaptosomes*

Ca^{2+} uptake was assayed for ATP-dependent Ca^{2+} uptake as described under Experimental Procedures. No Na^+ was present in the assay. The data points shown are the averages of three determinations. ATP, ○; ATP and 2 mM amiloride, □; no ATP, ●; and no ATP plus 2 mM amiloride, ■. The standard deviation for triplicate determinations varied from ±2.5% to ±20%, and the average for the above points was ±9.3%.

Amiloride inhibition of Ca^{2+} uptake by reconstituted vesicles. Membranes containing the Na^+-Ca^{2+}carrier from heart (23) and brain (22) can be solubilized in cholate/NaCl and reconstituted into phospholipid vesicles. The reconstituted vesicles catalyze Na^+-dependent Ca^{2+} uptake and exhibit many of the transport characteristics of the carrier in the original membrane (22, 23). Figure 7 shows the time course of Ca^{2+} uptake by the reconstituted carrier. Ca^{2+} is rapidly accumulated by the Na^+ oxalate-loaded vesicles when diluted into Na^+-free media, whereas much less Ca^{2+} is associated with the vesicles in the absence of a Na^+ gradient. Previous work (22) with this reconstituted preparation demonstrated that the Ca^{2+} associated with the vesicles in the presence of a Na^+ gradient is intravesicular and not just bound to the external surface of the membranes. Amiloride (1 mM) inhibits Na^+-dependent Ca^{2+} uptake in the reconstituted vesicles (Fig. 7); the initial rate of Na^+-dependent uptake measured at 10 sec was reduced by 57% (SD = 18%; n = 3) in the presence of the drug, and the equilibrium level of accumulated Ca^{2+} was reduced by 82% (SD = 6%; n = 3). Amiloride did not affect the basal level of Ca^{2+} associated with the vesicles in the absence of an outward Na^+ gradient.

DISCUSSION

The above work demonstrates that amiloride inhibits the plasmalemma Na^+-Ca^{2+} exchange system from rat brain. The drug does not appear to increase the passive permeability of the membranes to Ca^{2+}. Thus amiloride presumably acts directly on the Na^+-Ca^{2+} exchange carrier. Presently, amiloride is the only drug known that

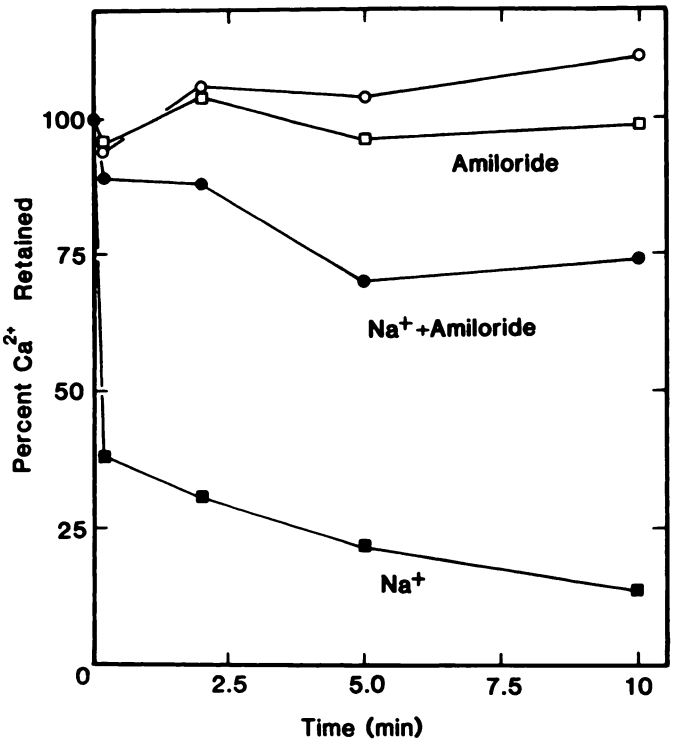

FIG. 5. *Amiloride inhibition of Na^+-dependent Ca^{2+} release from vesicles loaded with Ca^{2+} by ATP-dependent uptake*

Vesicles derived from lysed synaptosomes were loaded with $^{45}Ca^{2+}$ in the presence of ATP as described under Experimental Procedures. No Na^+ gradients were present. Uptake was terminated after 20 min by the addition of EGTA (15 mM). Ca^{2+} efflux was initiated (time zero) by the addition of Na^+ to some samples in the presence or absence of amiloride. Additions at time zero were none (○), 2.5 mM amiloride (□), 36 mM Na^+ (■), and 36 mM Na^+/2.5 mM amiloride (●). The data points shown are the averages of three determinations. The Ca^{2+} associated with vesicles loaded with Ca^{2+} in the absence of ATP under similar conditions was taken as background. The standard deviation for triplicate determinations varied from ±3.0% to ±14%, and the average of the above points was ±7.5%.

inhibits the brain Na^+-Ca^{2+} exchange system. Doxorubicin, which has been reported to inhibit Na^+-Ca^{2+} exchange in heart sarcolemma vesicles (28), does not inhibit Na^+-Ca^{2+} exchange in brain plasmalemma vesicles when the drug is present at 80 μM and at $^{45}Ca^{2+}$ concentrations of 0.5 or 2.0 μM.[2] Amiloride also inhibits Na^+-dependent uptake in the reconstituted vesicles (Fig. 7), indicating that the solubilized and reincorporated Na^+-Ca^{2+} carrier has the same properties as the carrier in the original membrane. The ATP-dependent Ca^{2+} transport system is not inhibited by the drug (Fig. 4). Although it is not presently possible to determine the exact mechanisms of amiloride inhibition, the observation that the drug is a competitive inhibitor with respect to Ca^{2+} uptake (Fig. 6) suggests that both Ca^{2+} and amiloride (a cation at pH 7.4, ref. 26) bind to the same site. Na^+ is also a competitive inhibitor of Ca^{2+} uptake (22). Thus the simplest explanation of substrate interaction with the carrier is that Na^+, Ca^{2+}, and amiloride bind to the same site and that, when amiloride is bound, the carrier is inhibited.

Interestingly, amiloride not only inhibits the initial rate of Ca^{2+} uptake but also reduces the amount of Ca^{2+} present in the vesicles after equilibrium has been reached (Figs. 1 and 2). Although the reduction in initial rates is consistent with amiloride's competing with cation binding at the substrate recognition site(s), the reduction in the equilibrium levels of vesicular Ca^{2+} cannot be explained by simple competitive inhibition. At equilibrium, the distribution of Ca^{2+} across the vesicular membrane is determined by intra- and extravesicular Na^+ and Ca^{2+} concentrations, the membrane potential, and the cou-

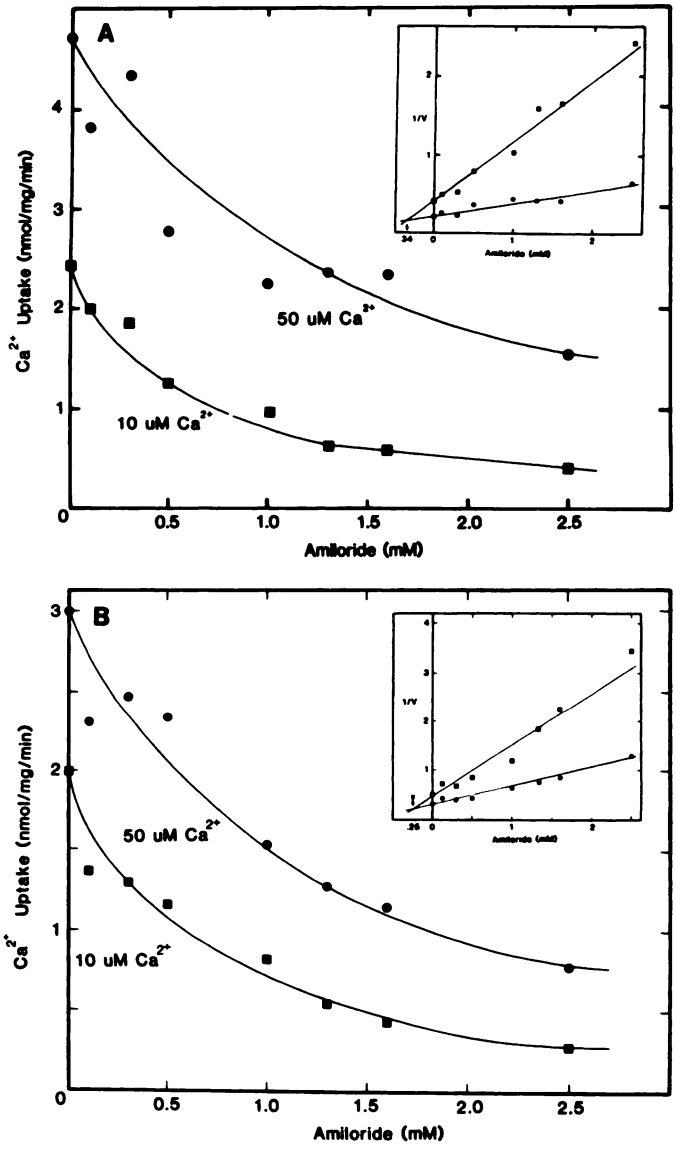

FIG. 6. *Concentration dependence of amiloride inhibition of Na^+-dependent Ca^{2+} uptake*

Ca^{2+} uptake was assayed in the presence of varied concentrations of amiloride by diluting Na^+-loaded microsomes (A) or Na^+-loaded vesicles from lysed synaptosomes (B) 30-fold into 160 mM KCl/20 mM Tris-HCl (pH 7.4) and either 10 μM (■) or 50 μM (●) $^{45}Ca^{2+}$. Uptake was terminated after 10 sec as described under Experimental Procedures. Ca^{2+} uptake by Na^+-loaded vesicles diluted into NaCl buffer rather than KCl buffer was taken as the background. Data points are the averages of three independent determinations. The *insets* present the amiloride inhibition data plotted by the linear transformation method of Dixon (27). I/V is the inverse of the Ca^{2+} uptake values.

[2] G. D. Schellenberg, unpublished data.

TABLE 1

Reversibility of amiloride inhibition

Microsomal vesicles were assayed for Na^+-dependent Ca^{2+} uptake in the presence or absence of 2 mM amiloride. Assays contained 50 μM $^{45}Ca^{2+}$. Uptake was determined at 23°C using 20-sec time points on three separate aliquots of the same microsomal preparation. Standard deviations are given in parentheses. Untreated vesicles were assayed without any processing. Control vesicles and vesicles preincubated in amiloride were initially incubated in 160 mM NaCl/20 mM Tris-HCl, ± 2 mM amiloride, for 10 min at 23°. The vesicles were then washed twice in 160 mM NaCl/20 mM Tris-HCl at room temperature. The final pellet was resuspended in the same buffer and incubated at 23° for 1 hr. Na^+-dependent Ca^{2+} uptake activity was then determined for both types of vesicles in the presence and absence of amiloride as described above.

	Ca^{2+} uptake		
	No addition	2 mM amiloride	
	nmoles/mg/min		
Untreated vesicles	5.1 (1.1)	0.20 (0.34)	4.9
Control vesicles	4.1 (0.58)	0.71 (0.82)	3.4
Vesicles preincubated in amiloride	4.0 (0.80)	0.84 (0.63)	3.2

pling ratio (number of Na^+ ions exchanged per Ca^{2+} ion) of the carrier. The Na^+-Ca^{2+} exchanger is thought to exchange three Na^+ ions per Ca^{2+} ion and is therefore electrogenic (29, 30). Thus, amiloride could conceivably alter the equilibrium Ca^{2+} content of the vesicles by reducing the coupling ratio of the carrier. This situation would occur if amiloride binds to a regulatory site which controls the coupling ratio. Alternately, the drug could replace one or more Na^+ ions during transport, resulting in less than three Na^+ ions transported per Ca^{2+} ion.

The Na^+-Ca^{2+} exchange system can exchange Na^+ for Ca^{2+} in either direction across the membrane. The direction of Ca^{2+} transport is determined by the Na^+ and Ca^{2+} gradients present (18, 19) and on the electrical potential across the membrane. Therefore, the Na^+ and Ca^{2+} interaction site(s) are present either simultaneously or sequentially on both the extravesicular surface of the carrier. If Na^+, Ca^{2+}, and amiloride are all recognized by the same site(s) on the carrier, then amiloride could potentially inhibit the carrier when present at either surface of the membrane. The data shown in Fig. 5 suggest that amiloride inhibits Na^+-Ca^{2+} exchange at the intracellular surface of the carrier. In this experiment, the presence of the ATP-dependent Ca^{2+} pump allowed us to load selectively inside-out vesicles with Ca^{2+}. Amiloride inhibited Na^+-dependent release of Ca^{2+} from these inside-out vesicles. However, the conclusion that amiloride acts at an intracellular site is based on the assumption that amiloride does not penetrate the vesicles over the time course of the experiment (10 sec–10 min). Indeed, the inhibitory effects of amiloride on Ca^{2+} uptake (Figs. 1 and 2) and Ca^{2+} release (Figs. 3 and 5) are observed at the most rapid assay time points (10–30 sec). Since in these experiments the vesicles are not preincubated in the presence of the drug, the rapid action of the drug suggests that amiloride does not have to equilibrate across the vesicle membrane and therefore acts at the external surface of the vesicles. Attempts to measure the amiloride permeability of the vesicles have so far been unsuccessful, since it is not presently possible to distinguish amiloride bound to the external surface of the membranes from that in the lumen of the vesicles.

Recently, Smith *et al.* (10) demonstrated that amiloride inhibits Na^+-Ca^{2+} exchange using intact murine erythroleukemia cells. In these studies, the cells were preincubated for several hours in the presence of 40 μM extracellular amiloride prior to Ca^{2+} uptake measurements. During this preincubation, the cells accumulated the drug to an intracellular concentration of approximately 1 mM. Na^+-Ca^{2+} exchange was inhibited only if the cells were preincubated with amiloride, and no inhibition occurred when 0.04 mM amiloride was added directly (no preincubation) to Ca^{2+} transport assays. These results led Smith *et al.* (10) to conclude that amiloride inhibited Na^+-Ca^{2+} exchange at a cytoplasmic site on the membrane. Since the K_I for amiloride is approximately 0.25–0.34 mM (Fig. 6), the presence of 0.04 mM amiloride at the extracellular surface of the cell would not be expected to inhibit uptake significantly, whereas the 1 mM intracellular amiloride would result in inhibition. Whether higher levels of the drug would inhibit Ca^{2+} transport without preincubation at an extracellular site remains to be seen.

Amiloride was originally characterized as a potassium-sparing diuretic and an inhibitor of Na^+ fluxes across Na^+-transporting epithelia (1–4). In these systems, the drug inhibits a facilitative diffusion Na^+ channel located on the mucosal surface of the cell. This epithelial Na^+ uptake system can be distinguished from the Na^+-Ca^{2+} carrier based on amiloride inhibition; the drug is a much more effective inhibitor of the epithelial system ($K_I \simeq$

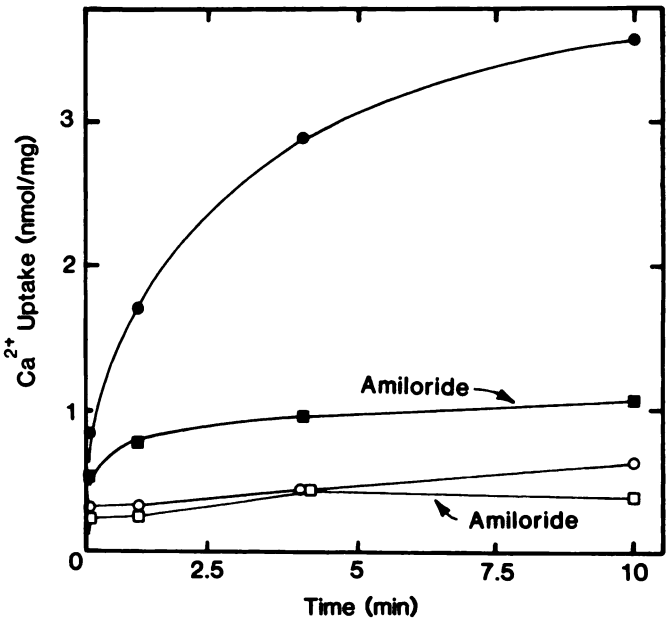

FIG. 7. *Amiloride inhibition of Na^+-dependent Ca^{2+} uptake by reconstituted phospholipid vesicles*

Ca^{2+} uptake was assayed in either the presence (■, □) or absence (●, ○) of 1 mM amiloride as described in Fig. 1. Sodium oxalate-loaded vesicles were assayed in 160 mM KCl (●), 160 mM KCl/1 mM amiloride (■), 160 mM NaCl (○), or 160 mM NaCl/mM amiloride (□). The data points shown are the averages of three determinations. The standard deviation for triplicate determinations varied from ±2.1% to ±21%, and the average for the above points was ±10%.

0.1–1 μM; refs. 31 and 32) than of Na^+-Ca^{2+} exchange (K_I = 0.25–0.34 mM; Fig. 6). Furthermore, unlike the Na^+-Ca^{2+} exchange system, the epithelial Na^+ carrier most likely does not transport Ca^{2+}. However, the amiloride-sensitive epithelial system does interact with Ca^{2+}. Extracellular Ca^{2+} at the mucosal surface of the cell layer partially inhibits Na^+ transport by this system (31–35). For example, 10 mM Ca^{2+} inhibits Na^+ transport (measured by short-circuit current) across bullfrog skin by a maximum of 20% (32). Additional Ca^{2+} does not result in additional inhibition. The inability of saturating Ca^{2+} to inhibit Na^+ flux completely suggests that Na^+ and Ca^{2+} do not interact competitively by binding to a common site on the Na^+-H^+ carrier. In contrast, Na^+ and Ca^{2+} appear to interact competitively with the Na^+-Ca^{2+} exchange carrier (22–23).

A second type of Ca^{2+} interaction with the Na^+ epithelial system has been described. In some amphibian preparations, Ca^{2+} alters the effectiveness of amiloride as an inhibitor. In toad skin preparations, the K_I of amiloride inhibition changes from 3.9 μM in the absence of Ca^{2+} to 0.44 μM in the presence of 1 mM Ca^{2+} (31).Cuthbert and Wong (34) proposed that inhibition occurs when Ca^{2+} and amiloride simultaneously bind to the Na^+ carrier. Recent work by Benos *et al.* (31) demonstrated that in bullfrog epithelial preparations, the K_I for amiloride is unaffected by Ca^{2+}, even though Ca^{2+} inhibits Na^+ fluxes in this preparation. Thus the Ca^{2+}-drug interaction may not be a universal property of the Na^+ epithelial system. However, the fact that both the epithelial Na^+ transport system and the Na^+-Ca^{2+} transport system recognize Na^+, Ca^{2+}, and amiloride suggests that the two carriers may have similar structural properties and may be the result of a gene duplication event.

A specific inhibitor of a Na^+-Ca^{2+} carrier would be extremely useful for determining the role of this transport system in regulating cytosolic Ca^{2+} levels. Amiloride is not ideal as a specific probe for Na^+-Ca^{2+} exchange for two reasons: (a) the relatively low affinity of amiloride for the carrier makes binding studies difficult, if not impossible; and (b) amiloride is not specific for the Na^+-Ca^{2+} exchange system and inhibits other non-Ca^{2+}-dependent Na^+ transport systems. However, we are presently examining structural analogues of amiloride in hopes of finding an amiloride-like drug with a higher affinity for the carrier and which is specific for the Na^+-Ca^{2+} transport system.

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REFERENCES

1. Biber, T. U. L. Effect of changes in transepithelial transport on the uptake of sodium across the outer surface of the frog skin. *J. Gen. Physiol.* **58**:131–144 (1971).
2. Bentley, P. J. Amiloride: a potent inhibitor of sodium transport across the toad bladder. *J. Physiol. (Lond.)* **195**:317–330 (1968).
3. Frizzel, R. A., and K. Turnheim. Ion transport by rabbit colon. II. Unidirectional sodium influx and the effects of amphotericin B and amiloride. *J. Membr. Biol.* **40**:193–211 (1978).
4. Schneyer, L. H. Amiloride inhibition of ion transport in perfused excretory duct of rat submaxillary gland. *Am. J. Physiol.* **219**:1050–1055 (1970).
5. Dörge, A., and W. Nagel. Effects of amiloride on sodium transport in frog skin. II. Sodium transport pool and unidirectional fluxes. *Pfluegers Arch. Eur. J. Physiol.* **321**:91–101 (1970).
6. Salako, L. A., and A. J. Smith. Changes in sodium pool and kinetics of sodium transport in frog skin produced by amiloride. *Br. J. Pharmacol.* **39**:99–109 (1970).
7. Cuthbert, A. W., and W. K. Shum. Binding of amiloride to sodium channels in frog skin. *Mol. Pharmacol.* **10**:880–891 (1974).
8. Aceves, J., and M. Cereijido. The effects of amiloride on sodium and potassium fluxes in red cells. *J. Physiol. (Lond.)* **229**:709–718 (1973).
9. Villereal, M. L. Sodium fluxes in human fibroblasts: effects of serum, Ca^{2+}, and amiloride. *J. Cell. Physiol.* **107**:359–369 (1981).
10. Smith, R. L., I. G. Macara, R. Levenson, D. Housman, and L. Cantley. Evidence that a Na^+-Ca^{2+} antiport system regulates murine erythroleukemia cell differentiation. *J. Biol. Chem.* **257**:773–780 (1982).
11. Koch, K. S., and H. L. Leffert. Growth control of differentiated adult rat hepatocytes in primary culture. *Ann. N. Y. Acad. Sci.* **349**:111–127 (1980).
12. Yamashita, S., S. Motomura, and N. Taira. Cardiac effects of amiloride in the dog. *J. Cardiovasc. Pharmacol.* **3**:704–715 (1981).
13. Pousti, A., and M. A. Khoyi. Effects of amiloride on isolated guinea pig atrium. *Arch. Int. Pharmacodyn. Ther.* **242**:222–229 (1979).
14. Seller, R. H., J. Greco, S. Banach, and R. Seth. Increasing the inotropic effect and toxic dose of digitalis by the administration of the antiklaiuretic drugs—further evidence for a cardiac effect of diuretic agents. *Am. Heart J.* **90**:56–67 (1975).
15. Waldorff, S., P. B. Hansen, H. Kjaergard, J. Buch, H. Egeblad, and E. Steiness. Amiloride-induced changes in digoxin dynamics and kinetics: abolition of digoxin-induced inotropism with amiloride. Clin. Pharmacol. Ther. **30**:172–176 (1981).
16. DiPolo, R., and L. Beaugé. Physiological role of ATP-driven calcium pump in squid axon. *Nature (Lond.)* **278**:271–273 (1979).
17. Caroni, P., and E. Carafoli. An ATP-dependent Ca^{2+} pumping system in dog heart sarcolemma. *Nature (Lond.)* **283**:765–767 (1980).
18. Schellenberg, G. D., and P. D. Swanson. Sodium-dependent and calcium-dependent calcium transport by rat brain microsomes. *Biochim. Biophys. Acta* **648**:13–27 (1981).
19. Gill, D. L., E. F. Grollman, and L. D. Kohn. Calcium transport mechanisms in membrane vesicles from guinea pig brain synaptosomes. *J. Biol. Chem.* **256**:184–192 (1981).
20. Bradford, H. F. Respiration *in vitro* of synaptosomes from mammalian cerebral cortex. *J. Neurochem.* **16**:675–684 (1969).
21. Gray, E. G., and V. P. Whittaker. The isolation of nerve endings from brain: an electron-microscopic study of cell fragments derived by homogenization and centrifugation. *J. Anat. (Lond.)* **96**:79–87 (1962).
22. Schellenberg, G. D., and P. D. Swanson. Solubilization and reconstitution of membranes containing the Na^+-Ca^{2+} exchange carrier from rat brain. *Biochim. Biophys. Acta* **690**:133–144 (1983).
23. Miyamoto, H., and E. Racker. Solubilization and partial purification of the Ca^{2+}/Na^+ antiporter from the plasma membrane of bovine heart. *J. Biol. Chem.* **255**:2656–2658 (1980).
24. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
25. Rahamimoff, H., and E. Abramovitz. Calcium transport in a vesicular membrane preparation from rat brain synaptosomes. *F. E. B. S. Lett.* **89**:223–226 (1978).
26. Cragoe, E. J. Structure-activity relationships in the amiloride series, in *Amiloride and Epithelial Sodium Transport* (A. W. Cuthbert, G. M. Fanelli, and A. Scriabine, eds.). Urban and Schwarzenberg, Baltimore and Munich, 1–20 (1979).
27. Dixon, M. The determination of enzyme inhibition constants. *Biochem. J.* **55**:170–171 (1953).
28. Caroni, P., F. Villani, and E. Carafoli. The cardiotoxic antibiotic doxorubicin inhibits the Na^+/Ca^{2+} exchange of dog heart sarcolemmal vesicles. *F. E. B. S. Lett.* **130**:184–186 (1981).
29. Pitts, B. J. R. Stoichiometry of sodium-calcium exchange in cardiac sarcolemmal vesicles. *J. Biol. Chem.* **254**:6232–6235 (1979).
30. Blaustein, M. P. Effects of internal and external cations and of ATP on sodium-calcium exchange in squid axons. *Biophys. J.* **20**:79–111 (1977).
31. Benos, D. J., L. J. Mandel, and R. S. Balabau. On the mechanisms of the amiloride-sodium entry site interaction in anuran skin epithelia. *J. Gen. Physiol.* **73**:307–326 (1979).
32. Takada, M., and H. Hayashi. Interaction of cadmium, calcium, and amiloride in the kinetics of active sodium transport through frog skin. *Jpn. J. Physiol.* **31**:285–303 (1981).
33. Curran, P. F., and J. R. Gill. The effect of calcium on sodium transport by frog skin. *J. Gen. Physiol.* **45**:625–641 (1962).
34. Cuthbert, A. W., and P. Y. D. Wong. The role of calcium ions in the interaction of amiloride with membrane receptors. *Mol. Pharmacol.* **8**:222–229 (1972).
35. Rabito, C. A., C. A. Rotunno, and M. Cereijido. Amiloride and calcium effect on the outer barrier of the frog skin. *J. Membr. Biol.* **42**:169–187 (1978).

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