Inhibition of Na^+/Ca^{2+} Exchange in Pituitary Plasma Membrane Vesicles by Analogues of Amiloride

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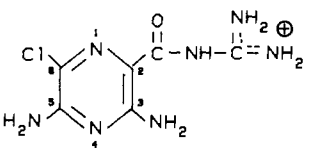
ABSTRACT: Amiloride is a weak inhibitor of Na^+/Ca^{2+} exchange in isolated plasma membrane vesicles prepared from GH_3 rat anterior pituitary cells. However, substitution on either a terminal guanidino nitrogen atom or the 5-amino nitrogen atom can increase inhibitory potency ca. 100-fold ($I_{50} \sim 10$ μM). A structure–activity study indicates that defined structural modifications of guanidino substituents are associated with increases in inhibitory activity. In contrast, analogues bearing 5-amino substituents generally increase in potency with increasing hydrophobicity of the substitution. Specificity in action of either class is indicated by several criteria. These inhibitors do not disrupt the osmotic integrity of the membrane, nor do they significantly interfere with plasmalemmal Ca^{2+}-ATPase-driven Ca^{2+} uptake, Na^+,K^+-ATPase enzymatic activity, or the function of Ca^{2+} or K^+ channels. Inhibition is freely reversible, further indicating a lack of nonspecific membrane effects. The mechanism by which each inhibitor class blocks exchange was found to be identical. Protonation of the guanidino moiety (i.e., cationic charge) is essential for activity. Analysis of transport inhibition as a function of Ca^{2+} concentration indicates noncompetitive kinetics. However, inhibition was reversed by elevating intravesicular Na^+, indicating a competitive interaction with this ion. These results suggest that the inhibitors function as Na^+ analogues, interact at a Na^+ binding site on the carrier (presumably the site at which the third Na^+ binds), and reversibly tie up the transporter in an inactive complex. In addition to blocking pituitary exchange, these analogues are effective inhibitors of the bovine brain and porcine cardiac transport systems.

Na^+/Ca^{2+} exchange activity has been demonstrated in a number of different excitable tissues including squid giant axon (Baker et al., 1967), heart (Reuter & Seitz, 1968; Reeves & Sutko, 1979), brain (Gill et al., 1981; Schellenberg & Swanson, 1981), and pituitary (Kaczorowski et al., 1984). Depending on tissue type, this transport reaction has been implicated in either the entry or removal of cell Ca^{2+} and is therefore thought to function in Ca^{2+} homeostasis (Barry & Smith, 1982; Langer, 1982). Unfortunately, most studies designed to assess this system's physiological role in controlling cellular Ca^{2+} flux have used indirect approaches, leaving the interpretation of data open to some controversy. Lack of potent specific transport inhibitors has prevented unequivocal resolution of this issue.

Amiloride [3,5-diamino-6-chloro-*N*-(diaminomethylene)-pyrazinecarboxamide], an antikaliuretic diuretic, has been used



PROTONATED AMILORIDE

to probe the function of a number of Na^+ transport systems (Benos, 1982). In high-resistance Na^+-transporting epithelial membranes, amiloride was described as a potent inhibitor of electrogenic Na^+ flux (Cuthbert & Fanelli, 1978; Benos, 1982). Various guanidine-substituted analogues have been reported to be more effective than amiloride itself in blocking Na^+ flux via this pathway (Cuthbert & Fanelli, 1978). It has been demonstrated in some epithelial systems that these compounds function as Na^+ analogues and interact competitively at a Na^+ binding site on the channel (Cuthbert, 1981; Benos, 1982). Amiloride has also been identified as an inhibitor of Na^+/H^+ exchange in several different cells (Kinsella & Aronson, 1980; Vigne et al., 1982; Frelin et al., 1983; Paris & Pouyssegur, 1983) although its potency varies with cell type. Recent studies with 5-amino nitrogen substituted analogues indicate that these molecules comprise a promising class of new potent inhibitors of Na^+/H^+ exchange (Vigne et al., 1984; L'Allemain et al., 1984; Zhuang et al., 1984). From a limited number of mechanistic investigations, amiloride also appears to act as a competitive inhibitor of Na^+ in blocking this transport reaction (Kinsella & Aronson, 1981; Vigne et al., 1982; Paris & Pouyssegur, 1983).

Using the strategy that the ability of amiloride to function as a Na^+ surrogate might be useful in blocking other Na^+-transporting systems, we have explored its efficacy in inhibiting pituitary Na^+/Ca^{2+} exchange. The results from these studies indicate that while amiloride is a rather poor inhibitor, various analogues bearing substituents on either a terminal guanidino nitrogen or the 5-amino nitrogen effectively block exchange in a specific mechanistically defined fashion. Moreover, these compounds effectively inhibit brain and cardiac Na^+/Ca^{2+} exchange. Thus, these amiloride analogues are the first relatively potent inhibitors of Na^+/Ca^{2+} exchange to be described. A preliminary report of these findings has been made

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in abstract form (Dethmers et al., 1983).

Experimental Procedures

Materials

Amiloride analogues bearing substituents on either a terminal nitrogen of the guanidino group or the 5-amino nitrogen were synthesized by methods previously described (Cragoe et al., 1967, 1971, 1981; Shepard et al., 1971; Cragoe, 1983). Stock solutions of inhibitors were prepared in dimethyl sulfoxide (Me_2SO)[1] typically at 100 mM. Inhibitors were added to aqueous buffers such that the final concentration of Me_2SO was never allowed to exceed 0.2%. Control experiments demonstrated that this concentration of Me_2SO had no effect on pituitary Na^+/Ca^{2+} exchange. $^{45}CaCl_2$ (800–1200 mCi/mmol) was purchased from the Radiochemical Center, Amersham, Inc. Ouabain, ATP, EGTA, and *p*-nitrophenyl phosphate were obtained from Sigma. Media components were purchased from Grand Island Biological Co. Nitrocellulose membrane filters (0.2 μm) were provided by Amicon, Inc. All other chemicals were of the highest purity available from commercial sources.

Methods

Growth of Cells and Preparation of Plasma Membrane Vesicles. GH_3 pituitary cells were grown in 8-L suspension cultures and used for the preparation of purified plasma membrane vesicles as previously described (Kaczorowski et al., 1984). For whole cell transport experiments, cells were grown in 500-mL suspensions, harvested and washed by centrifugation (200 *g* for 7 min), and suspended at a final concentration of 3×10^6 cells/mL in 135 mM NaCl, 5 mM KCl, 0.8 mM $MgCl_2$, 1 mg/mL glucose, 0.05% methylcellulose, and 50 mM Na-Hepes, pH 7.4. Porcine cardiac sarcolemmal membrane vesicles (Reeves & Sutko, 1979) and bovine brain plasma membrane vesicles (Schellenberg & Swanson, 1981) were prepared by established procedures with slight modifications. Because of the high transport activity of the heart preparation, these membranes were not routinely subjected to discontinuous gradient centrifugation for further purification. Brain vesicles highly enriched in plasma membrane were isolated by discontinuous sucrose density gradient centrifugation as described for pituitary plasma membrane vesicles (Kaczorowski et al., 1984). All vesicle preparations were quick frozen and stored at −80 °C.

Transport Assays. Na^+/Ca^{2+} exchange was monitored in pituitary, heart, and brain plasma membrane vesicle preparations as described (Kaczorowski et al., 1984). Vesicles were quick thawed at 37 °C, concentrated by centrifugation (150000*g* for 30 min), and resuspended in 100 mM NaCl, 300 mM mannitol, and 20 mM Tris–Hepes, pH 7.4, at a concentration of ca. 20 mg of protein/mL. After 30–60 min at 4 °C, Na^+ equilibration was complete, and the vesicles were used directly for transport studies. Aliquots (2 μL) were diluted 200-fold into 300 mM mannitol and 20 mM Tris–Hepes, pH 7.4, containing either 100 mM NaCl or KCl and 40 μM $^{45}CaCl_2$. After various times, the reaction was terminated by addition of 5 mL of K^+ reaction buffer containing 1 mM EGTA followed by immediate filtration onto membrane filters. Transport activity was determined from differences in Ca^{2+} uptake with and without an outwardly directed Na^+ gradient. For experiments where the pH of the medium was varied, vesicles were washed and resuspended in mixtures of 20 mM Tris–Hepes–Mes at the desired pH. In kinetic experiments monitoring Ca^{2+} transport, $^{45}CaCl_2$ was employed in the range 1–100 μM and initial rates of uptake monitored (15-s time point).

Ca^{2+} equilibration experiments were performed with GH_3 cells by adding 200 μM $^{45}CaCl_2$ to cells suspended in Ca^{2+} free media at 37 °C. Equilibration was monitred by quenching the reaction at various times with 5 mL of 300 mM mannitol and 20 mM Tris–Hepes, pH 7.4, containing 0.5 mM $LaCl_3$, followed by rapid filtration onto GF/C glass-fiber filters. $(Ca^{2+} + Mg^{2+})$-ATPase-dependent Ca^{2+} transport activity was measured in GH_3 vesicles as previously described (Barros & Kaczorowski, 1984).

Other Experimental Procedures. GH_3 cells were subjected to voltage clamp by using patch electrode methodology (Hagiwara & Ohmori, 1982; Dubinsky & Oxford, 1984). In experiments where amiloride inhibition of voltage-dependent Ca^{2+} or K^+ or Ca^{2+}-activated K^+ channel activities was measured, individual ionic conductances were isolated as described (Barros et al., 1984; Katz et al., 1984). Cells were maintained in a chamber at 25 °C by using a temperature-controlled buffer perfusion system, and inhibitors were dissolved in the perfusion buffer for application to the cells.

GH_3 cell respiratory activity was recorded by using a Clark-type oxygen electrode. Cells grown in culture were suspended at a concentration of 10^7 cells/mL in Dulbecco's phosphate-buffered saline containing 1 mg of glucose/mL and 0.05% methylcellulose and placed in a closed temperature regulated chamber at 37 °C. After basal rates of oxygen consumption [2 nmol of O_2 consumed min^{-1} $(10^6 \text{ cell})^{-1}$] were measured, various concentrations of amiloride analogues were added directly to the chamber. Effects on respiration were assessed relative to control rates of oxygen consumption.

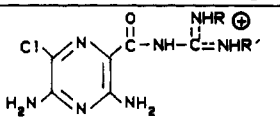
Binding of [^{3}H]thyrotropin–releasing hormone was assayed as described (Vandlen et al., 1981). Na^+,K^+-ATPase activity in GH_3 vesicles was determined by measuring ouabain-sensitive *p*-nitrophenyl phosphate hydrolytic activity (Kaczorowski et al., 1984). Protein was measured by the method of Lowry (Lowry et al., 1951).

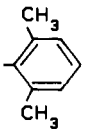
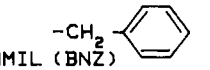
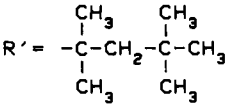
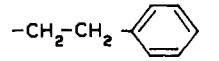
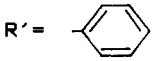
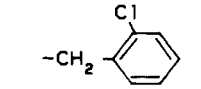
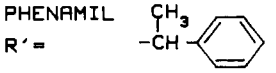
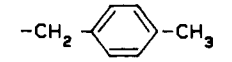
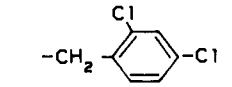
Results

Development of Inhibitors of Na^+/Ca^{2+} Exchange in Pituitary. A Na^+/Ca^{2+} exchange transport reaction has been characterized in GH_3 rat anterior pituitary cells (Kaczorowski et al., 1984). Purified plasma membrane vesicles prepared from these cells are a convenient model system for directly monitoring this transport activity. When vesicles are passively equilibrated with Na^+, concentrated, and then diluted into a Na^+-free medium containing $^{45}CaCl_2$, there is a rapid, time-dependent, concentrative uptake of Ca^{2+}. We have previously noted that amiloride can function as an inhibitor of this process (Kaczorowski et al., 1984). In order to define more clearly amiloride's ability to inhibit Na^+/Ca^{2+} exchange, vesicle transport experiments were performed under V_{max} conditions in the presence of varying concentrations of this compound. The data presented in Figure 1 indicate that amiloride will inhibit Na^+/Ca^{2+} exchange in a concentration-dependent fashion. Under these conditions, however, amiloride is a rather poor inhibitor ($I_{50} \simeq 1 - 2$ mM). Since some derivatives of amiloride have been shown to be more potent than the parent compound in inhibiting other Na^+ transport systems (Benos, 1982), an investigation was made to determine if any amiloride

[1] Abbreviations: Me_2SO, dimethyl sulfoxide; BNZ, *N*-benzylamiloride; DMB, *N*-(2,4-dimethylbenzyl)amiloride; CBA, N^5-(4-chlorobenzyl)amiloride; Tris, tris(hydroxymethyl)aminomethane; TRH, thyrotropin-releasing hormone; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ATPase, adenosinetriphosphatase.

Table I: Inhibition of Pituitary Na^+/Ca^{2+} Exchange by Guanidino-Substituted Analogues of Amiloride[a]



COMPOUND	I_{50}	COMPOUND	I_{50}
R, R′ = H AMILORIDE	1.1 mM	R′= 	150 uM
R = $-CH_2-CH_2-CH_2-CH_3$ R′= $-CH_2-CH_2-CH_2-CH_3$	330 uM	R′=  BENZAMIL (BNZ)	100 uM
R = H R′= 	140 uM	R′= 	90 uM
R′= 	200 uM	R′= 	20 uM
PHENAMIL R′= 	120 uM	R′= 	70 uM
		R′= 	12 uM

[a] Na^+/Ca^{2+} exchange activity was monitored in the presence of different concentrations of inhibitors under V_{max} conditions (i.e., 40 μM extravesicular $^{45}CaCl_2$). Inhibition was determined from initial rates of uptake relative to an untreated control [6 nmol of Ca^{2+} accumulated min^{-1} (mg of protein)$^{-1}$]. I_{50} values were determined graphically from titration profiles of inhibitor activity.

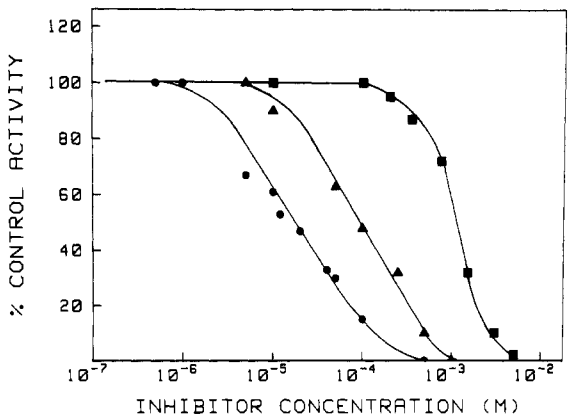


FIGURE 1: Concentration dependence of amiloride and amiloride analogue inhibition of pituitary Na^+/Ca^{2+} exchange. Vesicles (2 μL) were diluted 200-fold into a medium containing 300 mM mannitol, 100 mM KCl, 40 μM $^{45}CaCl_2$, 20 mM Tris–Hepes, pH 7.4, and various concentrations of amiloride (■), benzamil (▲), or DMB (●). The control rate of Ca^{2+} uptake was 6 nmol of Ca^{2+} accumulated min^{-1} (mg of protein)$^{-1}$.

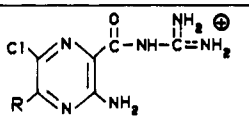
analogues possessed increased activity in blocking Na^+/Ca^{2+} exchange. Benzamil (BNZ), which bears a benzyl group on a terminal guanidino nitrogen, is more potent than amiloride in inhibiting epithelial Na^+ channels (Cuthbert & Fanelli, 1978; Aceves & Cuthbert, 1979; Cuthbert, 1981). Similarly, BNZ inhibits pituitary Na^+/Ca^{2+} exchange with a 10-fold increase in potency (I_{50} = 100 μM; Figure 1). The 2,4-dimethylbenzyl analogue of BNZ [i.e., *N*-(2,4-dimethylbenzyl)amiloride, DMB] yields an inhibitor which is 100 times more potent than amiloride (I_{50} = 10 μM; Figure 1). These results demonstrate that amiloride will block Na^+-dependent Ca^{2+} uptake in vesicles and that a range of inhibitory potencies are available with amiloride analogues.

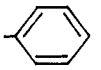
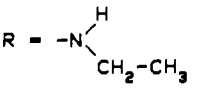
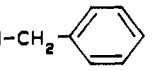
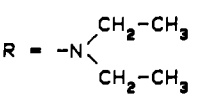
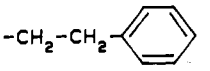
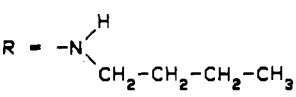
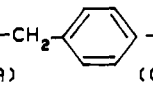
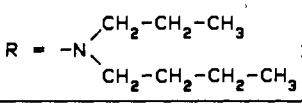
In order to search for those amiloride derivatives that can function as effective inhibitors of pituitary Na^+/Ca^{2+} exchange in a systematic fashion, two classes of analogues were investigated. The first group consists of molecules substituted on the terminal guanidino nitrogen. The results shown in Table I report the ability of selected analogues to block Na^+/Ca^{2+} exchange in pituitary. These data were obtained by titrating inhibitory activity under V_{max} conditions as described in Figure 1. The results indicate that there are marked structural constraints associated with achieving inhibitory potency. Derivitization with a benzyl group yields a greater increase in activity over that of amiloride than the corresponding alkyl, unsubstituted aryl, or aralkyl derivatives examined. Substitution of a chloro or methyl group at the 2-position of the benzyl group enhances potency another 5-fold over that of BNZ, but when these substituents are in the 3- or 4-position, the effects are less pronounced. This rank order of potency is much different from that characteristic of Na^+ channel inhibitors (Cuthbert & Fanelli, 1978; Benos, 1982). For example, *N*-(2,6-dimethylphenyl)amiloride, one of the most potent inhibitors of Na^+ channel activity (23-fold greater than amiloride;[2] K_i = ~2 × 10^{-8} M), is less active than BNZ against Na^+/Ca^{2+} exchange.

Another class of amiloride analogues is that bearing 5-amino nitrogen substituents. Some typical members of this class are shown in Table II and represent molecules of increasing hydrophobicity as the extent of the modification is increased. When the ability of these compounds to inhibit pituitary Na^+/Ca^{2+} exchange was examined (Table II), it was found that they, too, form a group of effective inhibitors. Unlike the derivatives bearing terminal guanidino substituents, however, there is much less structural constraint associated with

[2] Dr. George M. Fanelli, personal communication.

Table II: Inhibition of Pituitary Na^+/Ca^{2+} Exchange by 5-Amino Nitrogen Substituted Analogues of Amiloride[a]



COMPOUND	% INHIBITION @ 100 uM (I_{50})	COMPOUND	% INHIBITION @ 100 uM (I_{50})
R = $-NH_2$ (AMILORIDE)	5	R = 	53
R = 	19	R = 	77
R = 	65	R = 	90
R = 	45	R =  (CBA)	100 (C1) (20 uM)
R = 	100 (20 uM)	R = $-NH-CH_2-(CHOH)_4-CH_2OH$	0

[a] Na^+/Ca^{2+} exchange activity was monitored in the presence of a fixed concentration (100 μM) of various inhibitors under V_{max} conditions. Inhibition was assessed relative to control initial rates of uptake [6 nmol of Ca^{2+} accumulated min^{-1} (mg of protein)$^{-1}$]. In some cases, inhibitory activity was titrated, and I_{50} values were determined graphically from inhibition profiles.

increasing inhibitory potency. Apparently, increases in activity correlate generally with increases in the hydrophobicity of the substituent. Nonetheless, some members of this group are almost as potent as the best inhibitors of the other class.

Na^+-dependent transplasma membrane Ca^{2+} movements have previously been demonstrated in intact GH_3 pituitary cells (Kaczorowski et al., 1983a, 1984). To determine whether the inhibitors described above would block Na^+/Ca^{2+} exchange at the level of the whole cell, the experiment shown in Figure 2 was performed. Cells grown in suspension culture were harvested, washed, and placed in a normal Na^+,Ca^{2+}-free, minimal salts medium as described under Methods. Various concentrations of DMB (0–200 μM) were then added to the cell suspension followed by 200 μM $^{45}CaCl_2$, 1 min later. Ca^{2+} equilibration was allowed to proceed for 15 min at which time the cells were harvested by filtration, and Ca^{2+} accumulation in treated cells was compared with controls not exposed to DMB. Interestingly, low concentrations of DMB (1–50 μM) enhance total Ca^{2+} accumulation while higher concentrations begin to block Ca^{2+} uptake. These results suggest that DMB, at concentrations which block vesicle Na^+/Ca^{2+} exchange, enhances Ca^{2+} equilibration by inhibiting a Na^+-dependent Ca^{2+} clearance mechanism operating in the intact cell. Inhibition of Ca^{2+} uptake at higher DMB concentrations may be due to blockage of voltage-dependent Ca^{2+} channels (see below), the major pathway for Ca^{2+} influx into GH_3 cells (Hagiwara & Ohmori, 1982; Kaczorowski et al., 1983a).

Specificity of Amiloride Inhibition. To demonstrate that inhibition of vesicle Na^+/Ca^{2+} exchange is a specific phenomena, two approaches were taken. The effects of several structurally different, potent inhibitors were determined on a variety of unrelated membrane processes as was the mechanism by which these inhibitors function (see below).

When vesicles are passively equilibrated with either $^{45}CaCl_2$, [^{14}C]deoxyglucose, or [^{14}C]sorbitol and then diluted 200-fold into substrate-free buffer, loss of radiolabeled compound occurs via passive diffusion. The resulting $t_{1/2}$ of efflux at substrate concentrations of 100, 40, and 200 μM are 15, 11, and 10 min, respectively. Inclusion of 200 μM BNZ or 100 μM of either DMB or 5-*N*-(4-chlorobenzyl)amiloride (CBA, a 5-amino nitrogen derivative) in the dilution buffer has no effect whatsoever on these rates of passive diffusion. This is a strong indication that these analogues, at concentrations that completely abolish Na^+/Ca^{2+} exchange activity, do not disrupt the osmotic integrity of the vesicle membrane. Consistent with this notion are measurements of intravesicular H_2O volumes by ^3H_2O/[^{14}C]inulin exclusion techniques which are identical in the presence or absence of 100 μM DMB (data not shown). Complete inhibition of Na^+/Ca^{2+} exchange by the three analogues listed above is also easily reversed by washing vesicles, suggesting that no permanent membrane damage has occurred.

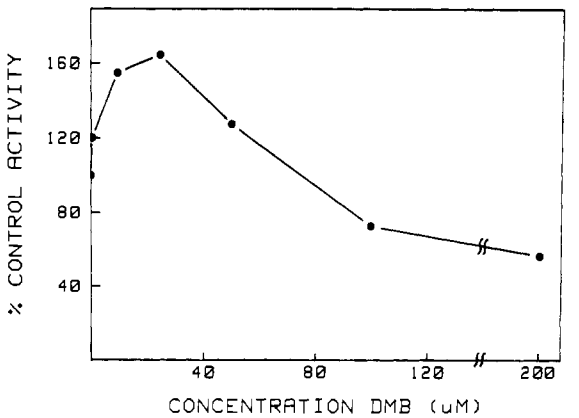


FIGURE 2: Effect of DMB on Ca^{2+} equilibration in GH_3 pituitary cells. Ca^{2+} equilibration was monitored as described under Methods. Various concentrations of DMB were added to cell suspensions 1 min before addition of 200 μM $^{45}CaCl_2$. Ca^{2+} uptake was terminated after 15 min and accumulation compared with untreated controls [200 pmol of Ca^{2+} equilibrated 15 min^{-1} (10^6 cells)$^{-1}$].

A number of plasma membrane ion flux pathways were next examined to determine if amiloride analogues interfere with their function. GH_3 vesicles possess a well-characterized (Ca^{2+} + Mg^{2+})-ATPase activity (Barros & Kaczorowski, 1984). Measurement of ATP-dependent Ca^{2+} transport in the presence of either 100 μM BNZ, 100 μM DMB, or 100 μM CBA indicates no pronounced inhibitory effects of these compounds (19%, 29%, and 26% inhibition, respectively). These vesicles also possess a ouabain-sensitive Na^+,K^+-ATPase (Kaczorowski et al., 1984). The K^+-dependent phosphatase activity of this enzyme was monitored as a guage of nonspecific interactions between these analogues and membrane-bound proteins. At 100 μM, neither BNZ, DMB, nor CBA significantly interfere with the pump's enzymatic activity (<20% inhibition). Moreover, these compounds have no effect on [^{3}H]ouabain binding, monitored in the presence of Na^+ and ATP, suggesting limited interaction with the ATPase in GH_3 vesicles (V. King and G. Kaczorowski, unpublished observations).

In addition to these active transport mechanisms, GH_3 cells display a number of ion flux pathways which have been characterized by electrophysiological techniques (Hagiwara & Ohmori, 1982; Dubinsky & Oxford, 1984; Barros et al., 1984; Katz et al., 1984). Voltage-dependent Ca^{2+} and K^+ and Ca^{2+}-activated K^+ conductances have been analyzed in GH_3 cells by whole cell voltage clamp using patch electrode methodology. Application of 100 μM DMB to voltage-clamped GH_3 cells results in a reversible 30–40% blockage of inward Ca^{2+} current, indicating some Ca^{2+} entry blocker activity of this compound at high concentrations (data not shown). This observation is consistent with data obtained in whole cell $^{45}Ca^{2+}$ flux experiments (see above). However, lower concentrations of DMB at which inhibition of exchange is clearly evident have no significant effect on the inward Ca^{2+} current. Similar analysis of K^+ conductances with either 100 μM DMB or CBA present indicates that these compounds have no inhibitory effect whatsoever on either type of K^+ channel. Furthermore, neither DMB nor CBA at this concentration has any effect on the holding current used in the voltage-clamp technique. Since resting membrane conductance did not increase, this further indicates that amiloride analogues do not disrupt the osmotic integrity of the membrane. Taken together, this lack of interference with transplasma membrane ion movements demonstrates that neither analogue class nonspecifically alters general membrane properties.

To explore further the interaction of amiloride analogues with systems which function at the level of the membrane, the effects of compounds on receptor binding and mitochondrial respiration were determined. GH_3 plasma membrane vesicles possess well-characterized high-affinity specific binding sites for thyrotropin-releasing hormone (TRH) (Vandlen et al., 1981; Kaczorowski et al., 1984). The TRH receptor–ligand interaction is exquisitely sensitive to membrane active agents. For example, agents that are detergents or possess local anesthetic activity (e.g., quinidine, bepridil, and lidocaine) interfere with binding activity at reasonably low concentrations (unpublished observations). It was, therefore, of interest to determine whether BNZ, DMB, or CBA would affect TRH binding in GH_3 membrane vesicles. In the range of concentrations at which Na^+/Ca^{2+} exchange activity is titrated (i.e., 0–50 μM DMB and CBA; 0–100 μM BNZ), none of these compounds seriously interfered with binding activity ($\leq$30% inhibition). However, at higher concentrations, TRH binding was significantly inhibited. In respiration assays, 100 μM DMB inhibited GH_3 whole cell oxygen consumption by 10–20% although neither CBA nor BNZ had any effect at this concentration. More extensive examination of the compounds listed in tables I and II indicate that at high concentrations some of the other analogues interfere with mitochondrial respiration. Taken together, these results indicate that caution must be exercised in assessing cellular responses to either analogue class when these compounds are used at high experimental concentrations or under conditions where intracellular accumulation may occur.

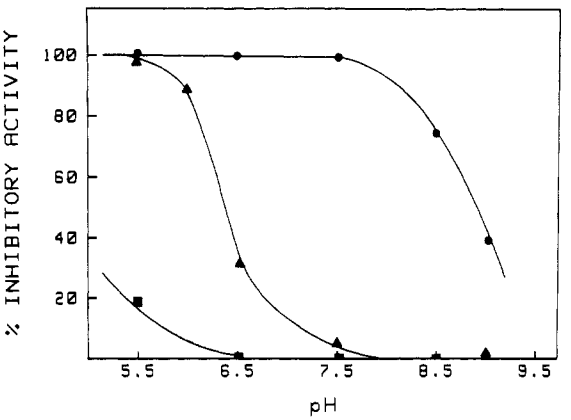


FIGURE 3: Effect of pH on inhibition of pituitary Na^+/Ca^{2+} exchange by amiloride analogues possessing different pK_a values. Vesicles were resuspended in a buffer containing 20 mM Tris–Hepes–Mes adjusted to a pH in the range 5.5–9.0 with either HCl or KOH. Ca^{2+} uptake was measured under V_{max} conditions in the presence of 100 μM DMB (●), 1 mM *N*-hydroxyamiloride (▲), or 100 μM 3-amino-6-chloro-*N*-[(diphenylamino)methylene]pyrazinecarboxamide (■) at varying pHs and initial rates of transport compared with untreated controls suspended in buffer at the equivalent pH. Control rates of uptake increased from 3 to 12 nmol of Ca^{2+} accumulated min^{-1} (mg of protein)$^{-1}$ as the pH was increased from 5.5 to 9.0.

The structural patterns of the two amiloride derivative classes which possess inhibitory activity reveal that defined structural constraints are associated with increases in potency. This serves as yet another indication of specificity in action and can be illustrated by considering the inhibitory effects of analogues bearing structurally similar substituents on the guanidino nitrogen. For example, analogues substituted at the 2-position of the benzyl group are more potent than analogues bearing similar 4-position substituents. Likewise, analogues bearing aryl or aralkyl substituents on the 5-amino nitrogen yield greater increases in activity than when the same series is substituted on the guanidino nitrogen. Thus, while derivatives of amiloride might have similar chemical structures or physical properties (e.g., hydrophobic characteristics), interactions with the Na^+/Ca^{2+} exchange carrier that result in increased inhibitory potency depend on a strictly defined set of molecular properties. This suggests a high degree of specificity in their mechanism(s) of action.

Mechanism of Inhibition. In order to probe the interaction of amiloride analogues with the GH_3 Na^+/Ca^{2+} exchange system, their mechanism of inhibition was investigated. If these inhibitors function as inorganic ion surrogates and interact at a cation binding site on the carrier, then the cationic form of the molecule (i.e., protonation of the guanidino group) should be absolutely required for activity. To test this, the inhibitory activity of three analogues with different pK_a's was determined as a function of pH (Figure 3). The pK_a of DMB is ca. 8.1, a value similar to that of amiloride (pK_a = 8.7). When initial rates of Na^+-dependent Ca^{2+} uptake are monitored at a fixed DMB concentration from pH 5.5 to pH 9.0 and compared with rates from untreated vesicles, there is a gradual decrease in inhibitory activity as the pH of the medium becomes more alkaline. This pattern corresponds with de-

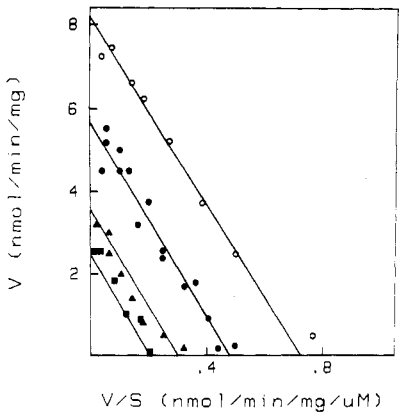


FIGURE 4: Kinetic analysis of DMB inhibition of pituitary Na^+/Ca^{2+} exchange. Initial rates of Na^+/Ca^{2+} exchange activity were measured as described under Methods in the presence of 1–100 μM $^{45}CaCl_2$. Transport was measured in the absence of inhibitor (○), or in the presence of 10 (●), 20 (▲), or 40 μM (■) DMB. Results are presented in an Eadie–Hofstee representation.

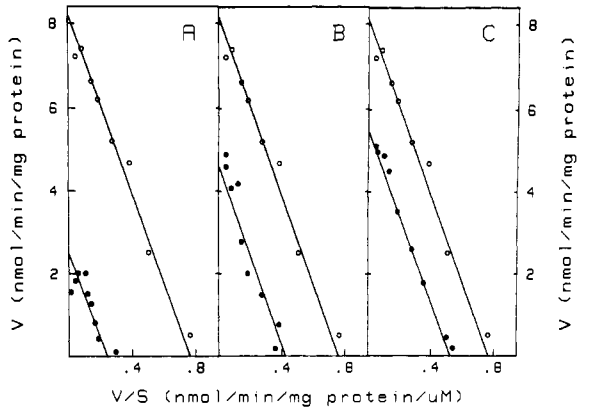


FIGURE 5: Kinetic analysis of inhibition of pituitary Na^+/Ca^{2+} exchange by amiloride, BNZ, and CBA. Initial rates of Na^+/Ca^{2+} exchange activity were monitored in the presence of 1–100 μM $^{45}CaCl_2$ and either 1 mM amiloride (A), 100 μM BNZ (B), or 20 μM CBA (C). Transport was determined in the absence (○) or the presence (●) of inhibitor. Results are represented in an Eadie–Hofstee plot.

protonation of DMB's guanidinium group at alkaline pH. Substitution of amiloride's terminal guanidino nitrogen with a hydroxyl group lowers the pK_a to 5.5. The pH profile of *N*-hydroxyamiloride, albeit a rather poor inhibitor of Na^+/Ca^{2+} exchange, indicates complete loss of activity at alkaline pH and recovery of activity as the pH of the medium becomes acidic. Similar results are obtained with other amiloride analogues. Substitution of both terminal guanidino nitrogen atoms with phenyl groups and replacement of the 5-amino nitrogen by a hydrogen [e.g., 3-amino-6-chloro-*N*-[(diphenylamino)methylene]pyrazinecarboxamide] lowers the pK_a below that of *N*-hydroxyamiloride (pK_a = 4.0). Examination of the pH profile of this inhibitor reveals that significant inhibition occurs only below pH 5.5 (Figure 3). These results are consistent with the notion that protonation of amiloride's guanidino group and hence the cationic form of the molecule is required for inhibitory activity.

Two classes of ion binding sites (i.e., a site that binds two Na^+ or one Ca^{2+} and another that binds the third Na^+ which is transported) have been proposed in the squid axon (Baker et al., 1969; Blaustein & Russell, 1975) and the cardiac (Reeves & Sutko, 1983; Slaughter et al., 1983) Na^+/Ca^{2+} exchange systems. Since Na^+/Ca^{2+} exchange in GH_3 vesicles has many similarities with the cardiac exchange system (e.g., both are electrogenic transport reactions, have similar apparent K_m's for Na^+ and Ca^{2+}, have identical pH profiles, and display a competitive interaction between Na^+ and Ca^{2+}; Kaczorowski et al., 1984), inhibitor action has been analyzed in terms of the model proposed for cardiac Na^+/Ca^{2+} exchange. A kinetic analysis of inhibitor action was undertaken to determine at which, if either, of the two cation binding sites these amiloride analogues interact. When initial rates of Na^+-dependent Ca^{2+} uptake were monitored as a function of extravesicular Ca^{2+} concentration, the data presented in Figure 4 were obtained. The initial rate data indicate that transport displays saturation kinetics with an apparent K_m for Ca^{2+} of 11 μM and V_{max} of 8.2 nmol of Ca^{2+} accumulated min^{-1} (mg of membrane protein)$^{-1}$. Addition of increasing amounts of DMB to the reaction medium prior to determining Ca^{2+} uptake results in a kinetic profile which displays parallel lines in an Eadie–Hofstee plot. This indicates that DMB is a noncompetitive inhibitor of transport with respect to Ca^{2+} (decrease in V_{max}; no change in apparent K_m) throughout a range of DMB concentrations. In addition, Ca^{2+} accumulations were monitored in the presence of concentrations of amiloride, BNZ, and CBA that produce ca. 50% inhibition of transport activity (Figure 5). In all cases, kinetic analyses indicate noncompetitive inhibition with respect to Ca^{2+} despite a 50-fold difference in inhibitor potencies. These results suggest that none of the inhibitors interact directly at the Ca^{2+} binding site on the carrier.

As previously noted, the Na^+/Ca^{2+} exchanger appears to possess two types of Na^+ binding sites. Since amiloride interacts noncompetitively with Ca^{2+}, it seems likely that any interaction at a Na^+ binding site on the pituitary carrier would occur where the third Na^+ is bound. To explore this possibility, several experimental approaches were taken. Examination of carrier-mediated Na^+/Na^+ exchange theoretically monitors the interaction between Na^+ and inhibitor. However, the passive leak of Na^+ in GH_3 plasma membrane vesicles is far greater than carrier-mediated Na^+ flux, making it technically difficult to analyze kinetically trans-Na^+ stimulated $^{22}Na^+$ fluxes. Measurements of Na^+-dependent Ca^{2+} efflux from Ca^{2+}-loaded vesicles could also be useful in analyzing Na^+-inhibitor interactions. Na^+-stimulated efflux in GH_3 vesicles is transient, probably due to rapid dissipation of the inwardly directed Na^+ gradient (Kaczorowski et al., 1984), again making it technically difficult to perform kinetic measurements. Nonetheless, in preliminary experiments, there was some indication that BNZ block of Na^+-induced Ca^{2+} efflux was reversed by elevating extravesicular Na^+. To quantitate the apparent protection by Na^+, GH_3 vesicles were passively equilibrated with varying concentrations of Na^+ from 50 to 400 mM, and aliquots of vesicles were then diluted into buffers containing equimolar K^+, saturating Ca^{2+} (40 μM), and a fixed concentration of DMB (10 μM). Measurement of initial transport rates as a function of intravesicular Na^+ indicates that as Na^+ is elevated, inhibition by DMB is progressively reversed (inhibition went from 70% to 5% in this range of ion concentrations). Similar data are obtained when the experiment is repeated in the presence of 20 μM CBA (data not shown). Control experiments performed with vesicles equilibrated in mixtures of Na^+ and K^+ (e.g., 100 mM Na^+ and 300 mM K^+) indicate that inhibition is not abolished by elevating K^+, indicating specificity in the reversal by Na^+ (i.e., this finding rules out relief from inhibition due to an ionic strength effect). These results provide evidence that amiloride-type compounds function as Na^+ analogues and competitively interact at a Na^+ binding site on the carrier. Given the kinetic data obtained with Ca^{2+}, it would appear that the interaction occurs at the third Na^+ binding site.

Inhibition of Na^+/Ca^{2+} Exchange in Other Membrane Vesicle Systems. To test whether the two classes of amiloride

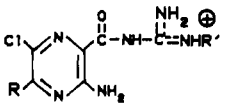
derivatives would have utility as inhibitors of the Na^+/Ca^{2+} antiport systems present in membranes of other tissues, a small number of compounds were selected for examination with bovine brain and porcine cardiac plasma membrane vesicles. Vesicles prepared from these tissues were passively equilibrated with 100 mM NaCl and initial rates of exchange activity monitored under V_{max} conditions in the presence of different amiloride analogues (Table III). Examination of these data reveals that amiloride analogues are effective in other systems. Comparison of inhibitory profiles in terms of a structure–activity relationship, however, reveals some small differences in inhibitor activity among the three tissues. While pituitary and heart are similar in terms of the maximum effectiveness of these compounds (i.e., an I_{50} of ca. 10 μM is achieved in each case), the rank order of inhibitor potencies is slightly different. For example, the analogue bearing a 1-naphthylmethyl group on the terminal guanidino nitrogen is the most effective inhibitor in heart vesicles but 3 times less potent in pituitary. In addition, the analogues bearing substituents on the terminal guanidino nitrogen are not quite as potent in inhibiting transport in brain as in pituitary vesicles, though the rank orders of activity are similar. Notable is the fact that the two analogues bearing substituents on the 5-amino nitrogen are markedly less potent in brain, thus indicating some differences between pituitary and brain Na^+/Ca^{2+} exchange. Nonetheless, in terms of the mechanism of inhibitor action, similarities exist between pituitary and heart (Slaughter et al., 1984; Cragoe et al., 1984) and between pituitary and brain (unpublished observations).

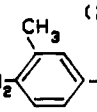
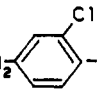
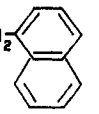
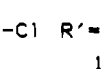
Discussion

The preceding study presents convincing evidence that analogues of amiloride are potent specific inhibitors of Na^+/Ca^{2+} exchange. These compounds clearly function as inhibitors in isolated pituitary plasma membrane vesicles. Data obtained by monitoring $^{45}Ca^{2+}$ equilibration in GH_3 cells are consistent with the interpretation that they are also effective in blocking Na^+-dependent Ca^{2+} clearance in the whole cell. Amiloride has previously been shown to inhibit Na^+/Ca^{2+} exchange in brain plasma membrane vesicles (Schellenberg et al., 1983), Friend Murine Erythroleukemia cells (Smith et al., 1982), and mitochondria derived from cardiac tissue (Jurkowitz et al., 1983). However, lack of potency and the proper selectivity has precluded it use in either mechanistic studies or evaluation of the physiological role of Na^+/Ca^{2+} exchange in intact tissues. With use of appropriate amiloride derivatives, these problems can be overcome. Two other compounds, adriamycin (Caroni et al., 1981) and amrinone (Mallov, 1983), have been reported to be inhibitors of cardiac Na^+/Ca^{2+} exchange, although the potency of amrinone was moderate at best. Neither of these compounds are active in blocking Na^+-dependent Ca^{2+} uptake in pituitary vesicles. In contrast, amiloride analogues function as inhibitors of exchange in heart sarcolemmal vesicles (Table III; Siegl et al., 1984) and in brain plasma membrane vesicles. This study thus represents the first description of potent inhibitors of Na^+/Ca^{2+} exchange which are functional in a number of different systems.

Two classes of amiloride analogues are effective inhibitors of pituitary Na^+/Ca^{2+} exchange. Analogues bearing substituents on the terminal guanidino nitrogen have been reported to be characteristically more active than amiloride in blocking passive Na^+ flux through non-tetrodotoxin-sensitive epithelial Na^+ channels (Cuthbert & Fanelli, 1978; Benos, 1982). Similarly, derivatives bearing substituents on the 5-amino nitrogen exceed amiloride in inhibitory potency when Na^+/H^+ exchange is monitored (Vigne et al., 1984; L'Allemain et al., 1984; Zhuang et al., 1984). Strikingly, both classes of analogues are effective inhibitors of Na^+/Ca^{2+} exchange, apparently without cross-reacting with the other Na^+ transport systems; i.e., guanidino-substituted derivatives usually do not

Table III: Effect of Various Amiloride Analogues on Na^+/Ca^{2+} Exchange in Rat Pituitary, Bovine Brain, and Porcine Heart Plasma Membrane Vesicles[a]



COMPOUND	% INHIBITION @ 100 uM (I_{50})		
	PITUITARY	BRAIN	HEART
R = $-NH_2$ R′ = H (AMILORIDE)	5	20	10
R = $-NH_2$ R′ = 	50	47	45
R = $-NH_2$ R′ = 	100 (20 uM)	80	100 (29 uM)
R = $-NH_2$ R′ =  (DMB)	100 (10 uM)	90 (40 uM)	100 (14 uM)
R = $-NH_2$ R′ = 	62	58	--
R = $-NH_2$ R′ = 	100 (30 uM)	100 (35 uM)	100 (17 uM)
R = $-NH_2$ R′ = 	100 (35 uM)	90 (45 uM)	100 (10 uM)
R =  R′ = H	100 (20 uM)	66	90 (40 uM)
R =  R′ = H	100 (20 uM)	57	90 (45 uM)

[a] Na^+/Ca^{2+} exchange activity was monitored in pituitary, brain, and heart preparations in the presence of different concentrations of amiloride analogues under V_{max} conditions. Inhibition was determined relative to control initial rates of transport of 7, 22, and 15 nmol of Ca^{2+} accumulated min^{-1} (mg of protein)$^{-1}$, respectively, in untreated vesicles. I_{50} values were determined graphically from titrations of inhibitor activity.

inhibit Na^+/H^+ exchange (Vigne et al., 1984; L'Allemain et al., 1984; Zhuang et al., 1984), and 5-amino-substituted analogues show reduced potency against Na^+ channels (Li & De Sousa, 1979; Benos, 1982). There are clear differences with respect to structure–activity relationships and potency of amiloride analogues in inhibiting each of the three Na^+-transporting systems. Amiloride itself is 3 orders of magnitude more potent in inhibiting Na^+ channels (Benos, 1982) than Na^+/Ca^{2+} exchange. Many of the guanidino-substituted analogues are effective at submicromolar concentrations in blocking Na^+ channels in frog skin and display a much different structural specificity for inhibitory action in this system (Cuthbert & Fanelli, 1978). The derivatives bearing 5-amino nitrogen substituents are different in terms of rank order and absolute potency of activity when inhibitory profiles for Na^+/H^+ exchange (Vigne et al., 1984; L'Allemain et al., 1984; Zhuang et al., 1984) and Na^+/Ca^{2+} exchange are compared. However, results obtained with Na^+/H^+ exchange have not been confirmed by direct measurements of inhibition in isolated plasma membrane vesicles and rely principally on whole cell flux data. The differences in activity among the various analogues most probably reflect individual characteristics of the three distinct Na^+ transport systems, and it is, therefore, quite remarkable that both guanidino and 5-amino nitrogen substituted analogues block Na^+/Ca^{2+} exchange.

It is apparent that the substituent changes on the guanidino and 5-amino nitrogen atoms which produce increased inhibitory activity are completely different. The guanidino derivatives show a much more rigid structural specificity for increased potency than the 5–amino nitrogen substituted analogues, whose activities increase with the general hydrophobicity of the substituent. Somewhat surprising is the fact that the mechanism by which these two inhibitor classes function seems to be indentical (i.e., kinetically they both appear to interact at the third Na^+ binding site, B site, of the carrier). A possible explanation arises from considering the nature of the interaction between inhibitor and carrier. If the cationic form of amiloride binds at one of the carrier's Na^+ binding sites, it might be expected that terminal guanidino nitrogen derivatives interact with those active site residues which comprise this binding site because of their proximity to the protonated guanidino group. Hence, the spatial or structural tolerance of guanidino derivatives that interact at the B site may be strict. In contrast, inhibitory patterns of the 5-amino nitrogen derivatives may be a consequence of a lipophilic interaction between inhibitors and the membrane. As hydrophobicity of the substitution increases, more inhibitor may partition into the membrane, resulting in a high local concentration of Na^+ analogue at the surface of the carrier. Germain to this point is the fact that these substitutions are distal to the guanidinium moiety so it is less likely that they are involved in a direct interaction with residues at the carrier's Na^+ binding site. Thus, the two structural classes may show increased activity over amiloride for different reasons, although, in the end, better inhibitory activity of each class simply reflects better competition with Na^+ for binding to the carrier.

The question of specificity in amiloride's action has been addressed in other systems. Amiloride itself, albeit at high concentrations, has been reported to be an inhibitor of protein synthesis (Lubin et al., 1982; Zhuang et al., 1984), Na^+,K^+-ATPase (Soltoff & Mandel, 1983; Zhuang et al., 1984), and protein kinase activity (Holland et al., 1983) as well as acting as an agent that efficiently collapses transmembrane ΔpH (interior acid) (Dubinsky & Frizzell, 1983). These properties could effectively obscure the interpretation of results with amiloride analogues in long-term metabolic studies using intact cells (Zhuang et al., 1984). However, except for the last mode of action, the studies with pituitary vesicles would not be affected by these complicating facets of amiloride action. If Na^+ dependent Ca^{2+} uptake in vesicles was due to the coupled action of Na^+/H^+–Ca^{2+}/H^+ exchange and, therefore, directly dependent on the formation of an intermediate ΔpH (interior acid), then the ability of amiloride to block Ca^{2+} uptake could be due to either collapse of the ΔpH or direct inhibition of Na^+/H^+ exchange. In pituitary (Kaczorowski et al., 1984) as in heart and brain, protonophores do not inhibit Na^+/Ca^{2+} exchange, thus ruling out the involvement of an intermediate ΔpH in this transport reaction. This would indicate that amiloride acts directly at the level of the Na^+/Ca^{2+} exchange carrier. The experiments in which passive membrane permeability or membrane conductance was monitored support the conclusion that amiloride analogues do not disrupt the osmotic integrity of the membrane. Inhibition by amiloride analogues is also reversed completely either by washing away inhibitor or by elevating Na^+ concentration. Reversibility is a strong indication that exposure of vesicles to these inhibitors does not result in premanent membrane damage. At inhibitor concentrations that completely block exchange, other transport systems, ionic conductance pathways, and membrane-bound receptor systems are not significantly affected. These observations, taken together with the defined structure–activity relationship for inhibitor action and the postulated mechanism of action, provide strong indications that amiloride inhibition of Na^+/Ca^{2+} exchange is a specific phenomenon which occurs by direct association of inhibitor with the antiporter.

The results of this study suggest that all amiloride derivatives function as Na^+ analogues, specifically interacting at the B site of the pituitary carrier and tying up the transporter in an inactive complex. The following lines of evidence support this idea. In order to function as an inhibitor, the acylguanidino moiety of amiloride must be protonated to the acylguanidinium form since only the cationic species is active. This is clearly apparent when analogues with low pK_a's were investigated and found to be functional only at acidic pHs. A similar requirement for the cationic form of the molecule has been shown in inhibition of epithelial Na^+ channels (Benos, 1982) and Na^+/H^+ exchange (Vigne et al., 1984). When the kinetics of the inhibition were determined, amiloride analogues functioned solely as noncompetitive inhibitors with respect to Ca^{2+}. Under the conditions of these experiments, mixed types of inhibition patterns were never observed. Thus, amiloride's interaction must be at a site distal to the Ca^{2+} binding site (A site). The finding that elevated Na^+ concentration can reverse inhibition suggests that inhibition is competitive with respect to Na^+. Given the model proposed for Na^+/Ca^{2+} exchange in heart (Reeves & Sutko, 1983), these data are most easily interpreted as an interaction between inhibitor and the B site of the carrier. Recent experiments from this laboratory with a photoaffinity label based on an amiloride-type structure are consistent with this interpretation (Kaczorowski et al., 1983b; unpublished results). These results can be contrasted with other data obtained by using quinacrine which has recently been shown to inhibit Na^+/Ca^{2+} exchange in cardiac sarcolemmal vesicles (De La Pena & Reeves, 1984). Quinacrine inhibition has a different spectrum of properties. In these studies, inhibition was shown to be competitive with respect to both Ca^{2+} and Na^+, and the interaction was localized to the A site of the carrier.

The mechanism by which amiloride analogues function to block Na^+/Ca^{2+} exchange in heart and brain appears to be similar to the one proposed from studies with pituitary vesicles. Data presented herein indicate that amiloride derivatives are indeed effective inhibitors in heart and brain. A detailed kinetic analysis of the action of benzamil in inhibiting Na^+/Ca^{2+} exchange in cardiac sarcolemmal vesicles supports our proposed mechanism of amiloride action (Slaughter et al., 1984; Cragoe et al., 1984; unpublished results). Moreover, kinetic studies with purified brain plasma membrane vesicles also clearly indicate that amiloride analogues are noncompetitive inhibitors with respect to Ca^{2+} (M. L. Garcia and G. Kaczorowski, unpublished observations). This last result should be contrasted with other reports where amiloride (Schellenberg et al., 1983) and various moderately effective amiloride analogues (Schellenberg et al., 1985) have been indicated to be competitive inhibitors with respect to Ca^{2+} in brain. The reason for the discrepency between these studies and our own is not clear at present. In any event, the utility of amiloride analogues as inhibitors of Na^+/Ca^{2+} exchange in a number of different systems suggests that these compounds could be useful tools in probing the physiological role of Na^+/Ca^{2+} exchange in intact tissues. Studies along these lines with cardiac muscle have begun (Siegl et al., 1984).

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Registry No. Amiloride (R = R′ = H), 2609-46-3; amiloride (R = R′ = Bu), 94889-77-7; amiloride [R = H, R′ = $C(CH_3)_2CH_2C(CH_3)_3$], 87243-91-2; amiloride (R = H, R′ = C_6H_5), 2038-35-9; amiloride [R = H, R′ = $-CH(CH_3)C_6H_5$], 1634-15-7; amiloride (R = H, R′ = 2,6-$Me_2C_6H_3$), 87243-92-3; amiloride (R = H, R′ = $CH_2C_6H_5$), 2898-76-2; amiloride (R = H, R′ = $CH_2CH_2C_6H_5$), 1163-46-8; amiloride (R = H, R′ = $CH_2C_6H_4$-*o*-Cl), 1163-44-6; amiloride (R = H, R′ = $CH_2C_6H_4$-*p*-CH_3), 1163-45-7; amiloride (R = H, R′ = $CH_2C_6H_3$-2,6-Cl_2), 90689-42-2; amiloride (N^5 = Et), 2235-96-3; amiloride (N^5 = Et_2), 2086-31-9; amiloride (N^5 = Bu), 1151-56-0; amiloride (N^5 = Pr, Bu), 1160-22-1; amiloride (N^5 = C_6H_5), 1158-04-9; amiloride (N^5 = $CH_2C_6H_5$), 1160-51-6; amiloride (N^5 = $CH_2CH_2C_6H_5$), 89270-03-1; amiloride (N^5 = $CH_2C_6H_4$-*p*-CH_3), 1163-58-2; amiloride [N^5 = $CH_2(CHOH)_4CH_2OH$], 1170-52-1; amiloride (R = H, R′ = $CH_2C_6H_3$-2,4-Me_2), 2093-13-2; amiloride (R = H, R′ = $CH_2C_6H_3$-3,4-Cl_2), 1166-01-4; amiloride (R = H, R′ = 1-$CH_2C_{10}H_7$), 14236-52-3; sodium, 7440-23-5; calcium, 7440-70-2.

References

Aceves, J., & Cuthbert, A. W. (1979) *J. Physiol. (London) 295*, 491–504.

Baker, P. F., Blaustein, M. P., Hodgkin, A. L., & Steinhardt, R. A. (1967) *J. Physiol. (London) 192*, 43p–44p.

Baker, P. F., Blaustein, M. P., Hodgkin, A. L., & Steinhardt, R. A. (1969) *J. Physiol. (London) 200*, 431–458.

Barros, F., & Kaczorowski, G. J. (1984) *J. Biol. Chem. 259*, 9404–9410.

Barros, F., Katz, G., Vandlen, R., Kaczorowski, G., & Reuben, J. P. (1984) *Biophys. J. 45*, 307a.

Barry, W. H., & Smith, T. W. (1982) *J. Physiol. (London) 325*, 243–260.

Benos, D. J. (1982) *Am. J. Physiol. 242*, C131–C145.

Blaustein, M. P. & Russell, J. M. (1975) *J. Membr. Biol. 22*, 285–312.

Caroni, P., Villani, F., & Carafoli, E. (1981) *FEBS Lett. 130*, 184–186.

Cragoe, E. J., Jr. (1983) in *Diuretics, Chemistry, Medicine and Pharmacology*, Chapter 6, pp 303–342, Wiley, New York.

Cragoe, E. J., Jr., & Shepard, K. L. (1971) U.S. Patent 3 577 418.

Cragoe, E. J., Jr., Woltersdorf, O. W., Jr., Bicking, J. B., Kwong, S. F., & Jones, J. H. (1967) *J. Med. Chem. 10*, 66–75.

Cragoe, E. J., Jr., Woltersdorf, O. W., Jr., & De Solms, S. J. (1981) U.S. Patent 4 246 406.

Cragoe, E., Kaczorowski, G. J., Reeves, J. P., & Slaughter, R. S. (1984) *J. Physiol. (London) 353*, 74P.

Cuthbert, A. W. (1981) in *Ion Transport by Epithelia* (Schultz, S. G., Ed.) pp 181–196, Raven Press, New York.

Cuthbert, A. W., & Fanelli, G. M. (1978) *Br. J. Pharmacol. 63*, 139–149.

De La Pena, P., & Reeves, J. P. (1984) *Biophys. J. 45*, 81a.

Dethmers, J. K., Cragoe, E. J., Jr., & Kaczorowski, G. J. (1983) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2847.

Dubinsky, J. M., & Oxford, G. S. (1984) *J. Gen. Physiol. 83*, 309–339.

Dubinsky, W. P., Jr., & Frizzell, R. A. (1983) *Am. J. Physiol. 245*, C157–C159.

Frelin, C., Vigne, P., & Lazdunski, M. (1983) *J. Biol. Chem. 258*, 6272–6276.

Gill, D. L., Grollman, E. F., & Kohn, L. D. (1981) *J. Biol. Chem. 256*, 184–192.

Hagiwara, S., & Ohmori, H. (1982) *J. Physiol. (London) 331*, 231–252.

Holland, R., Woodgett, J. R., & Hardie, D. G. (1983) *FEBS Lett. 154*, 269–273.

Jurkowitz, M. S., Altschuld, R. A., Brierley, G. P., & Cragoe, E. J., Jr. (1983) *FEBS Lett. 162*, 262–265.

Kaczorowski, G. J., Vandlen, R. L., Katz, G. M., & Reuben, J. P. (1983a) *J. Membr. Biol. 71*, 109–118.

Kaczorowski, G. J., Dethmers, J. K., & Cragoe, E. J., Jr. (1983b) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 42*, 2849.

Kaczorowski, G. J., Costello, L., Dethmers, J. K., Trumble, M. J., & Vandlen, R. L. (1984) *J. Biol. Chem. 259*, 9395–9403.

Katz, G., Barros, F., Kaczorowski, G., Vandlen, R., & Reuben, J. P. (1984) *Biophys. J. 45*, 307a.

Kinsella, J. L., & Aronson, P. S. (1980) *Am. J. Physiol. 238*, F461–F469.

Kinsella, J. L., & Aronson, P. S. (1981) *Am. J. Physiol. 241*, F374–F379.

L'Allemain, G., Franchi, A., Cragoe, E. J., Jr., & Pouyssegur, J. (1984) *J. Biol. Chem. 259*, 4313–4319.

Langer, G. A. (1982) *Annu. Rev. Physiol. 44*, 435–450.

Li, J. H., & De Sousa, R. C. (1979) *J. Membr. Biol. 46*, 155–169.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Lubin, M., Cahn, F., & Coutermarsh, B. A. (1982) *J. Cell. Physiol. 113*, 247–251.

Mallov, S. (1983) *Res. Commun. Chem. Pathol. Pharmacol. 41*, 197–201.

Paris, S., & Pouyssegur, J. (1983) *J. Biol. Chem. 258*, 3503–3508.

Reeves, J. P., & Sutko, J. L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 590–594.

Reeves, J. P., & Sutko, J. L. (1983) *J. Biol. Chem. 258*, 3178–3182.

Reuter, H., & Seitz, N. (1968) *J. Physiol. 195*, 451–470.
Schellenberg, G. D., & Swanson, P. D. (1981) *Biochim. Biophys. Acta 648*, 13–27.
Schellenberg, G. D., Anderson, L., & Swanson, P. D. (1983) *Mol. Pharmacol. 24*, 251–258.
Schellenberg, G. D., Anderson, L., Cragoe, E. J., Jr., & Swanson, P. D. (1985) *Mol. Pharmacol.* (in press).
Shepard, K. L., & Cragoe, E. J., Jr. (1971) U.S. Patent 3 573 306.
Siegl, P. K. S., Cragoe, E. J., Jr., Trumble, M. J., & Kaczorowski, G. J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3238–3242.
Slaughter, R. S., Sutko, J. L., & Reeves, J. P. (1983) *J. Biol. Chem. 258*, 3183–3190.
Slaughter, R. S., De La Pena, P., Reeves, J. P., Cragoe, E. J. Jr., & Kaczorowski, G. J. (1984) *Biophys. J. 45*, 81a.
Smith, R. L., Macara, I. G., Levenson, R., Housman, D., & Cantley, L. (1982) *J. Biol. Chem. 257*, 773–780.
Soltoff, S. P., & Mandel, L. J. (1983) *Science (Washington, D.C.) 20*, 957–959.
Vandlen, R. L., Sarcione, S. L., & Telakowski, C. A. (1981) *Biochim. Biophys. Acta 649*, 595–607.
Vigne, P., Frelin, C., & Lazdunski, M. (1982) *J. Biol. Chem. 257*, 9394–9400.
Vigne, P., Frelin, C., Cragoe, E. J., Jr., & Lazdunski, M. (1984) *Mol. Pharmacol. 25*, 131–136.
Zhuang, Y.-X., Cragoe, E. J., Jr., Shaikewitz, T., Glaser, L., & Cassel, D. (1984) *Biochemistry 23*, 4481–4488.

Kinetics of Cholesterol and Phospholipid Exchange from Membranes Containing Cross-Linked Proteins or Cross-Linked Phosphatidylethanolamines†

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ABSTRACT: Mono- and dipalmitoylphosphatidylethanolamine derivatives have been synthesized and used to evaluate the role of cross-links between the amino groups of two phospholipid molecules in the rate of cholesterol movement between membranes. Incorporation of the cross-linked phospholipids into small unilamellar vesicles (the donor species) decreased the rate of spontaneous cholesterol exchange with acceptor membranes (small unilamellar vesicles or *Mycoplasma gallisepticum* cells). These results suggest that the cross-linking of aminophospholipids by reactive intermediates, which may be one of the degenerative transformations associated with peroxidation of unsaturated lipids and cellular aging, can inhibit cholesterol exchangeability in biological membranes. The rates of spontaneous [^{14}C]cholesterol and protein-mediated ^{14}C-labeled phospholipid exchange from diamide-treated mycoplasma and erythrocyte membranes have also been measured. The formation of extensive disulfide bonds in the membrane proteins of *M. gallisepticum* enhanced the ^{14}C-labeled phospholipid exchange rate but did not affect the rate of [^{14}C]cholesterol exchange. The rates of radiolabeled cholesterol and phospholipid exchange between erythrocyte ghosts and vesicles were both enhanced (but to different extents) when ghosts were treated with diamide. These observations suggest that diamide-induced oxidative cross-linking of sulfhydryl groups in membrane proteins does not lead to random defects in the lipid domain.

Lipid peroxidation and protein cross-linking are two factors that influence the rates of lipid translocation in membranes. Peroxidation of unsaturated fatty acyl chains alters the physical properties of the lipids and can also cause covalent modifications in proteins and lipids by reactions involving reactive intermediates. If critical levels of peroxidation products are present, the rates of lipid transbilayer movement are stimulated (Shaw & Thompson, 1982). It has been proposed that nonbilayer lipid structures are produced from cross-linking of membrane lipids (Barsukov et al., 1980). Cholesterol was found to reduce the rate of lipid movement in some membranes exposed to peroxidation, possibly by stabilizing the bilayer (Shaw & Thompson, 1982) or suppressing the extent of peroxidation (Mowri et al., 1984). Malondialdehyde, which is formed during peroxidation of unsaturated lipids, can cross-link a variety of biological macromolecules, including the amino groups of phospholipids and proteins through Schiff base formation (Jain & Hochstein, 1980; Rice-Evans & Hochstein, 1981; Jain & Shohet, 1982). There has been considerable interest in the effects of malondialdehyde on biological systems since it has been suggested that cross-linking of macromolecules contributes to cellular aging and other degenerative processes [e.g., Bland (1978)]. Although the structure of the product from the reaction of phosphatidylethanolamine (PE)[1] with malondialdehyde has not been established (Bidlack & Tappel, 1973; Shimasaki et al., 1984),

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[1] Abbreviations: FNPS, bis(4-fluoro-3-nitrophenyl) sulfone; PBS, 150 mM NaCl, 5 mM KCl, 5.5 mM Na_2HPO_4, 0.8 mM NaH_2PO_4, and 0.5 mM $CaCl_2$, pH 7.4; PDIT, 1,4-phenylene diisothiocyanate; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; nitrosulfone-mono-PE, 4-(α,β-dipalmitoylphosphatidylethanolamino)-3,3′-dinitro-4′-hydroxydiphenyl sulfone; nitrosulfone-di-PE, bis[4-(α,β-dipalmitoylphosphatidylethanolamino)-3-nitrophenyl] sulfone; α,β- or α,γ-PDIT-mono-PE, *N*-[4-[(α,β- or α,γ-dipalmitoylphosphatidylethanolamino)thioformamido]phenyl]thiourea; α,β- or α,γ-PDIT-di-PE, *N*,*N*′-1,4-phenylenebis[(α,β- or α,γ-dipalmitoylphosphatidylethanolamino)thioformamide]; 1,4-xylylene-di-PE, *N*,*N*′-1,4-xylylenebis(α,β-dipalmitoylphosphatidylethanolamine); STM, 0.40 M sucrose, 50 mM tris(hydroxymethyl)aminomethane, and 20 mM $MgCl_2$, pH 7.4; SUV, small unilamellar vesicles; SDS, sodium dodecyl sulfate.

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