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Inhibition of Synaptosomal Membrane Na^+-Ca^{2+} Exchange Transport by Amiloride and Amiloride Analogues

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SUMMARY

Na^+-Ca^{2+} exchange in rat brain synaptosomal plasmalemma vesicles is reversibly inhibited by amiloride (3,5-diamino-6-chloro-*N*-(diaminomethylene)pyrazinecarboxamide). This drug (pK_a = 8.7) inhibits Na^+-dependent Ca^{2+} uptake more effectively at basic pH values than at neutral pH values, indicating that the positively charged form of amiloride is the active moiety. Twenty amiloride analogues were examined for ability to inhibit Na^+-Ca^{2+} exchange. These studies demonstrate that the 6-chloro group, the 5-amino substituent, and the carbonyl guanidinium moiety are essential for drug inhibition of Na^+-Ca^{2+} exchange. *N*-Benzyl amiloride derivatives such as 3,5-diamino-6-chloro-*N*-(benzylamino-aminomethylene)pyrazinecarboxamide (benzamil) and 3,5-diamino-6-chloro-*N*-(2-phenethylamino-aminomethylene)pyrazinecarboxamide are more potent inhibitors of Na^+-dependent Ca^{2+} uptake than is amiloride. The amiloride analogue pattern of interaction with the Na^+-Ca^{2+} exchange system is distinct from the inhibition patterns of the epithelial Na^+ channel and the Na^+-H^+ exchange transport system.

INTRODUCTION

The synaptic plasma membrane contains a Na^+-Ca^{2+} exchange transport mechanism that is thought to be involved in the regulation of cytoplasmic Ca^{2+} levels. This transport system is capable of mediating Ca^{2+} efflux coupled to an inward directed Na^+ gradient. Recently, using vesicles derived from rat brain synaptic plasma membranes, we demonstrated that amiloride inhibits Na^+-Ca^{2+} exchange but not ATP-dependent Ca^{2+} transport (1). The drug acts as a competitive inhibitor with respect to Ca^{2+} (K_i = 300 μM) and probably interacts directly with the Na^+-Ca^{2+} exchange carrier. Amiloride also inhibits other Na^+ transport systems (2). Na^+ fluxes across frog skin (3), toad bladder (4), and other Na^+-transporting tight epithelia (5, 6) are amiloride sensitive. In these systems, the drug acts at the mucosal surface of the cells by inhibiting a Na^+ channel. Drug binding to this channel is extremely tight with K_i values ranging from 1.0 to 0.1 μM (7), depending on the assay conditions. Benzamil, a more potent benzyl derivative of amiloride, appears to inhibit epithelial Na^+ fluxes by the same mechanism (8, 9). Other transport systems affected by amiloride include the ubiquitous Na^+-H^+ exchange mechanism (10) and the Na^+,K^+-ATPase (11).

In the present study, we examined the effects of amiloride, benzamil, and other amiloride analogues on synaptic plasmalemma Na^+-Ca^{2+} exchange transport. This work was undertaken to determine the mechanism by which these drugs inhibit the Na^+-Ca^{2+} exchange carrier and to define the characteristics of the amiloride-Na^+-Ca^{2+} carrier interaction site. Structural analogues of amiloride were examined for inhibition of Na^+-Ca^{2+} exchange to determine which constituents are necessary for drug interactions with the carrier. Several amiloride analogues were found which are more potent inhibitors of Na^+-Ca^{2+} exchange transport than is amiloride. Studies of the inhibitory properties of the compounds as a function of pH indicate that the positively charged forms of these drugs inhibit Na^+-Ca^{2+} exchange.

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EXPERIMENTAL PROCEDURES

Preparation of membrane vesicles. Synaptosomes were prepared from whole rat brain by the procedure of Bradford (12). Synaptosomal plasma membrane vesicles were obtained by osmotically lysing synaptosomes using the procedure of Gill *et al.* (13).

Na^+-Ca^{2+} exchange transport assay. Na^+-dependent $^{45}Ca^{2+}$ transport was assayed in synaptosomal plasmalemma vesicles essentially as previously described (14). Vesicles 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 vesicle membranes. Unless indicated otherwise, 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/nmol) in a final volume of 150 μl. For experiments where the assay pH was varied, Tris-maleate was used in place of Tris/HCl as the buffer. 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/assay) into the reaction media. Uptake was terminated by the addition of 5 mM EDTA followed by

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rapid filtration through a nitrocellulose filter (Schleicher and Schuell, 0.45 μm). The filter was washed three times with 3-ml aliquots of 160 mM KCl, 20 mM Tris/HCl (pH 7.4). At 23° and 10 μM $^{45}Ca^{2+}$, uptake was linear for approximately 5–10 sec. In most experiments for the sake of reproducibility, initial rates were approximated by measuring uptake at 10 sec.

ATP-dependent Ca^{2+} uptake by synaptosomal membrane vesicles was assayed in 160 mM KCl, 20 mM Tris/HCl (pH 7.4), 2.5 mM $MgCl_2$, 10 μM $^{45}Ca^{2+}$, 0.1 mM ouabain, 0.2 mM dinitrophenol, 0.2 mM NaN_3, 0.15 μg/ml oligomycin, and either 2 mM Tris/ATP, 2 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 (14).

The concentration of vesicles used in uptake assays (0.05–0.1 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.* (15) as modified by Bailey (16). Bovine serum albumin was used as a standard.

Materials. The compounds used in the study were prepared by previously described methods (17–23).

RESULTS

Na^+-Ca^{2+} exchange transport was studied in membrane vesicles prepared from osmotically shocked cerebral cortex synaptosomes. These vesicles are resealed membrane fragments derived from the synaptic plasma membrane (13, 24). When Na^+-loaded vesicles are diluted into Na^+-free media, Ca^{2+} is rapidly taken up by a time-dependent process. Little Ca^{2+} is accumulated if no Na^+ gradient is present. Control experiments using EGTA[1] and A23187 show that the Ca^{2+} associated with the membranes in response to a Na^+ gradient is present in the lumen of the vesicles (data not shown). We recently demonstrated that amiloride inhibits Na^+-dependent Ca^{2+} uptake and Na^+-dependent Ca^{2+} release using synaptosomal plasma membrane vesicles (1).

Inhibition of Na^+-Ca^{2+} exchange by amiloride analogues. The effects of amiloride analogues on Na^+-Ca^{2+} exchange were examined to determine which constituents of the drug are required for inhibition and to find more potent Na^+-Ca^{2+} exchange inhibitors. Na^+-dependent Ca^{2+} uptake was measured at pH 7.4 in the presence of 10 μM $^{45}Ca^{2+}$, and $K_{0.5}$ values were determined (Fig. 1 and Table 1). Under these conditions, the $K_{0.5}$ for amiloride is 0.4 mM.

The importance of the 6-chloro substituent of amiloride was examined using compounds B–E (Table 1). Replacement of the 6-chloro group of amiloride by a 6-H, 6-fluoro, 6-bromo, or 6-iodo (compounds B–E) results in reduced inhibition with $K_{0.5}$ values of 1.5 mM or greater (Table 1). Although the pK_a values of these derivatives range from 8.7 to 9.3, all are essentially fully protonated at the pH of the uptake assay. Thus, differing degrees of protonation do not explain the observed differences in inhibition. Further, the order of inhibition (Cl ≫ Br ≅ I > H ≅ F) does not follow the order of the pK_a values (H > F > I > Br > Cl).

Analogues with different substituents at the 5-position of the pyrazine ring also were tested for inhibitory activity. Addition of methyl groups to the 5-amino moiety has little effect on drug activity; the 5-*N*,*N*-dimethyl derivative of amiloride, compound J ($K_{0.5}$ = 0.5 mM), is only slightly less potent than amiloride. Complete removal of the 5-amino group and replacement with a hydrogen

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

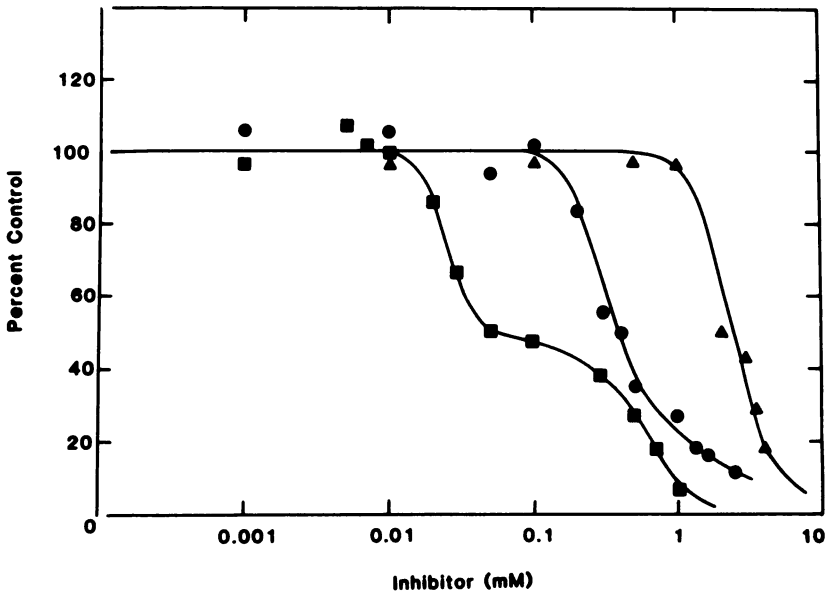


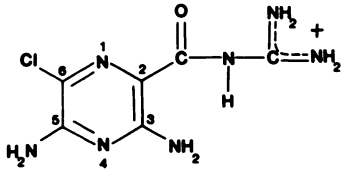
FIG. 1. *Inhibition of Na^+-Ca^{2+} exchange by compound R, amiloride, and compound B*

Na^+-dependent Ca^{2+} uptake was assayed in the presence of varying concentrations of compound R (■), amiloride (●), or compound B (▲) by diluting Na^+-loaded vesicles into 10 μM $^{45}Ca^{2+}$, 20 mM Tris-HCl (pH 7.4), and either 160 mM KCl or 160 mM NaCl. Ca^{2+} accumulated in the presence of NaCl was subtracted as the control from uptake in the presence of KCl. Assays were performed at 23° and terminated after 10 sec as described under Experimental Procedures. Each data point is the average of three determinations performed on the same day. The above data are representative of two other experiments performed on different days with other vesicle preparations.

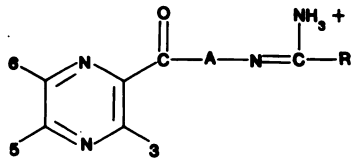
TABLE 1

Amiloride analogue inhibition of Na^+-Ca^{2+} exchange in synaptosomal membrane vesicles

Na^+-dependent Ca^{2+} uptake was assayed as described in Fig. 1 and under Experimental Procedures.



Amiloride

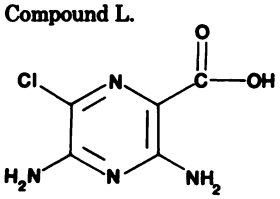


Compound	Pyrazine ring constituents			A	R	pK_a[a]	$K_{0.5}$[b]
	6	5	3				
							mM
A (amiloride)	Cl	NH_2	NH_2		NH_2	8.67	0.4
B	H	NH_2	NH_2		NH_2	9.30	3.5
C	F	NH_2	NH_2		NH_2	9.0	4.0
D	Br	NH_2	NH_2		NH_2	8.72	1.5
E	I	NH_2	NH_2		NH_2	8.85	1.6
F	Cl	H	NH_2		NH_2	7.03	3.0
G	Br	H	NH_2		NH_2	7.1	2.7
H	H	H	NH_2		NH_2		5.0
I	H	H	H		NH_2		>5.0
J (MK-685)	Cl	$N(CH_3)_2$	NH_2		NH_2		0.5
K (guanidine)							>5.0
L[c]	Cl	NH_2	NH_2				3.5
M[d]	Cl	NH_2	NH_2				4.0
N	Cl	NH_2	NH_2	NH	NH_2	9.00	No inhibition
O	Cl	H	NH_2		$NHCH_3$		4.5
P (phenamil)	Cl	NH_2	NH_2		NHC_6H_5		0.40[e]
Q (benzamil)	Cl	NH_2	NH_2		$NHCH_2C_6H_5$	8.10	0.27[e]
R	Cl	NH_2	NH_2		$NHCH_2CH_2C_6H_5$		0.05[e]
S	Cl	NH_2	NH_2		$NHCHC_6H_5$ (CH$_3$)		0.25[e]
T	Cl	H	NH_2		$NHCH_2CH_2C_6H_5$		>2
U	Cl	$NHCH(CH_3)_2$	NH_2		$N(CH_3)_2$	7.85	3.0

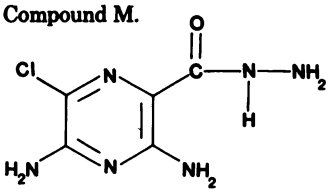
[a] The pK_a values of the amiloride analogues were determined in 30% ethanol by Merck Sharp and Dohme. The pK_a values of guanidine (36) and amiloride (27) were determined in aqueous solution.

[b] $K_{0.5}$ is the drug concentration giving half-maximal inhibition.

[c] Compound L.



[d] Compound M.



[e] Inhibition curves were biphasic.

atom result in a loss of inhibitory activity. Analogues F, G, and H are all less active compared to amiloride (Table 1). Similarly, experiments with compounds bearing a 2-phenethyl group on the terminal guanidine nitrogen atom (derivatives R and T) also indicate that the 5-amino group is important for drug-carrier interaction; analogue T, the 5-H derivative, is a very poor inhibitor compared to the analogous 5-amino compound, derivative R (Table 1).

The role of the guanidino moiety in the inhibition observed with amiloride and its analogues was examined using a variety of compounds. Guanidine (compound K) itself is not an inhibitor. Replacement of the positively charged carbonyl guanidinium moiety with a carboxyl group (compound L) or a hydrazinocarbonyl group (compound M) reduces the amount of inhibition observed. Insertion of a NH group between the carbonyl and the guanidino groups (compound N) abolishes the inhibitory activity of the compound without greatly altering the pK_a. These data indicate that the guanidino moiety is essential for inhibition and that the distance of this moiety from the pyrazine ring is critical.

Compounds bearing aryl or aralkyl substituents on the terminal nitrogen of the guanidino group (compounds P–S) are better inhibitors of Na^+-Ca^{2+} exchange than is amiloride (Table 1). The most potent drug tested was

compound R, the 2-phenethyl derivative. Inhibition by these compounds was consistently biphasic (for example, see Fig. 1). When inhibition by compounds P (phenamil), Q (benzamil), R, and S was analyzed by the linear transformation method of Dixon (Ref. 25, using analogue concentrations between 0.05 and 1 mM and Ca^{2+} concentrations of 10 and 50 μM), biphasic plots were obtained and valid K_i values could not be calculated (data not shown). Similar experiments with amiloride as the inhibitor yielded linear plots at drug concentrations up to 2.5 mM (1). The cause of the observed biphasic inhibition pattern is not presently known and is under study. The biphasic nature of the curves may indicate that drug interaction with the carrier is cooperative.

One possible explanation for the increased potency of the aryl and aralkyl derivatives is their increased hydrophobicity. For example, the distribution ratios of amiloride and benzamil in a chloroform/aqueous pH 7.4 buffer mixture are 0.01 and 2.01, respectively (26). The hydrophobic nature of these aryl and aralkyl derivatives could result in increased partitioning of these drugs into the vesicle membranes, thereby allowing better drug access to the Na^+-Ca^{2+} carrier. However, compound T, a hydrophobic 2-phenethyl derivative which lacks a 5-amino group on the pyrazine ring, has a chloroform/buffer distribution ratio of 1.22 (26) but is not a good inhibitor. Likewise, compound U, which partitions almost exclusively into chloroform (26) but does not have an aryl or aralkyl group, is a poor inhibitor. Therefore, hydrophobicity alone is not sufficient for effective inhibition.

The aryl and aralkyl amiloride derivatives presumably inhibit Na^+-Ca^{2+} exchange by interacting with the Ca^{2+} carrier. However, these compounds could inhibit uptake by increasing the permeability of the vesicles to Ca^{2+}, thereby causing accumulated Ca^{2+} to be released. To test this possibility, vesicles were loaded with $^{45}Ca^{2+}$ by Na^+-Ca^{2+} exchange. Uptake was terminated by the addition of EDTA, and Ca^{2+} efflux was monitored for 10 min. As shown in Fig. 2, analogue R did not affect the rate of passive Ca^{2+} efflux. Ca^{2+} efflux from these loaded vesicles could be induced by adding extravesicular Na^+ (Fig. 2). This Ca^{2+} efflux is due to Na^+-Ca^{2+} exchange operating in the reverse direction. Compound R inhibited Na^+-dependent Ca^{2+} efflux. Similar results have also been obtained with amiloride (1).

Effect of pH on analogue inhibition of Na^+-Ca^{2+} exchange. The pK_a of amiloride is 8.7 (27) and at pH 7.4 the drug is primarily present as a positively charged moiety. However, at neutral pH, a finite fraction of the compound is present in the uncharged form. To determine whether synaptosomal plasmalemma Na^+-Ca^{2+} exchange is inhibited by the charged or uncharged drug, we examined amiloride inhibition of Na^+-Ca^{2+} exchange as a function of pH. As previously reported for Na^+-Ca^{2+} exchange for heart sarcolemma vesicles (28), Na^+-dependent Ca^{2+} uptake by synaptosomal membrane vesicles is highly dependent on the pH of the assay medium; Na^+-dependent Ca^{2+} uptake increases dramatically with increasing pH while Na^+-independent Ca^{2+} uptake is not affected (data not shown). Amiloride inhibition is also pH-dependent. At pH 7.0, when the drug is primarily in the cationic form, 0.5 mM amiloride inhibits uptake by 44%, while at pH 9.5 the same concentration of drug had little effect on uptake (Table 2). Analogue R was also a significantly better inhibitor at pH 7 then at pH 9.5. The other analogues tested (Q, T, and U) were also more effective at the lower pH value but the difference in drug activity was not statistically significant (Table 2). These data indicate that the protonated forms of these compounds are the Na^+-Ca^{2+} exchange inhibitors. Since the positive charge is localized on the guanidino moiety of amiloride, the guanidinium side chain must be involved in drug binding to the Na^+-Ca^{2+} carrier.

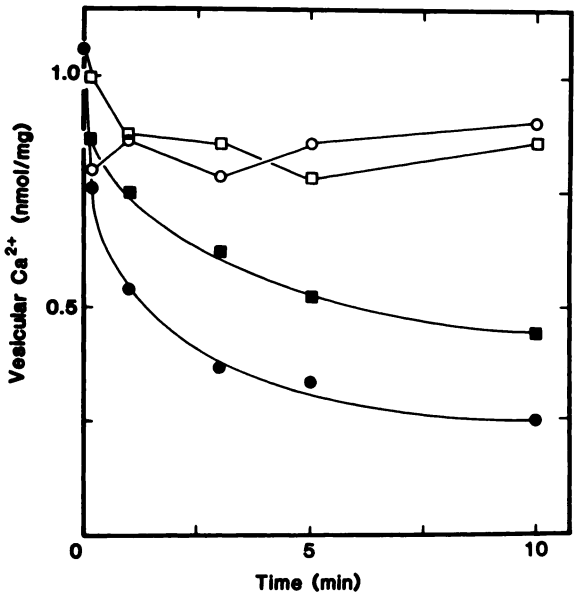


FIG. 2. *Effect of compound R on vesicular Ca^{2+} permeability and on Na^+-dependent Ca^{2+} efflux*

Na^+-loaded synaptosomal membrane vesicles were diluted 30-fold into Na^+-free media (160 mM KCl, 20 mM Tris/HCl, pH 7.4, 2 μM $^{45}Ca^{2+}$) and allowed to accumulate $^{45}Ca^{2+}$ for 20 min. Uptake was terminated by the addition of EDTA (5 mM final concentration). The $^{45}Ca^{2+}$ content of the vesicles was assayed over the next 10 min at the indicated times. Ca^{2+} efflux was stimulated in some samples by the addition of NaCl (36 mM final concentration). Additions at time zero were none (○), 100 μM compound R (□), 36 mM NaCl (●), and 36 mM NaCl, 100 μM compound R (■). The background was taken as the $^{45}Ca^{2+}$ associated with Na^+-loaded vesicles diluted into Na^+-containing media under similar conditions. The values presented are the average of three determinations performed on the same day with the same vesicle preparation. The above data are representative of two other experiments performed on different days with other vesicle preparations.

Effect of membrane potential on analogue inhibition of Na^+-Ca^{2+} exchange. In heart sarcolemma vesicles, Na^+-dependent Ca^{2+} uptake is electrogenic (29) and 3 Na^+ ions are exchanged per Ca^{2+} ion (30). Conditions (or drugs) which generate an interior negative potential inhibit Na^+-dependent Ca^{2+} uptake. A possible mechanism by which amiloride and aryl and aralkyl amiloride analogues could inhibit Na^+-dependent Ca^{2+} uptake is by altering the potential across the vesicle membrane. To test whether membrane potential affects drug inhibition, we examined the effects of valinomycin on Na^+-dependent Ca^{2+} uptake in the presence and absence of amiloride, benzamil, and compound R (Table 3). In the standard

TABLE 2

Effect of pH on amiloride and amiloride analogue inhibition of Na^+-Ca^{2+} exchange in synaptosomal membrane vesicles

Na^+-dependent Ca^{2+} uptake was determined as described under Experimental Procedures. Na^+-loaded synaptosomal membrane vesicles were diluted into 10 μM $^{45}Ca^{2+}$, 20 mM Tris-maleate buffer (pH 7.0 or 9.5) and either 160 mM KCl or NaCl. Stock solutions of the added drugs were adjusted to the indicated pH prior to addition to the assay. Uptake was assayed over a period of 10 sec. Ca^{2+} uptake by Na^+-loaded vesicles diluted into NaCl media was taken as the background. The values presented are the average of data from three different vesicle preparations assayed on the same day ± the standard deviation. The data were analyzed by two-way variance analysis. The effect of each drug at the two pH values was compared by contrasts. Amiloride and compound R were significantly less effective at pH 9.5 than at pH 7.0 and the level of significance is indicated in the footnotes.

Addition	Concentration	Na^+-dependent Ca^{2+} uptake			
		pH 7.0		pH 9.5	
	mM	*nmol/mg/min*	*% control*	*nmol/mg/min*	*% control*
None		2.37 ± 0.27	100	3.91 ± 0.67	100
Amiloride[a]	0.5	1.05 ± 0.03	44	3.74 ± 0.51	96
Q	0.5	0.40 ± 0.12	17	1.04 ± 0.16	27
R[b]	0.25	0.70 ± 0.04	30	2.23 ± 0.21	57
T	2	1.58 ± 0.30	67	3.38 ± 0.43	86
U	2	1.38 ± 0.11	58	3.16 ± 0.12	81

[a] $p > 0.01$.
[b] $p > 0.025$.

TABLE 3

Effect of valinomycin on the inhibition of synaptosomal membrane vesicle Na^+-Ca^{2+} exchange by amiloride, benzamil, and compound R

Na^+-dependent Ca^{2+} uptake in synaptosomal membrane vesicles was measured as described under Experimental Procedures. Uptake was assayed in the presence of 10 μM $^{45}Ca^{2+}$ over a period of 5 sec. Valinomycin dissolved in ethanol was added to a final concentration of 10 μM. An equal volume of ethanol was added to control assays. Na^+-dependent uptake in the absence of a Na^+ gradient was subtracted as the control. The values presented are the average of data from three different vesicle preparations assayed on the same day ± the standard deviation.

Addition	Na^+-dependent Ca^{2+} uptake			
	−Valinomycin		+Valinomycin	
	nmol/mg/min	*% control*	*nmol/mg/min*	*% control*
None	3.49 ± 0.70	100	4.60 ± 0.64	132
Amiloride	1.15 ± 0.18	33	1.63 ± 0.33	47
Q (benzamil)	0.44 ± 0.52	13	−0.048[a] ± 0.27	−1.4
R	−0.096[a] ± 0.33	−2.8	0.29 ± 0.19	8.3

[a] The above values are Ca^{2+} uptake in KCl media minus Ca^{2+} uptake in NaCl media. Negative values were obtained when the NaCl value was larger than the KCl value.

Ca^{2+} uptake assay, an inward-directed K^+ gradient is generated when the vesicles are diluted into KCl medium; addition of valinomycin generates an interior positive diffusion potential which should stimulate Na^+-Ca^{2+} exchange and destroy any drug-induced negative potential. As shown in Table 3, valinomycin stimulates Na^+-Ca^{2+} exchange, indicating that uptake is electrogenic. Amiloride, benzamil, and compound R are inhibitory in the presence of valinomycin, demonstrating that these drugs do not inhibit Ca^{2+} uptake by altering the membrane potential. Valinomycin does stimulate Ca^{2+} uptake slightly in the presence of these compounds which is consistent with the stimulation seen in the absence of inhibitors.

ATP-dependent Ca^{2+} transport in membrane vesicles. In addition to the Na^+-Ca^{2+} exchange system, synaptosomal plasma membranes contain an ATP-dependent Ca^{2+} transport mechanism. Both Ca^{2+} transport systems

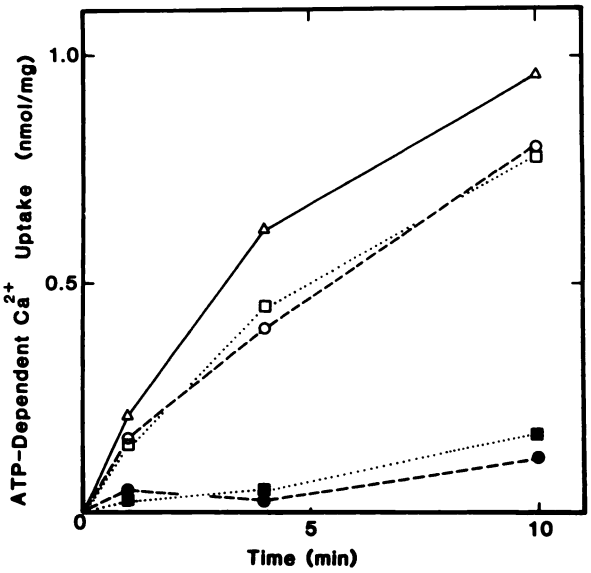


FIG. 3. *The effects of compound R and benzamil on ATP-dependent Na^+ uptake by synaptosomal membrane vesicles*

Ca^{2+} uptake was assayed for ATP-dependent Ca^{2+} uptake as described under Experimental Procedures. No Na^+ was present in the assay. The values presented are the average of three assays performed on the same day using a single vesicle preparation. The above data are representative of two other experiments performed on different vesicle preparations on different days. Ca^{2+} uptake in the absence of ATP in the presence or absence of compound R or benzamil was taken as the background and subtracted from Ca^{2+} uptake in the presence of ATP. Control (no additions), △; 0.05 mM Q, ○; 0.5 mM Q, ●; 0.05 mM R, □; and 0.5 mM R, ■.

are present in the vesicles derived from these membranes (13). Amiloride (2 mM) does not inhibit ATP-dependent uptake (1). Fig. 3 illustrates the time course of ATP-dependent Ca^{2+} uptake into synaptosomal plasma membranes. The aryl and aralkyl derivatives R and benzamil (compound Q) both inhibit uptake when present at 0.5 mM but are only slightly inhibitory at 0.05 mM (Fig. 3). These analogues presumably inhibit uptake by interacting directly with the Ca^{2+}-ATPase. Alternatively, the

uncharged form of the drugs could cross the vesicular membrane as permeant weak bases and become protonated in the lumen of the vesicles. This process would make the intravesicular pH more basic and potentially inhibit ATP-dependent Ca^{2+} uptake. However, in experiments where benzamil was tested in the presence of nigericin (a Na^+-H^+ exchange ionophore), no potentiation of drug inhibition was observed (data not shown).

DISCUSSION

We previously demonstrated that amiloride inhibits Na^+-Ca^{2+} exchange transport in rat brain synaptosomal plasmalemma vesicles (1). In the present paper, we examined the ability of a number of amiloride analogues to inhibit Na^+-Ca^{2+} exchange transport. The results can be summarized as follows. 1) Most of the substituents of amiloride are essential for drug activity and the majority of modifications result in either no change or reduced drug activity. The 6-chloro and 5-amino residues are essential for good drug activity as is the guanidinium side chain. 2) Addition of *N*-benzyl groups to the terminal amino moiety of the guanidinium side chain results in enhanced drug activity. 3) Inhibition by amiloride and by *N*-benzyl derivatives is pH-dependent, and protonation of the guanidinium side chain appears to be necessary for drug activity. Recently Siegl *et al.* (31) examined the effects of some amiloride analogues on Na^+-Ca^{2+} exchange in heart sarcolemma vesicles. In agreement with the work described above, the most potent inhibitors were the derivatives with a benzyl substituent including benzamil (IC_{50} = 120 μM), *N*-(1-naphthylmethyl)-amiloride (IC_{50} = 11 μM), and 3′,4′-dichlorobenzamil (IC_{50} = 120 μM) (31).

Although the exact mechanism by which amiloride inhibits Na^+-Ca^{2+} exchange is not known, several lines of evidence suggest that these compounds act directly on the carrier. First, these drugs act as competitive inhibitors with respect to Ca^{2+}. Since Na^+ is also a competitive inhibitor of Ca^{2+} transport, the simplest explanation of these data is that Ca^{2+}, Na^+, and amiloride-like drugs bind to a common site on the carrier. Second, inhibition by amiloride-based compounds is highly dependent on the structure of the analogue (Table 1); small changes in structure have large effects on drug activity. These data are consistent with these compounds interacting directly with a specific binding site on a carrier which has definite steric constraints dictating inhibitor interaction. Further, these analogues do not appear to alter Na^+-Ca^{2+} exchange by indirect mechanisms. For example, previous work has shown that amiloride does not inhibit uptake by increasing the passive Ca^{2+} leakiness of the vesicle membranes; amiloride does not alter the rate of Ca^{2+} efflux from vesicles (1). Similarly, the inhibitors benzamil and phenamil described in this study do not alter the permeability of the membranes to Ca^{2+}. Therefore, an effect of these drugs on the permeability properties of the lipid bilayer is unlikely. In addition, the data in Table 3 show that analogue inhibition is not related to alterations of the membrane potential. Lastly, these compounds could conceivably indirectly affect Na^+-Ca^{2+} exchange by altering the intravesicular pH. Frizzell and Dubinsky (32) reported that amiloride can act as a permeant weak base and diffuse across the membrane in the uncharged form. Once inside the vesicle, amiloride could associate with H^+ ions and increase the intravesicular pH. Amiloride does not appear to inhibit Na^+-Ca^{2+} exchange by this mechanism since increased pH stimulates rather than inhibits Na^+-Ca^{2+} exchange (Table 2 and Ref. 28). Additionally, if the drug was acting as a permeant weak base, it should be a more effective inhibitor at basic pH values where more of the uncharged form of the drug would be present to cross the membrane. As shown in Table 2, amiloride is more effective at lower rather than higher pH values.

Previous work from our laboratory (1) and work by Smith *et al.* (33) suggest that amiloride can inhibit Na^+-Ca^{2+} exchange at the cytoplasmic surface of the plasmalemma. Since the preparation used in the above study is most certainly a mixed population of right side-out and inside-out vesicles, it is not presently possible to determine whether these drugs can also inhibit transport at the extracellular side of the membrane.

Amiloride was originally described as an inhibitor of the passive Na^+ entry mechanism found in tight epithelial tissues and the effects of amiloride analogues on this Na^+ channel have been analyzed using frog skin preparations (8, 9, 34, 35). Results from the present study suggest that the Na^+-Ca^{2+} exchange system is distinct from the epithelial Na^+ system in several aspects. Although Ca^{2+} does affect the activity of the epithelial system, Ca^{2+} is not a substrate for the epithelial Na^+ transport mechanism and Ca^{2+} is not exchanged for Na^+. In addition, amiloride is a more potent inhibitor (K_i = 1.0 to 0.1 μM; Ref. 7) of the epithelial system compared to the Na^+-Ca^{2+} exchange system described here (K_i = 300 μM; Ref. 1). The pattern of amiloride analogue interaction is similar but not identical for these two transport systems. Drug inhibition of both systems requires the presence of a 6-halo substituent (9, 34, 35). Similarly, a 5-amino or 5-substituted amino group is also required (9, 34, 35). Both systems are inhibited by the protonated form of amiloride. A guanidino side chain is also required for inhibition (34). Finally, analogues with aryl or aralkyl substituents on the terminal nitrogen atom of the guanidino moiety such as benzamil are the most effective inhibitors of both transport systems (9). Three differences in the drug interaction patterns for these two transport systems are evident from the data shown in Table 1. First, in the epithelial system, amiloride and the 6-bromo derivative (compound D in Table 1) are almost equally potent inhibitors of Na^+ transport (9). In contrast, compound D is a much less effective inhibitor of Na^+-Ca^{2+} exchange than is amiloride. Second, compound J (MK-685) which is a 5-*N*,*N*-dimethyl derivative of amiloride, is a weak inhibitor of Na^+-Ca^{2+} exchange (Table 1) but stimulates Na^+ transport in frog skin preparations (35). Third, the compound produced by lengthening of the guanidinium side chain by the insertion of a -NH- group between the carbonyl and guanidino moiety (compound N) is comparable to amiloride as an inhibitor of epithelial Na^+ transport (34, 35) but has no inhibitory effect on Na^+-Ca^{2+} exchange (Table 1).

Amiloride is also an inhibitor of the Na^+-H^+ exchange transport system found in a number of tissues including cardiac cells (10). The $K_{0.5}$ of amiloride inhibition of this system is 7 μM. Recently, Vigne *et al.* (10) examined the pharmacology of amiloride analogue inhibition of Na^+-H^+ exchange. The pattern of inhibition of Na^+-H^+ exchange differs from that of the Na^+-Ca^{2+} exchange system in two ways. First, the addition of a *N*-benzyl group to amiloride results in an analogue which is a more potent inhibitor of Na^+-Ca^{2+} exchange than is amiloride (Table 1). In contrast, *N*-benzyl derivatives are less active than amiloride as inhibitors of Na^+-H^+ exchange transport ($K_{0.5}$ = 7 μM for amiloride, 100 μM for benzamil; Ref. 10). Second, replacement of the protons on the 5-amino group with methyl groups (compound J, Table 1) or other alkyl or alkenyl groups results in a more active inhibitor of Na^+-H^+ exchange compared to amiloride (for example, $K_{0.5}$ = 0.3 μM for compound J versus 7 μM for amiloride; Ref. 10). Conversely, compound J is a slightly less active inhibitor of Na^+-Ca^{2+} exchange compared to amiloride itself (Table 1).

In summary, the above work demonstrates that amiloride and some of its analogues inhibit Ca^{2+} transport presumably by interacting directly with the carrier. Inhibition is highly dependent on the structure of the analogue since small changes can drastically alter the inhibitory properties of these compounds. The data presented demonstrate that the pattern of amiloride analogue inhibition of Na^+-Ca^{2+} exchange is distinct from the inhibition pattern of the epithelial Na^+ channel or the Na^+-H^+ exchange system. Several aryl and aralkyl derivatives were found to be more potent inhibitors than amiloride, but none can be regarded as completely specific for the Na^+-Ca^{2+} exchange system.

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