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Structure-Activity Relationships of Amiloride and Certain of Its Analogues in Relation to the Blockade of the Na^+/H^+ Exchange System

P. VIGNE,[1] C. FRELIN,[1] E. J. CRAGOE, JR.,[2] AND M. LAZDUNSKI[1]

Centre de Biochimie du Centre National de la Recherche Scientifique, Faculté des Sciences, Parc Valrose, 06034 Nice Cedex, France, and Merck Sharp & Dohme Research Laboratories, West Point, Pennsylvania 19486

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SUMMARY

Amiloride and 38 amiloride analogues were tested for their inhibitory action on the Na^+/H^+ exchanger of chick skeletal muscle cells. The unsubstituted guanidino group of amiloride is essential for the activity of the molecule, since substitution of its results in almost inactive molecules. Selected modification of position 3 and 5 substitutents of amiloride have a less dramatic effect on its potency. Substitution of the 5-amino group of amiloride with alkyl or alkenyl groups produced compounds that were up to 140 times more potent than amiloride in inhibiting the Na^+/H^+ exchanger. Such molecules would appear to be preferable to use in place of amiloride in biochemical and physiological studies of the Na^+/H^+ exchanger.

INTRODUCTION

The Na^+/H^+ exchange system is a major mechanism for the regulation of internal pH in eukaryotic cells (1, 2). It has been identified in mouse and chick skeletal muscle cells (3, 4), sheep cardiac cells (5), renal proximal tubule cells (6), neuroblastoma cells (7), MDCK cells (8), and fibroblastic cell lines (9–11). The Na^+/H^+ exchanger is inhibited by amiloride (3,5-diamino-6-chloro-*N*-(diaminomethylene)pyrazinecarboxamide), a well-known diuretic drug (1–11) which also inhibits Na^+ channels in electrogenically transporting epithelia, and the (Na^+, K^+)ATPase and the Na^+/Ca^{2+} exchange systems at high concentrations (12–15).

The amiloride-sensitive Na^+/H^+ exchanger seems to have the same properties in muscle cells, fibroblasts, and kidney cells (4, 9–11, 16). Inhibition of the Na^+/H^+ exchanger by amiloride is competitively antagonized by Na^+; therefore, very high concentrations of amiloride are required to inhibit the activity of the Na^+/H^+ exchanger under physiological conditions (2, 4). This paper describes the structure-activity relationships of amiloride and 38 of its analogues in relation to their property of blocking the Na^+/H^+ exchanger of chick skeletal muscle cells. It is demonstrated that there are analogues of amiloride which are much more potent than amiloride itself.

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[1] Centre de Biochimie du Centre National de la Recherche Scientifique, Faculté des Sciences, Parc Valrose, 06034 Nice Cedex, France.

[2] Merck Sharp & Dohme Research Laboratories, West Point, Pa. 19486.

MATERIALS AND METHODS

Amiloride analogues were synthetized as previously described (17–21). Aqueous solutions of amiloride and its analogues were prepared by using either a preformed salt or conversion to a salt using an equivalent of hydrochloric and/or isethionic acid. DMEM,[3] M199 culture medium, and fetal calf serum were obtained from GIBCO (Grand Island, N. Y.). $^{22}NaCl$ was obtained from CEA (Saclay, France). Ouabain was from Sigma Chemical Company (St. Louis, Mo.), and nigericin from Calbiochem (La Jolla, Calif.).

Myoblasts from 9- to 12-day-old chick embryo pectoralis muscle were prepared according to the method of Fiszman and Fuchs (22) and grown in DMEM/M199 (3:1) culture medium supplemented with 5% fetal calf serum, penicillin (200 units/ml), and streptomycin (50 μg/ml). Gelatin-coated 24-well tissue culture clusters were seeded with 5×10^5 cells/well. Cultures were maintained at 37° in a water-saturated atmosphere of air/CO_2 (95:5). Cultures consisting of differentiated muscle cell (myotubes) were used for $^{22}Na^+$ flux experiments after 3–4 days of culture. 3T3 fibroblasts were grown as previously described (9).

$^{22}Na^+$ flux experiments were determined as follows. Cells were incubated for 15 min at 37° in a Na^+-free medium consisting of 140 mM choline chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM glucose, and 25 mM Hepes-Tris at pH 7.4 and supplemented with nigericin (1 μg/ml) and varying concentrations of amiloride or amiloride analogue. Under these conditions, the internal Na^+ concentration, measured by flame photometry, decreased to less than 1 mM. Nigericin, an electroneutral K^+/H^+ ionophore (23), was used to acidify the internal space, which increases the activity of the Na^+/H^+ exchange system by 2- to 3-fold (9) and renders its inhibition by amiloride and its analogues easier to study. Na^+-depleted muscle cells were then shifted to a medium consisting of 137 mM choline chloride, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 3 mM NaCl, 5 mM glucose buffered at pH 7.4 with 25 mM Hepes-Tris and supplemented with 1–2 μCi/ml

[3] The abbreviations used are: DMEM, Dulbecco-Vogt modification of Eagle's medium; Hepes, 4-(2-hydroxyethyl)-piperazineethanesulfonic acid.

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of $^{22}Na^+$, 0.2 mM ouabain, and the same concentration of amiloride or amiloride analogue as in the preincubation medium. For convenience, a 3-min time of uptake was chosen. It was checked that the values of half-maximal inhibitions of the rate of $^{22}Na^+$ uptake by amiloride or by some selected amiloride analogues were the same in experiments using a 3-min or a 1-min period of uptake. At the end of the uptake period, the cells were quickly rinsed with a Na^+- and K^+-free medium (4) and digested in 2 ml of 0.1 N NaOH. The radioactivity incorporated by the cells was determined using a *gamma* counter. The rate of amiloride-sensitive $^{22}Na^+$ uptake is defined as the difference in the rate of $^{22}Na^+$ uptake measured in the absence and in the presence of 0.2 mM amiloride. The rate of amiloride-sensitive $^{22}Na^+$ uptake increased linearly with time up to 3 min. It was checked that the rate of $^{22}Na^+$ uptake measured in the presence of a saturating amount of amiloride was identical with the rate of $^{22}Na^+$ uptake measured in the presence of a saturating concentration of amiloride analogue. Protein contents were determined according to the method of Hartree (24).

RESULTS

Figure 1 shows the time course of $^{22}Na^+$ accumulation by chick skeletal muscle cells in the presence and absence of 0.2 mM amiloride. The linear relationship shown in the *inset* of Fig. 1 shows that the uptake of $^{22}Na^+$ ions via the amiloride-sensitive Na^+/H^+ exchanger follows first-order kinetics. For reasons of convenience we chose to measure routinely the rates of $^{22}Na^+$ uptake after a 3-min period. Under these conditions, the amiloride (0.2 mM)-sensitive component of the $^{22}Na^+$ flux represents 70% of the total rate of $^{22}Na^+$ uptake. The concentration-response curve for amiloride inhibition of the rate of $^{22}Na^+$ uptake by chick myotubes that have been treated with nigericin is presented in Fig. 2. The half-maximal effect for inhibition is observed at 7 μM amiloride; this value is identical with the value previously reported for chick myotubes that had not been treated with nigericin (4). The same value of 7 μM amiloride was found for the half-maximal inhibition when the rate of $^{22}Na^+$ uptake was measured after a 1-min period instead of a 3-min period.

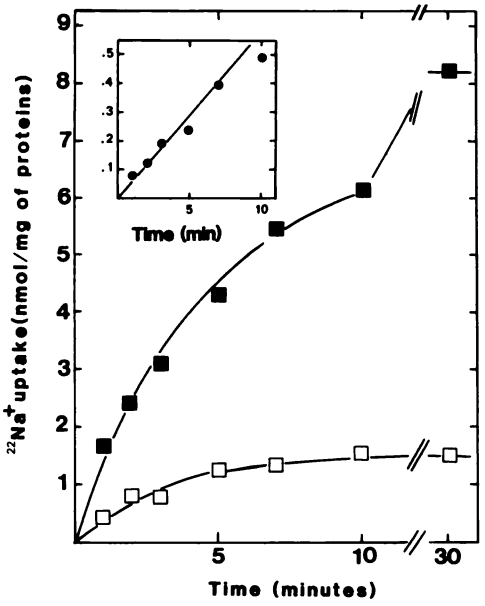


FIG. 1. *Effect of amiloride on the time course of $^{22}Na^+$ accumulation by chick myotubes*

Na^+-depleted chick muscle cells were incubated for various periods of time in a 3 mM Na^+ medium supplemented with 0.2 mM ouabain in the presence (□) or in the absence (■) of 0.2 mM amiloride. Cells had been pretreated with nigericin (1 μg/ml) in a 5 mM K^+, Na^+-free medium for 15 min prior to the uptake experiment. *Inset*: kinetics of the amiloride-sensitive $^{22}Na^+$ uptake were analyzed using a first-order scheme described by the following equation:

$$\log[\Delta Na^+\infty/(\Delta Na^+\infty - \Delta Na_t^+)] = kt$$

where ΔNa_t^+ and $\Delta Na^+\infty$ represent the difference between the intracellular $^{22}Na^+$ concentration in cells that have been incubated in the absence and in the presence of 0.2 mM amiloride at time t and at equilibrium, respectively. $\Delta Na^+\infty$ = 6.7 nmoles/mg of protein.

Concentration-response curves for the inhibition exhibited by 38 different amiloride analogues on the rate of amiloride-sensitive $^{22}Na^+$ uptake were obtained in the presence of 3 mM external Na^+. These conditions of low external Na^+ concentrations were chosen because Na^+ ions inhibit in a competitive manner the binding of amiloride to its receptor site on the Na^+/H^+ exchanger (2, 4, 11, 16).

Typical concentration-response curves for the inhibition of the rate of amiloride-sensitive $^{22}Na^+$ uptake by various amiloride analogues are presented in Fig. 2.

Table 1 gives the $K_{0.5}$ values for two amiloride analogues bearing substituents on the terminal nitrogen atom of the guanidino group. Replacement of one of the protons on the terminal nitrogen atom of the guanidino moiety by a benzyl group (No. 1, benzamil) or of both protons by methyl groups (No. 2) produced compounds that were 10 times less active than amiloride. Insertion of a nitrogen atom between the carbonyl carbon and the guanidino moiety of amiloride (No. 3, MK-875) also produced an almost inactive molecule.

Results presented in other tables indicate that the selected modifications of substituents in positions 3, 5, and 6 of amiloride generally had less detrimental effects on the potency.

Table 2 gives $K_{0.5}$ values obtained for four 6-halo compounds. The 6-chloro (amiloride), 6-bromo (No. 5), and 6-iodo (No. 6) compounds inhibited the rates of $^{22}Na^+$ uptake in the same range of concentrations. The 6-fluoro

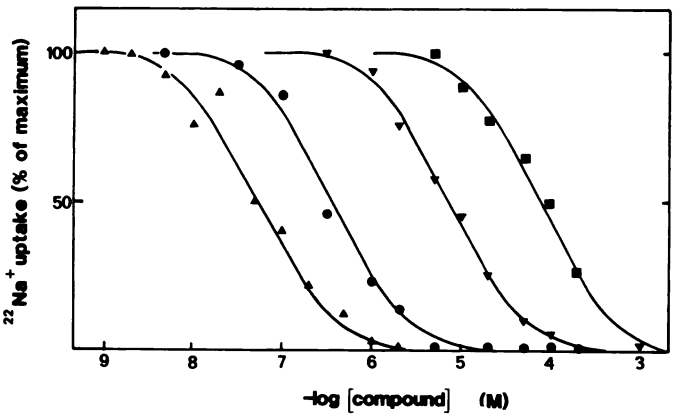
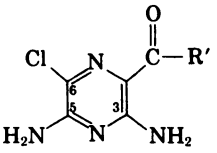


FIG. 2. *Concentration-response curve for the inhibition of the initial rate of $^{22}Na^+$ uptake by amiloride and amiloride analogues*

Compounds used were benzamil (No. 1, ■), amiloride (▼), 5-*N*-dimethylamiloride (No. 22, ●), and 5-*N*-ethylisopropylamiloride (No. 33, ▲). $^{22}Na^+$ uptake (percentage of maximum) refers to the amiloride-sensitive component of Na^+ uptake.

TABLE 1

Effects of substitution on the guanidino group of amiloride on its potency for inhibiting the amiloride-sensitive $^{22}Na^+$ flux by cultures of chick skeletal muscle cells



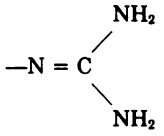
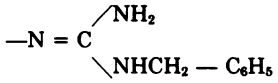
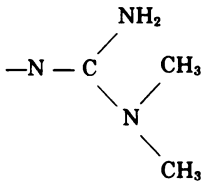
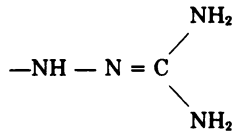
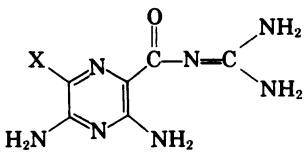
	R′	$K_{0.5}$
		μM
Amiloride		7
Compound 1		100
Compound 2		100
Compound 3		100

TABLE 2

Effect of the nature of the halogen in position 6 on the potency of amiloride derivatives for inhibiting the amiloride-sensitive $^{22}Na^+$ flux by cultures of chick skeletal muscle cells



	X	$K_{0.5}$
		μM
Compound 4	F	70
Amiloride	Cl	7
Compound 5	Br	6
Compound 6	I	5

compound (No. 4) was 10 times less active than amiloride.

The contribution of the substituent in position 5 to the activity of amiloride is shown in Table 3. Replacement of the 5-amino group by protons (No. 35) resulted in a compound that was 10 times less active than amiloride (Table 4). Replacement of one of the protons of the 5-amino group by a variety of substituents (Nos. 7–21) produced compounds that had about the same potency as amiloride. Replacement of the two protons of the 5-amino group by a methyl and a methoxy group (No. 27), by a methyl and an amino group (No. 28), or a tetramethylene radical (No. 34) slightly decreased the potency of the molecule as compared with amiloride. However, when the two substituents on the nitrogen of the 5-amino group were alkyl or alkenyl moieties, compounds with a higher potency than amiloride were generated (Nos. 22–26 and Nos. 29–33). The most active compound was the ethyl isopropyl derivative (No. 33), which was 140 times more potent than amiloride for $^{22}Na^+$ uptake. Branching of one alkyl group appears to be important, since the ethyl isopropyl compound (No. 33) was much more active than the compounds bearing an ethyl and a straight-chain alkyl or alkenyl group (Nos. 29–32). The concentration-response curves for the ethyl isopropyl derivative and the dimethyl derivative for inhibition of the rate of amiloride-sensitive $^{22}Na^+$ uptake are shown in Fig. 2.

The effects of variations of the substituents on the 3-amino group are shown in Table 4. In a series of compounds that have a 5-H group, replacement of one of the protons of the 3-amino group by a benzyl (No. 37) or by a furfuryl group (No. 36) did not significantly change the potency of the molecule (compare with No. 35). However, when a 5-ethylamino group was present, the substitution of one of the protons of the 3-amino group by a 2-methoxyethyl-ethoxymethyl moiety produced a 10-fold decrease in potency (compare No. 38 and No. 14). In order to determine whether the rank order of activity of amiloride and analogues is the same for the Na^+/H^+ exchange system in skeletal muscle cells as compared with other cells, the effects of a number of the compounds presented in the various tables were also studied using 3T3 fibroblasts. $K_{0.5}$ values obtained in that case are shown in Table 5. Independent studies carried out in this Institute by Pouysségur *et al.*[4] have shown that the Na^+/H^+ exchanger is present in various fibroblastic, epithelial, and endothelial cells and that the order of efficiency on the Na^+/H^+ exchanger of different amiloride derivatives is the same on different cell types.

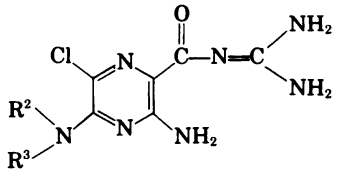
DISCUSSION

In this paper we have compared the potency of 38 amiloride analogues with regard to their ability to inhibit the uptake of $^{22}Na^+$ ions by chick skeletal muscle cells. The following evidence indicates that the rate of the amiloride-sensitive $^{22}Na^+$ uptake represents a measure of the activity of the Na^+/H^+ exchanger of the plasma membrane: (a) Amiloride inhibits both a Na^+ uptake component and a H^+ efflux component with the same dose-response curves (4), and (b) the amiloride-sensitive Na^+ influx component and the amiloride-sensitive H^+ efflux component show similar biochemical properties in relation to their dependence on external Na^+ and pH (4). A Na^+/H^+ exchanger with properties identical with those found in chick skeletal muscle is also present at the plasma membrane of a variety of cell types, including fibroblasts (9–11) and kidney cells (8, 16). Chick skeletal muscle cells were chosen because of their high rates of

[4] G. L'Allemain, A. Franchi, E. J., Cragoe, and J. Pouysségur, manuscript in preparation.

TABLE 3

Effect of substituent on the 5-amino group of amiloride on its potency for inhibiting the amiloride-sensitive $^{22}Na^+$ flux by cultures of chick skeletal muscle cells



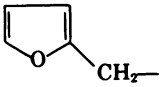
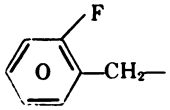
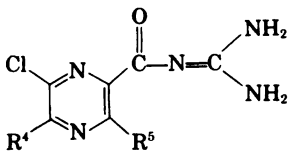
	R^2	R^3	$K_{0.5}$
			μM
Amiloride	H	H	7
Compound 7	$C_6H_5—(CH_2)_2—$	H	10
Compound 8	$CH_2OH—(CHOH)_4—CH_2—$	H	10
Compound 9	$CF_3—CH_2—$	H	10
Compound 10	$C_6H_5—C_6H_2—$	H	2
Compound 11	$CH_3—C_6H_4—CH_2—$	H	20
Compound 12		H	5
Compound 13	$NH_2—C(=NH)—$	H	2
Compound 14	$CH_3—CH_2—$	H	4
Compound 15	$(CH_3)_2—CH—CH_2—$	H	1
Compound 16	$CH_3—CH_2—CH_2—$	H	3
Compound 17	$(CH_3)_2—CH—$	H	2
Compound 18	$CH_3—(CH_2)_4—$	H	3
Compound 19	$CH_3—CH_2—CH(CH_3)—$	H	3
Compound 20		H	5
Compound 21	$CH_3—(CH_2)_2$ $CH_3 \quad CH—$	H	5
Compound 22	$CH_3—$	$CH_3—$	0.3
Compound 23	$CH_3—$	$CH_3—CH_2—$	0.2
Compound 24	$CH_3—$	$CH_3—CH_2—CH_2—$	0.2
Compound 25	$CH_3—$	$CH_3—(CH_2)_2—CH_2—$	0.5
Compound 26	$CH_3—$	$(CH_3)_2—CH—$	0.2
Compound 27	$CH_3—$	$CH_3—O—$	20
Compound 28	$CH_3—$	$NH_2—$	2
Compound 29	$CH_3—CH_2—$	$CH_3—CH_2—$	0.1
Compound 30	$CH_3—CH_2—$	$CH_3—CH_2—CH_2—$	0.3
Compound 31	$CH_3—CH_2—$	$CH_3—(CH_2)_2—CH_2—$	0.3
Compound 32	$CH_3—CH_2—$	$CH_2=CH—CH_2—$	0.1
Compound 33	$CH_3—CH_2—$	$(CH_3)_2—CH—$	0.05
Compound 34	$—(CH_2)_4—$		1

TABLE 4

Effect of substituents on the 3-amino group of amiloride on its potency for inhibiting the amiloride-sensitive $^{22}Na^+$ flux by cultures of chick skeletal muscle cells



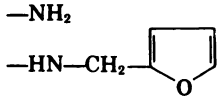
	R^4	R^5	$K_{0.5}$
			μM
Amiloride	NH_2	$—NH_2$	7
Compound 35	H	$—NH_2$	50
Compound 36	H		100
Compound 37	H	$—HN—CH_2—C_6H_5$	20
Compound 38	$CH_3—CH_2—NH—$	$—HN—CH_2—CH_2—O—CH_3$	50

TABLE 5

Comparison of the $K_{0.5}$ values of selected amiloride analogues for the Na^+/H^+ exchanger of chick skeletal muscle cells and 3T3 fibroblasts

Compound	$K_{0.5}$	
	Chick skeletal muscle	3T3 fibroblasts
	μM	
1 (amiloride)	7	5
5	6	2.5
6	5	2
7	10	10
8	10	10
12	5	2
13	2	0.8
22	0.3	0.5
29	0.1	0.2
33	0.05	0.1

amiloride-sensitive $^{22}Na^+$ uptake as compared with other cell types that have been investigated and also because these cells, unlike cardiac cells, are apparently completely devoid of a Na^+/Ca^{2+} exchange system (25) that could also be inhibited by amiloride and by its derivatives (14, 15).

The results presented in Table 1–4 can be summarized as follows:

1. The unsubstituted guanidino group is important for high activity of the molecule. Its modification results in almost inactive molecules. The guanidino group is essential for the activity of tetrodotoxin, a well-known inhibitor of voltage-dependent Na^+ channels (21). We suggest that, as with the interaction of tetrodotoxin with the Na^+ channel, the guanidino group of amiloride recognizes a Na^+ site on the Na^+/H^+ exchanger. This hypothesis is suggested by the fact that Na^+ ions act as competitive inhibitors of amiloride's action on the Na^+/H^+ exchanger of chick skeletal muscle cells (4) and of kidney cells (8, 16). The analogy between amiloride and tetrodotoxin cannot be extended further, since it has also been shown that amiloride, at concentrations up to 1 mM, does not prevent the binding of labeled tetrodotoxin to its receptor site on the Na^+ channel (26) and, conversely, that tetrodotoxin, at concentrations up to 30 μM, does not modify the activity of the Na^+/H^+ exchanger in chick myotubes (4).

2. The nature of the 6-halo group is not important for the activity of amiloride. Only when fluoro was substituted for chloro did the compound lose some activity.

3. The potency of amiloride derivatives can be increased by substitution of the 5-amino group. Monosubstituted derivatives generally had about the same activity as amiloride irrespective of the size of the substituent. On the other hand, disubstituted derivatives exhibited as much as 140 times the potency of amiloride.

4. The role of the 3-amino group substituents is less clear, mainly because only a few analogues have been tested. However, it seems that substitution of the 3-amino group leaves activity unchanged or produces a decrease. The relative potencies of amiloride and selected analogues in mouse 3T3 fibroblasts were comparable to those observed in chick myotubes, suggesting that the order of potency is probably the same for Na^+/H^+ exchangers in many different cell membranes (Table 5). Our previous studies (4, 9) have, indeed, indicated that the two systems are very similar.

Before being recognized as a potent inhibitor of the Na^+/H^+ exchanger, amiloride was known to inhibit Na^+ channels of tight epithelia, such as those found in frog skin. The activity on frog skin of several amiloride analogues used in this work has been previously reported (12, 27, 28). The pharmacological properties of the two Na^+ permeation systems can thus be compared: (a) Several values of $K_{0.5}$ for amiloride action on frog skin have been reported. They are between 10 and 100 nM in the absence of external Na^+ (12, 27). This value is only 5 μM for the Na^+/H^+ exchanger of chick myotubes, fibroblasts, and kidney cells (4, 9–11, 16). (b) Benzamil (compound No. 1) is 10 times more potent than amiloride on frog skin (12); it is almost inactive on the Na^+/H^+ exchanger of chick myotubes (Fig. 2). (c) Substitution of the 5-amino nitrogen atom results in almost inactive molecules for inhibiting Na^+ channels in frog skin (12, 27). By contrast, 5-*N*-substituted amiloride derivatives are up to 140 times more active than amiloride in inhibiting the Na^+/H^+ exchanger (Fig. 3). (d) The nature of the 6-halo group appears to be more important for the inhibition of the skin Na^+ channel than for the inhibition of the Na^+/H^+ exchanger. Substitution of chloro by iodo reduced the potency of the molecule for the skin Na^+ channel by one order of magnitude (12, 27); it did not modify its inhibiting action on the Na^+/H^+ exchanger (Table 2).

These differences strongly suggest that the Na^+ channel in frog skin and the Na^+/H^+ exchanger, even though both are inhibited by amiloride at a Na^+ coordination site (4, 8, 11, 16, 29), are distinct Na^+ permeation systems. In addition to its effect on the Na^+/H^+ exchanger and on the Na^+ channels of frog skin, amiloride was reported to inhibit both (Na^+,K^+)ATPase in kidney tubules (13) and the Na^+/Ca^{2+} exchanger system in brain cells (14, 15) at high concentrations (between 0.1 and 1 mM). Such effects could not have affected the significance of our results (a) since $^{22}Na^+$ uptake experiments were performed in the presence of ouabain to block (Na^+,K^+)ATPase and (b) no Na^+/Ca^{2+} exchange system seems to be present at the plasma membrane of chick skeletal muscle cells (25). Interestingly, the pharmacology of the Na^+/Ca^{2+} exchange system of brain cells seems to be different from the pharmacology of the Na^+/H^+ exchanger in skeletal muscle cells. Swanson *et al.* (14) reported that substitutions at position 5 of the pyrazine ring reduced the ability of the amiloride analogue to inhibit Ca^{2+} uptake. Conversely, Table 3 shows that any substitution on position 5 of the pyrazine ring increased the potency of the molecule for inhibiting the Na^+/H^+ exchanger. One class of amiloride analogues with a phenyl residue attached to the terminal guanidino nitrogen group of amiloride was more effective than amiloride itself in inhibiting Na^+/Ca^{2+} exchange. Conversely, we found (Table 1) that substitution of the guanidino moiety of amiloride produced compounds that were almost inactive for inhibition of Na^+/H^+ exchanger in chick skeletal muscle cells and 3T3 cells.

Cragoe *et al.* (17, 21, 30) reported that substitution of

the guanidino moiety by a variety of substituents generally produced compounds that had the same diuretic properties as amiloride, yet Table 1 shows that these compounds are almost inactive on the Na^+/H^+ exchanger of chick myotubes. We have observed that one of these compounds (benzamil, No. 1) is also much less active than amiloride in inhibiting the Na^+/H^+ exchanger in dog kidney cells (MDCK cell line) (data not shown). This finding suggests that the diuretic properties of amiloride derivatives bearing substituents on the terminal guanidino nitrogen atom are not due to a direct inhibitory action on the Na^+/H^+ exchanger.

An important result of this work is that there are many analogues that are more active than amiloride itself, and that the best of them, with dissociation constants between 50 nM and 200 nM, could serve as better and more specific inhibitors than amiloride for studying the role of the Na^+/H^+ exchanger under physiological conditions. Moreover, such analogues can be radiolabeled and used to titrate and identify biochemically the Na^+/H^+ exchanger protein.

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Send reprint requests to: Dr. P. Vigne, Centre de Biochimie du Centre National de la Recherche Scientifique, Faculté des Sciences, Parc Valrose, 06034, Nice Cedex, France.