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Structure–activity relations of amiloride derivatives, acting as antagonists of cation binding on Na^+/K^+-ATPase

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In a search for an organic analogue of K^+ or Na^+ ions that binds to the cation binding sites of Na^+/K^+-ATPase with high affinity, the potency of the diuretic amiloride and its derivatives in blocking Rb^+ occlusion has been tested. Although amiloride itself has a low affinity (> 200 μM), insertion of short alkyl chains in position 5 of the pyrazine ring of the molecule dramatically increased the affinity of the compound. For example, 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) competes with a $K_i \approx 10$ μM. In derivatives lacking a halogen in position 6 of the ring, a 6-fold decrease in affinity was found. Substitutions in the guanidinium moiety did not produce high affinity inhibitors of Rb^+ occlusion. Several derivatives at positions 5 and 6 of the pyrazine ring were found to be strictly competitive inhibitors with respect to Rb^+ ions. The highest affinity was observed around pH 8.0–8.2, and low temperature. EIPA and 5-(*N*-methyl-*N*-isobutyl)amiloride (MIBA) stabilized the E_1 form of FITC[1]-labelled Na^+/K^+-ATPase, behaving as Na^+ analogues. The present findings are similar to our previous results, showing that alkyl- and arylguanidinium derivatives are competitive Na^+-like antagonists in cation sites. Conclusions concerning the structural features of amiloride derivatives which are necessary to produce the highest binding affinity, are being exploited in synthesis of competitive cation analogues. Derivatives with sufficiently high affinity (0.1–1 μM) will be converted to affinity and photoaffinity reagents.

Introduction

We have initiated a search for an organic analogue that interacts with the cation binding site of the Na^+/K^+-ATPase with sufficiently high affinity to warrant its conversion to an affinity or photoaffinity reagent. This led to the finding that alkyl- and arylguanidinium compounds inhibit occlusion of Rb^+ and Na^+ with a relatively high affinity [1]. In a Rb^+ occlusion assay, *m*- and *p*-xylylenebisguanidinium (mXBG and pXBG) were found to compete with Rb^+ or Na^+ with intrinsic affinities of 7.7 and 8.2 μM, respectively, the highest reported to date. All guanidinium derivatives stabilize the E_1 conformation of fluorescein-labelled Na^+/K^+-ATPase, acting as competitive Na^+ analogues. Guanidinium compounds block ATPase activity and Na^+-dependent phosphorylation from ATP. Thus they are Na^+-like antagonists. Guanidinium derivatives are probably not occluded [1].

From a consideration of binding affinities and available concentrations of enzyme, one can deduce that a useful affinity or photoaffinity label will have a dissociation constant of 0.1–1 μM.

The diuretic drug amiloride and its derivatives have proven to be useful for elucidating the molecular basis of Na^+ transport mechanisms, particularly epithelial Na^+ channels and Na^+/H^+ exchangers [2–5]. Amiloride have a positively charged guanidinium group which has been suggested to interact with Na^+ channels [2]. Amiloride and its derivatives inhibit Na^+ channels (< 1 μM) and Na^+/H^+ exchangers [6], with high potency. Attempts have been made to label and identify Na^+ channels [7,8] or the Na^+/H^+ exchanger(s) [9,10].

Amiloride and its analogs can also inhibit Na^+/K^+-ATPase activity with low affinities [11–14]. Because a large number of derivatives of amiloride have been synthesized, systematic screening of amiloride derivatives as potential competitors of Rb^+ and Na^+ in

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Abbreviation: FITC, fluorescein 5-isothiocyanate.

occlusion sites was performed. It was thought this might reveal structural features necessary for high binding affinity to the cation sites.

Materials and Methods

Na^+/K^+-ATPase was prepared from fresh pig kidney red outer medulla as in Ref. 15. Before use, the enzyme was dialysed at 4°C for 16 h against 1000 volumes of a solution containing 25 mM histidine (pH 7.0). Protein was determined by the modified Lowry assay [16], using bovine serum albumin as a standard.

Rb^+ occlusion assay. The Rb^+ occlusion assay was performed as described in Refs. 17 and 18 and in short as follows: 40 μg of enzyme were incubated for 3 min at room temperature in 50 μl of a standard reaction mixture containing 100 mM Tris-HCl (pH 7.0), or 100 mM choline plus 10 mM Tris-HCl (pH 8.5) (unless otherwise indicated), Rb^+ up to 4 mM plus $0.5 \cdot 10^6$ cpm $^{86}RbCl$. 0.5 ml of ice-cold 200 mM sucrose was added to dilute and cool the suspension, which was immediately transfered to Dowex 50W-X8 columns. 1 ml of ice-cold sucrose was added to elute the enzyme with the occluded $^{86}Rb^+$. $^{86}Rb^+$ was counted by Cerenkov radiation. For screening amiloride derivatives, duplicate samples were incubated at each concentration tested. Differences in $^{86}Rb^+$ occlusion between duplicates were no greater than 10%.

Fluorescence measurements. FITC-labelling of the Na^+/K^+-ATPase was as described in Refs. 19 and 20. Equilibrium fluorescence measurements were performed at room temperature using a Perkin-Elmer MPF 4A spectrofluorimeter. Excitation and emission wavelengths were 495 and 520 nm, respectively, the slit width was 10 nm and the time constant was 0.3 s. Unless specified otherwise, 6 μg of labelled enzyme were suspended in 1.5 ml of 100 mM choline-HCl plus 10 mM Tris-HCl (pH 8.5) under constant magnetic stirring. For equilibrium titrations, increasing amounts of RbCl, NaCl and guanidinium compounds were added until the signal became constant. The amplitude of the fluorescence changes was measured and then corrected for the dilution due to the added volume.

Calculations. Linear and nonlinear regression analysis were done using a personal computer and the programme Enzfitter (Elsevier-BIOSOFT). The results are given in parameter values ± S.E.

Materials. Dowex 50W-X8, 50–100 mesh, was from Fluka. $^{86}Rb^+$ was obtained from Du Pont-New England Nuclear. FITC (isomer I) was purchased from Sigma.

Results

Are amiloride derivatives competitors of Rb^+ in an occlusion assay?

Previously, we have established that alkyl- and arylguanidinium derivatives competitively inhibit Rb^+ and Na^+ occlusion in a wide range of conditions [1]. Fig. 1 represents a typical Rb^+ dose response curve in the presence or absence of the amiloride derivative 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA). 15 μM EIPA reduced the apparent affinity of the enzyme for Rb^+, from 319 ± 70 to 653 ± 150 μM Rb^+, with no significant difference in the maximal Rb^+ binding capacity (3.76 ± 0.3 and 3.72 ± 0.23 nmol Rb^+/mg protein, respectively). The binding affinity of EIPA, calculated from a simple competitive model ($K_{0.5,app} = K_{0.5} \cdot (1 + I/K_i)$), was found to be 14.32 μM. Therefore, EIPA, like the guanidinium derivatives described previously,

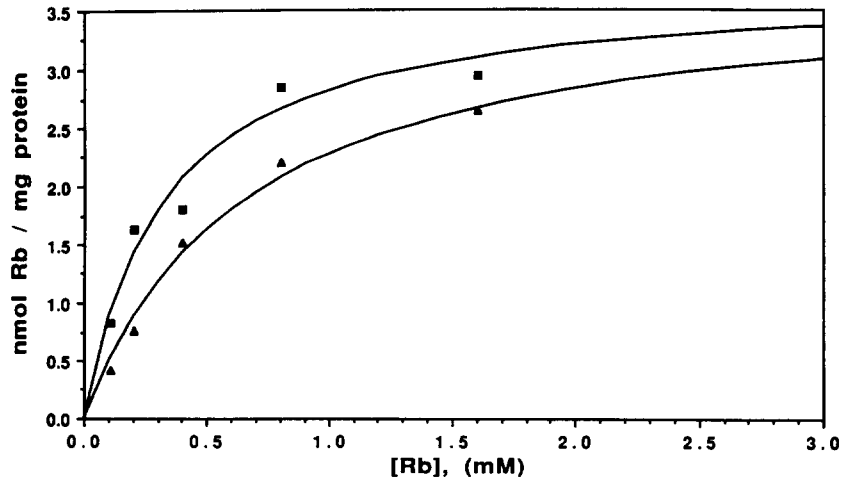


Fig. 1. Competitive inhibition by EIPA of Rb^+ occlusion. (A) Rb^+ occlusion at different Rb^+ concentrations in the absence or presence of EIPA. Enzyme (25 μg) was incubated in 50 μl reaction mixture containing 100 mM choline chloride + 10 mM Tris-HCl pH 8.0, with 0.108 mM to 4.0 mM Rb^+ (■) and with 15 μM EIPA (▲). The continuous line is that using the best-fit of binding to a single site. $B_{max} = 3.72 \pm 0.23$, 3.76 ± 0.30 nmol Rb^+/mg protein, respectively, and $K_{0.5} = 0.32 \pm 0.07$, 0.65 ± 0.15 mM Rb^+, respectively. The point at 4 mM Rb^+ is not shown in the figure in order to allow expansion of the scale and convenient observation of the competitive effect of the inhibitor.

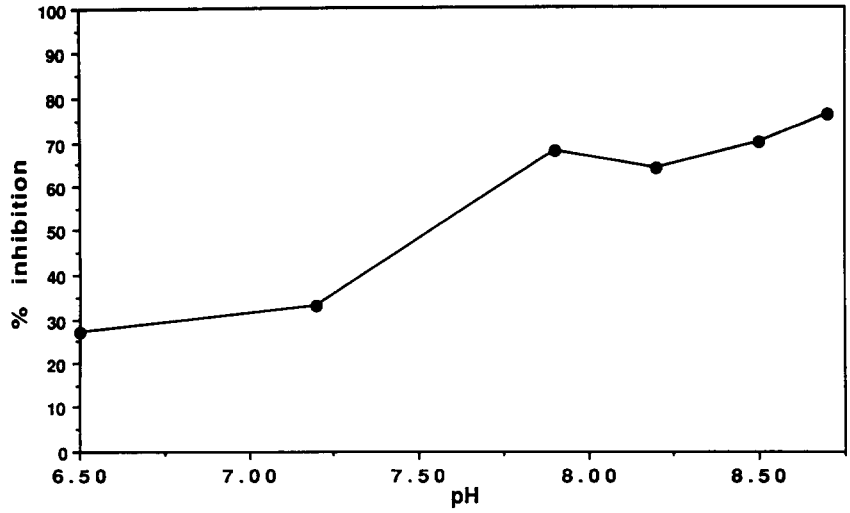


Fig. 2. The effect of pH on the inhibitory potency of EIPA. Enzyme (0.8 mg/ml) was incubated in 50 μl reaction mixture containing 100 mM choline chloride plus 10 mM Tris-HCl (pH 7.0–8.75), with 108 μM RbCl + $^{86}Rb^+$ in the presence and absence of 25 μM of EIPA.

acts as a simple competitor of Rb^+, with behaviour compatible with binding to a single site. Similar behaviour was found for all other amiloride derivatives tested in this type of competition assay, but not all derivatives were tested for competition, see (Table I).

Effect of pH on the affinity of the amiloride derivatives

The inhibitory potency of EIPA rose about 2.5-fold over a pH range of 6.5–8.7 when measured at a fixed concentration of EIPA (25 μM), as seen in Fig. 2 which depicts one of two similar experiments. The apparent affinity for Rb^+ decreased (1.5-fold) in the pH range 7.0–8.2 (not shown). Thus, the intrinsic affinity for the inhibitor rose only about 1.5-fold in this range. At pH values above 8.2 the intrinsic affinity of EIPA is reduced compared to that at pH 8.2.

Effect of temperature on the inhibitory potency of the amiloride derivatives

The inhibitory potency of EIPA increased as the temperature decreased. As seen in Fig. 3, which represents one of three experiments, the percentage inhibition with 15 μM EIPA and 68 μM Rb^+ was 35% at 37°C, 45% at 23°C, and 69% at 0°C respectively. The Rb^+ affinity also increases as the temperature decreases (not shown). Thus the intrinsic affinity of EIPA is elevated even more than appears to be the case from Fig. 3.

Structure-activity relations of amiloride derivatives

Rb^+ occlusion was measured at a concentration close to the $K_{0.5}$ value, and variable amounts of the different amiloride derivatives were added. The in-

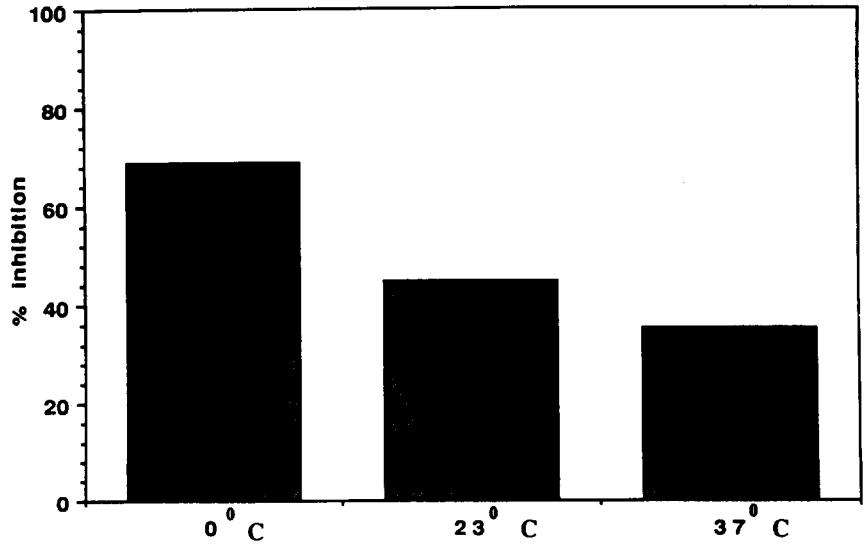


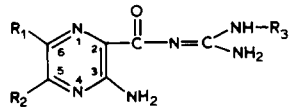
Fig. 3. The effect of temperature on the inhibitory potency of EIPA. Enzyme (0.8 mg/ml) was incubated in 50 μl reaction mixture containing 100 mM choline chloride plus 10 mM Tris-HCl (pH 8.0), with 68 μM $^{86}Rb^+$ in the presence and absence of 15 μM of EIPA. The mixture was incubated for 15 min 0, 23, or 37 °C. The reaction was stopped by a 10-fold dilution with ice-cold sucrose, as described in Methods.

hibitory potency of each of the compounds (IC_{50}) was estimated and is summarized in Table I. The amiloride derivatives can be divided into three main groups: (a) derivatives of position 6 of the pyrazine ring (R_1), (b) derivatives of position 5 of the pyrazine ring (R_2), and (c) derivatives of the guanidine group (R_3). All derivatives inhibited Rb^+ occlusion. Full competition curves were also performed for 6 of the 15 derivatives tested.

TABLE I

The structure of amiloride derivatives and their potency to inhibit Rb^+ occlusion

The inhibitory potency of each of the amiloride derivative were determined in different sets of experiments, with reaction mixtures containing 100 mM choline chloride + 10 mM Tris-HCl (pH 8.0) and Rb^+ concentration at its $K_{0.5}$ (~ 50 μM). The values followed by (+) indicate that full competition experiments were performed, and the compound was found to be competitive with Rb^+. The numbers followed by (?) indicate that competition experiments were not performed. In all cases the results were fitted to a single site competitive inhibitor model. The IC_{50} of the compound can be transformed to the intrinsic affinity (K_i), by dividing the value by two.



Amiloride derivatives

Compound (R_2 substitution)	Name	Abbreviation	R_1	R_3	IC_{50} (μM) competitive	
H_2N—		amiloride	Cl	H	> 200	+
CH_3CH_2 N— $(CH_3)_2CH$	5-(N-ethyl-N-isopropyl)amiloride	EIPA	Cl	H	16	+
	6-iodo- -"-	A31	I	H	22	+
	6-dechloro- -"-	A30	H	H	104	+
H N— S=C=N	5-N-(phenyl-3-isothiocyanate)amiloride		Cl	H	75	+
CH_3 N— $(CH_3)_2CHCH_2$	5-(N-isobutyl-N-methyl)amiloride	MIBA	Cl	H	23	+
$(CH_3)_2N$—	5-(N,N-dimethyl)amiloride	DMA	Cl	H	34	?
N—	5-(N,N-hexamethylene)amiloride	HMA	Cl	H	36	?
N H NH_2	5-N-(3-aminophenyl)amiloride	A35	Cl	H	62	?

Compound (R_3 substitution)	Name	Abbreviation	R_1	R_2	IC_{50} (μM)	
		phenamil	Cl	NH_2	70	?
		benzamil	Cl	NH_2	50	?
$-CH_2$	6-bromo-benzamil		Br	NH_2	> 200	?
CH_3O $-CH_2$ NO_2	2'-methoxy-5'-nitrobenzamil	NMBA	Cl	NH_2	> 200	?
	6-iodo- -"-		I	NH_2	> 200	?
$-CH_2$	(N-naphthylmethyl)amiloride		Cl	NH_2	40	?

As can be seen, substitution with alkyl moieties in the R_2 position led to the highest inhibitory potencies. The derivative with the highest affinity is EIPA. Aryl substitutions at the R_2 were less effective. Removal of the halogen in the R_1 substantially decreased the inhibitory potency of the amiloride derivatives (EIPA ≈ A31 ≫ A30). Substitutions on the guanidinium group (R_3) also yielded much less potent inhibitors. Since the latter derivatives were of less interest for present purposes, full competition curves were not performed.

EIPA is a Na^+-like compound in stabilizing the E_1 conformation;

We have shown previously that alkyl- and arylguanidinium derivatives stabilize the E_1 conformation of fluorescein-labelled enzyme [1]. The amiloride derivatives EIPA and MIBA, like Na^+ ions, was found also to stabilize the E_1 conformation of the pump, and EIPA like Na^+ ions were found to compete with Rb^+ ions in the fluorescence assay (experiments not shown). The intrinsic affinity of EIPA calculated from competition curves was 5–10 μM, a range of values which is close to that calculated from occlusion experiments (Fig. 1 and Table I). Therefore the fluorescence experiments confirm that EIPA, the amiloride derivative with the highest affinity, is a Na^+-like analogue, competing with Rb^+ ions on the E_1 form.

Discussion

The search for an organic analogue with a sufficiently high binding affinity to make it worth while to convert it to an affinity label, requires knowledge of structural features and conditions which determine the binding affinity. The availability of the range of amiloride derivatives has permitted us to infer a number of important properties of potential high affinity cation analogues. The amiloride derivative cannot itself fulfil that role, for the highest affinity observed as yet, for EIPA, is only about 10 μM (Fig. 1 and Table I).

Binding conditions

(1) Elevation of the pH from 6.5 to 8.2 increased the inhibitory potency of EIPA by 2.5-fold. When Rb^+ affinity is taken into account, this increase reflects a real rise of 1.5-fold in intrinsic affinity of EIPA. The intrinsic affinity of EIPA is reduced as the pH is elevated above 8.2. For alkyl- and arylguanidinium compounds, we observed an increase in affinity in a similar pH range of about 5-fold, with no decrease in affinity at high pH [1]. The pK_a of these guanidinium derivatives is around 12, and they might be expected to be fully protonated at all pH values below 9. The latter findings, taken together with the relatively small rise in binding affinity of the amiloride derivative $pK \approx 8.5$ (Fig. 2), indicate that it is the protonated form of the guanidinium group which interacts with the cation binding domain. These findings support the possibility of direct competition of protons with the guanidinium group in a binding site with a $pK_a \approx 7.5$. A similar pK_a was inferred from effects of pH on modification of carboxyl residues by DCCD [18], or on electrogenicity of reconstituted pumps sustaining so-called 'uncoupled Na^+ flux' [21].

(2) The inhibitory potency of EIPA increased as the temperature was reduced. This increase in the inhibitory potency also reflects a real increase in the EIPA intrinsic affinity since Rb^+ affinity also increased as the temperature drops. It is likely that the dissociation rate of the bound EIPA is reduced, as is the case for Rb^+ ions [18].

Structure–activity relations of amiloride derivatives

Amiloride itself exhibited a low affinity for the Na^+/K^+-ATPase. Introduction of an aliphatic moiety in position 5 of the pyrazine ring (Table I), dramatically increased the potency of the molecule. The affinities observed for EIPA, A31, MIBA are all about 10 μM. This is about the same as observed previously for *p*- and *m*-xylylenebisguanidinium [1]. Considering that EIPA contains only a single guanidinium group, the importance of the aliphatic tail at the R_2 can be appreciated. Removal of a halogen group in position 6 of the pyrazine ring had a profound effect in decreasing the affinity. For example, the affinity of EIPA or A31 with Cl or I at position R_1 was about 6-fold higher than that of A30, with H at R_1. Future design of cation analogues will have to take into account the role of halogen insertion in an aromatic ring. Finally, derivatives with aryl substitutions in the R_3 (guanidyl group) position were all a little more effective than amiloride itself, but all showed lower affinity than with EIPA. A comparison of say A35 with phenamil, which are about equal potency does not suggest that adding an additional aryl ring at either position R_2 or R_1 is of much consequence for the binding interaction.

Comparison with studies on inhibition of Na^+/K^+-ATPase

There are several reports of inhibition by amiloride and derivatives of Na^+/K^+-ATPase activity, active transport of Rb^+ or ouabain-sensitive O_2 consumption, using parially purified renal enzyme or intact liver cells or kidney tubules [11–14]. Published studies have been restricted largely to examination of derivatives at the amino group in position 5. Where these and the present studies can be compared, the observed order of apparent affinities is compatible. For example, the order of potency EIPA > DMA > amiloride is a reproducible finding [12–14]. The absolute values of IC_{50} for inhibition of Na^+/K^+-ATPase activity are all much

lower than those for inhibition of Rb^+ occlusion. (Compare for example the IC_{50} of ≈ 300 μM for inhibition of Na^+/K^+-ATPase by EIPA in Ref. 14. with the value of 16 μM reported in Table I for inhibition of Rb^+ occlusion). A similar large difference for inhibition of Na^+/K^+-ATPase activity and Rb^+ occlusion was observed for the guanidinium derivatives we described recently [1]. The lower affinity in Na^+/K^+-ATPase assays is partly attributable to the presence of higher concentrations of competing cations, but also to the fact that the form of the protein binding the inhibitor with the highest affinity (E_1) is not the major steady-state intermediate. This is therefore additional, if indirect, evidence that the amiloride and guanidinium derivatives in Ref. 1 have a similar mode of action, namely they compete with Na^+ ions on the E_1 form.

Conclusion

For purposes of designing a high-affinity Na^+ analogue, the provisional conclusions which can be drawn from the present study of amiloride derivatives and also from the aryl and alkyl derivatives described in Ref. 1, can be summarised as follows:

(1) The pK of the guanidinium moiety should be ≈ 12 as for the guanidinium ion and derivatives in Ref. 1, rather than ≈ 8.5 as in amiloride derivatives [4], as deduced from relative effects of pH on potency of the inhibitors see Fig. 2, and Ref. 1.

(2) The guanidinium need not be substituted with extra groups, as in phenamil or benzamil (Table I).

(3) The potency of the guanidinium derivative is proportional to the number of guanidium groups in the molecule [1].

[4] The affinity is greater in compounds with an aryl ring as in *p*- or *m*-xylylenebisguanidinium or the pyrazine ring of amiloride derivatives, rather than for the most potent aliphatic guanidinium derivatives.

(5) The hydrophobicity of the derivative may be an important factor, as deduced from the large increase in potency of derivatives with aliphatic substitutions at the 5-NH_2 position in amiloride.

(6) A halogen substituent in the pyrazine ring is important (Table I).

These conclusions are now being exploited in the design and synthesis of Na^+ analogues with affinities in the 0.1–1.0 μM range. Appropriate derivatives will be converted to affinity or photo-affinity reagents.

Acknowledgments

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