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Biochemical Pharmacology, Vol. 36, No. 5, pp. 778–780, 1987.
Printed in Great Britain.
0006-2952/87 $3.00 + 0.00

Inhibition of cardiac phosphodiesterases by amiloride and its *N*-chlorobenzyl analogues

(*Received* 14 *August* 1986; *accepted* 29 *September* 1986)

Amiloride (3,5-diamino-6-chloro-*N*-(diaminomethylene)-pyrazinecarboxamide) is widely employed as an antikaliuretic agent, and has recently gained importance as an inhibitor of numerous plasmalemmal Na^+ transport systems. These include epithelial Na^+ channels, the Na^+/H^+ exchange (for review see ref. 1), and the Na^+/Ca^{2+} exchange [2–4]. The diuretic effect of the drug has been ascribed to the inhibition of one or more of these systems. Other pharmacological effects of amiloride such as the increase in cardiac inotropism [4–6], the antagonism toward the toxic effects of digitalis both *in vivo* [7] and *in vitro* [4, 6], and the vascular smooth muscle relaxing effect [8], have been tentatively ascribed to inhibition of the Na^+/Ca^{2+} exchange system [4, 6, 8].

Amiloride has also been observed to inhibit other enzymatic systems, including the Na^+/K^+ ATPase [9], protein-kinases [10–13], and adenylate cyclase [14]. These effects are not strictly related to the action of the drug on kidney and heart.

The amiloride molecule can be modified to obtain derivatives that show more selective inhibitory effects on the different Na^+ transport or exchange systems. In particular, the 5-amino nitrogen substituted analogues are potent inhibitors of Na^+/H^+ exchange while compounds bearing substituents on terminal nitrogen of the guanidino group block the Na^+/Ca^{2+} exchange. Of these, benzamil, chloro-, dichloro-, and dimethyl-benzyl derivatives of amiloride are the most potent agents [15–17].

In the present paper we describe the inhibition of partially purified cardiac bovine soluble phosphodiesterases by amiloride, *o*-chlorobenzyl amiloride and 3′,4′-dichlorobenzyl amiloride.

Materials and methods

Amiloride and 3′,4′-dichlorobenzyl amiloride were kindly supplied by Merck-Sharp and Dohme (Roma, Italy, and Rahway, NJ, respectively); *o*-chlorobenzyl amiloride was prepared according to Cragoe *et al.* [18–19].

Preparation of phosphodiesterase. Soluble cAMP and cGMP phosphodiesterases were partially purified from bovine heart by the procedure of Butcher and Sutherland as modified by Carpenedo *et al.* [20] to the stage of DEAE-cellulose column. The 0.2 M KCl fraction used as the source of phosphodiesterase showed good hydrolyzing activity against both cAMP and cGMP, contained calmodulin, and required micromolar concentrations of exogenous Ca^{2+} for full activity. A Lineweaver–Burk plot of the enzyme preparation was downward curvilinear. A low K_m of 3 μM and a high K_m of 40 μM for cAMP and a low K_m of 5 μM and a high K_m of 30 μM for cGMP were obtained by extrapolation. When cAMP phosphodiesterase activity was assayed in the presence of increasing concentrations of cGMP, its activity was completely blocked by concentrations of cGMP that were only slightly higher than the K_m value of the enzyme for cAMP, but when cGMP phosphodiesterase was assayed in the presence of increasing concentrations of cAMP, amounts 80 times higher than the K_m of the enzyme for cGMP had to be added to obtain a 50% inhibition of the hydrolyzing activity.

Assay of phosphodiesterase activity. Phosphodiesterase activity was assayed at 37° for 10 min using the two step procedure of Thompson *et al.* [21]. Tritiated cyclic nucleotides were converted to [^{3}H] labelled 5′-nucleotides in a 0.4 ml reaction mixture containing 40 mM Tris · HCl, pH 8, 5 mM $MgCl_2$, 20 μM $CaCl_2$, 12 U calmodulin (bovine brain, Sigma) and a proper amount of enzyme. The reaction was stopped by freezing the sample in liquid nitrogen (30 sec) and then thawing it in boiling water (90 sec). The 5′-nucleotides were then converted to [^{3}H] nucleosides with 0.3 U of 5′-nucleotidase (Crotalus atrox, grade II, Sigma). The radioactive nucleosides were separated from labelled cyclic nucleotides using Dowex 1 × 2 anion-exchange resin in two volumes of ethanol–water 1:1. When assaying for Mg^{2+}-dependent phosphodiesterase activity, $CaCl_2$ and calmodulin were omitted and 1 mM EGTA pH 8 was added.

Amiloride and the *N*-chlorobenzyl analogues were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in incubation medium never exceeded 1%. This amount did not affect phosphodiesterase activity.

Protein assay. Protein content was determined according to Lowry *et al.* [22] by using bovine serum albumin as standard.

Results and discussion

IC_{50} values calculated from the concentration–response curves of the inhibition of cardiac soluble Ca^{2+}-calmodulin activated phosphodiesterase by amiloride and the *N*-chlorobenzyl analogues (Table 1) show that amiloride was a weak inhibitor of the enzyme when both cAMP and cGMP were employed as substrates (IC_{50}s in the millimolar range).

Table 1. Inhibition of cardiac soluble cAMP and cGMP Ca^{2+}-calmodulin activated phosphodiesterase by amiloride and its -chlorobenzyl analogues.

	IC_{50}s (μM)	
	cAMP phosphodiesterase	cGMP phosphodiesterase
Amiloride	3000.0	>4000.0
o-Chlorobenzyl amiloride	65.0	550.0
3′,4′-Dichlorobenzyl amiloride	8.5	26.0
1-Methyl-3-isobutylxanthine	7.0	5.0
Theophylline	220.0	340.0

Incubation medium: 40 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 1 μM [^3H] cyclic nucleotides, 20 μM $CaCl_2$, 12 U calmodulin and about 10 μg enzymatic proteins (volume 0.4 ml).

The substitution of an H on the terminal nitrogen of the guanidino group of amiloride molecule with an *o*-chlorobenzyl or with a 3′,4′-dichlorobenzyl group increased the inhibitory potency of the parent compound. The IC_{50} values of these derivatives were in the low micromolar range. IC_{50} values of 1-methyl-3-isobutylxanthine and theophylline are reported for comparison. All the compounds showed greater inhibition of cAMP than cGMP phosphodiesterase activity and did not act through the calmodulin component of the enzyme, since their IC_{50} values on Mg^{2+}-dependent and on Ca^{2+}-calmodulin activated forms were quite similar. Moreover, when isolated cardiac sarcolemmal vesicles prepared according to Floreani and Luciani [4] were used as source of the membrane-bound phosphodiesterase, the inhibitory effect of the compounds was still evident (e.g. IC_{50} of 3′,4′-dichlorobenzyl analogue = 35 μM).

Amiloride and the chlorobenzyl analogues inhibited the cyclic nucleotide hydrolyzing enzymes with the same order of potency as they inhibit the Na^+/Ca^{2+} exchange in cardiac sarcolemmal vesicles [17] and in pituitary cell membranes [15]. It is notable that the 3′,4′-dichlorobenzyl analogue inhibited the phosphodiesterases at lower concentrations than those that inhibit the Na^+/Ca^{2+} exchange [17]. Furthermore, while 3′,4′-dichlorobenzyl amiloride is about equipotent with *o*-chlorobenzyl amiloride in inhibiting the Na^+/Ca^{2+} exchange [17], it was 8 times and 20 times more potent than the *o*-chlorobenzyl derivative as inhibitor of cAMP and cGMP hydrolyzing enzymes, respectively.

The increased inhibitory potency of the hydrophobic substitutions at the terminal nitrogen of the guanidino group of amiloride may be the result of increased hydrophobic bonding with the enzyme. On the other hand, the addition of a *o*-chloro- or a 3′,4′-dichlorobenzyl group to the amiloride molecule might result in a steric conformation of the parent compound that increases its affinity for the enzyme. It should be noted that 0.5 mM 3′,4′-dichlorotoluene (the added moiety in 3′,4′-dichlorobenzyl amiloride) was devoid of any inhibitory effect.

The enzyme preparation that we used showed a typical non-linear kinetic plot. Although the explanation of this downward curvature is still debated and caution must be used when extrapolating to apparent K_m values [23], we identified two linear regions corresponding to the high and low substrate concentrations. We investigated the kinetics of inhibition of the Ca^{2+}-calmodulin activated phosphodiesterase by the two-chlorobenzyl analogues of amiloride in the linear region of cAMP concentrations ranging from 1 to 50 μM cAMP. From the Lineweaver–Burk plot reported in Fig. 1, both *o*-chlorobenzyl and 3′,4′-dichlorobenzyl analogues appeared to be non-competitive inhibitors of cAMP.

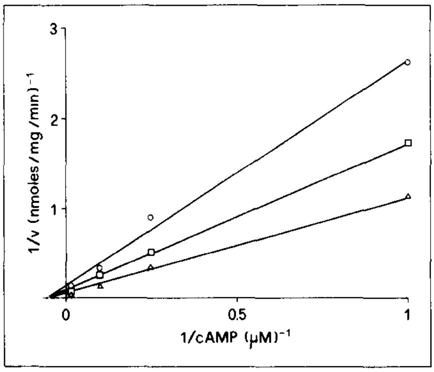

Fig. 1. Lineweaver–Burk plot of the Ca^{2+}-calmodulin activated cAMP phosphodiesterase activity in the absence (△—△) and in the presence of 50 μM *o*-chlorobenzyl amiloride (□—□) and 25 μM 3′,4′-dichlorobenzyl amiloride (○—○). Incubation medium and conditions as described in the Materials and Methods.

These agents and other structurally similar compounds are known to inhibit other enzymatic systems. For instance, Siegl *et al.* [17] report that 3′,4′-dichlorobenzyl amiloride reduces cardiac sarcolemmal Ca^{2+} ATPase activity, and Schellenberg *et al.* [24] have found that benzamil markedly affects calcium movements across the mitochondrial membrane and significantly decreases the mitochondrial respiratory ratio.

In conclusion, our results indicate that *o*-chlorobenzyl and 3′,4′-dichlorobenzyl analogues of amiloride are good inhibitors of cardiac phosphodiesterases. Whereas millimolar concentrations of amiloride are necessary to affect the soluble cAMP and cGMP phosphodiesterases, micromolar concentrations of the derivatives are sufficient to produce significant inhibition of enzyme activity. The analogues show a greater inhibitory effect on the cAMP than cGMP hydrolyzing enzyme and are non-competitive with respect to cAMP. They cannot therefore be considered specific inhibitors of the Na^+/Ca^{2+} exchange system alone. As a consequence, the interpretation of the mechanism of the pharmacological effects of these compounds only in terms of Na^+/Ca^{2+} exchange inhibition is unjustified.

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