The interaction of amiloride with acetylcholinesterase and butyrylcholinesterase

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The diuretic drug amiloride was found to be a powerful inhibitor of the reaction of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) with their specific choline ester substrates. The inhibition constant is in the micromolar range. On the other hand, when added to a mixture of cholinesterase (AChE and BChE) and neutral substrates, amiloride, in some cases, enhanced the reaction rate. The rate of the reaction of butyrylcholinesterase with *p*-nitrophenyl butyrate was increased up to 12 fold by amiloride.

Acetylcholinesterase; Butyrylcholinesterase; Amiloride; Inhibition; Rate enhancement

1. INTRODUCTION

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) have been extensively studied during the last five decades, and a great number of compounds have been investigated as substrates or inhibitors of the enzymes.

Interestingly, several quaternary nitrogen compounds of hydrophobic nature were found to be inhibitory of the reaction between cholinesterases and their specific choline substrates, but also to induce enhanced reaction rate between the enzymes and nonionic substrates or covalent inhibitors [1–7].

Here we report on the effect of the diuretic drug amiloride (Scheme 1) on the activity of AChE and BChE.

Amiloride contains a heterocyclic hydrophobic acyl moiety, derived from pyrazine, to which a guanidinium group is attached (pK = 8.7). It was of interest to examine whether the drug has an effect on the activity of cholinesterases.

Amiloride is known as a powerful inhibitor of the H^+/Na^+ exchange with K_i = 0.3 μM [8,9]. Recently it has been found that the drug inhibits also other systems such as Na^+/Ca^{2+} exchange with K_i = 10 μM [10], phosphorylation by a variety of protein kinases with K_i = 0.1 mM [11,12] and phosphorylation of the regulatory myosin light chain by myosin light chain kinase, K_i = 1 μM [13].

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Abbreviations: AChE, acetylcholinesterase; BChE, butyrylcholinesterase; pNP, *p*-nitrophenyl; oNP, *o*-nitrophenyl; pNPB, *p*-nitrophenyl butyrate; MSF, methanesulfonyl fluoride; 2-PAM, pyridine-2-aldoxime methiodide

2. MATERIALS AND METHODS

AChE (electric eel) and BChE (bovine serum) were products of Worthington and Sigma. Acetylcholine chloride, butyrylcholine iodide, *p*-nitrophenyl esters and amiloride were obtained from Sigma.

2.1. *Assay of enzyme activity*

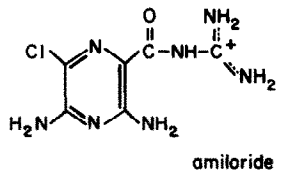
To a solution of acetylcholine chloride or butyrylcholine iodide (1 mM in 10 ml containing 0.1 N NaCl) AChE (final concentration 10^{-10} M) or BChE (final concentration 10^{-8} M) was added. The reaction took place in the pH-stat at pH 7.5, 25°C. The released protons were titrated with 0.05 N NaOH, and the reaction course was followed by a recorder conjugated to the pH stat.

The reactivity of cholinesterase with *p*-nitrophenyl esters (0.1 mM) was determined at pH 7.5 (0.1 M phosphate buffer, 2% v/v acetonitrile), 25°C, and followed spectrophotometrically at 400 nm. From the course of alkali uptake in the pH stat or from the absorbance change in the spectrophotometer the reaction rate was calculated.

2.2. *Effect of amiloride*

Amiloride in different concentrations (0.1–100 μM) was present in the reaction solution of enzyme and substrate, and the reaction rate was measured. In the case of inhibition the double reciprocal plot ($1/v$ vs $1/[S]$) or the Dixon plot ($1/v$ vs inhibitor concentration) [14] yielded K_i, the inhibition constant.

The effect of amiloride on the irreversible inactivation of AChE by methanesulfonyl fluoride (MSF) [1] was also studied. Enzyme solu-



amiloride

Scheme 1

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tion was incubated with MSF (1 mM). At timed intervals an aliquot was removed and added to a substrate solution and the reaction rate measured. From the decline of enzyme activity the inactivation rate was calculated.

3. RESULTS AND DISCUSSION

When amiloride was added to a solution containing AChE and acetylcholine the enzymatic reaction was inhibited. Analysis of the reaction kinetics indicated that the inhibition was competitive with $K_i \simeq 5.5 \pm 0.5$ μM. However, when amiloride was added to a solution containing AChE and the poor non-ionic substrate ethyl acetate, there was no marked effect on the reaction rate. On the other hand, the addition of amiloride to a solution of AChE with 1 mM of the covalent inhibitor MSF enhanced the rate of enzyme inactivation. 4 μM amiloride increased the inactivation rate by a factor of 3.

Amiloride was found to be a potent competitive inhibitor of the reaction of BChE with butyrylcholine, with $K_i \simeq 0.95 \pm 0.1$ μM. On the other hand, amiloride considerably enhanced the reaction rate between BChE and the neutral substrate *p*-nitrophenyl butyrate (pNPB). The effect of amiloride on activation of BChE is depicted in Fig. 1. Maximum rate enhancement is 12 fold and it is achieved at approximately 10 μM of amiloride, whereas half maximal rate enhancement occurs at 1.1 μM amiloride.

Amiloride affected also the reaction rate between BChE and a series of nitrophenyl ester derivatives of various acids, but the rate enhancement was smaller than that found with pNPB (Table I).

The fact that amiloride is a competitive inhibitor of the reaction between acetylcholine and AChE suggests that the guanidinium group of the inhibitor occupies the anionic site of the enzyme, thus excluding from it the cationic moiety of the substrate. The finding that amiloride enhances the rate of the covalent inhibition of AChE by MSF implies that, in the ternary complex AChE-amiloride-MSF formed, binding of amiloride induces conformational change of the enzyme which leads to the increased rate of inhibition by MSF.

Rate enhancement of covalent inhibition of AChE induced by binding of quaternary ammonium derivatives has been described in the literature. Wilson and coworkers showed that the tetraethyl ammonium cation increased the rate of AChE inhibition by MSF up to 33 fold [1]. In the presence of tetramethyl ammonium salt the rate of inhibition of AChE by dimethylcarbamoyl fluoride was increased up to 14 fold [2].

It is interesting that quaternary ammonium derivatives were found in few cases to accelerate the inhibition of AChE caused by chemical modification of amino acid residues outside the active site of the enzyme. Thus, Wilson and Silman demonstrated that pyridine-2-aldoxime methiodide (2-PAM) dramatically

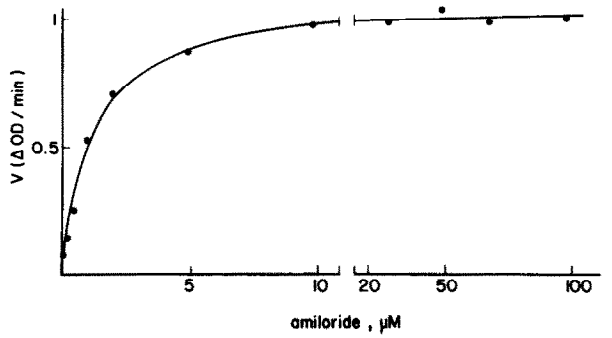

Fig. 1. Amiloride induced activation of the reaction between BChE and pNPB (0.1 mM) at pH 7.5, 25°C. The rate is expressed in the change of optical density units at 400 nm/min.

accelerated the rate of inhibition of AChE by arsenite, with maximum enhancement of 220 fold [3].

Blumberg and Silman found that 0.1 mM methyl pyridinium increased several fold the rate of inhibition of AChE caused by *N*-hydroxysuccinimide ester of *N*-acetyl-*p*-(2,4-dinitroanilino)-*L*-phenylalanine. A tyrosine residue was modified by the ester [4]. The effect of quaternary ammonium salts on AChE catalyzed hydrolysis of substrates has also been reported in the literature. Metzger and Wilson showed that 3 mM tetraethyl ammonium provided 3-fold acceleration of acetyl fluoride [5]. Bartlett and Rosenburry found that 1-methyl acridinium induced 5-fold enhancement of the rate of ethyl acetate hydrolysis by AChE [6]. Desire and Saint-Andre have studied the effect of a series of quaternary ammonium heterocyclic compounds on AChE catalyzed hydrolysis of alkyl acetates. The largest rate enhancement, 6.5 fold, occurred where *N*-methyl benzo-7,8-quinolinium was added to a solution containing AChE and ethyl acetate [7].

The finding that amiloride can accelerate the inactivation of AChE by MSF adds the guanidinium moiety to the ligands which on binding to the enzyme enhance its activity.

The effect of amiloride on BChE was more pronounced than that on AChE. Amiloride inhibited the

Table I

Ratio (r) of the rate of reaction between BChE and 0.1 mM substrate in the presence of 4 μM amiloride to the rate of the same reaction without the drug, at pH 7.5, 25°C

Substrate	r[a]
pNP acetate	2.6
pNP propionate	3.8
pNP butyrate	8.5
pNP pentanoate	4.4
pNP hexanoate	2.1
pNP octanoate	1.5
oNP acetate	0.6
oNP butyrate	0.4

[a] $r < 1$ indicates inhibition

BChE catalyzed hydrolysis of butyrylcholine with $K_i \simeq 0.95 \pm 0.1$ μM, compared with $K_i = 5.5 \pm 0.5$ μM for the parallel reaction of AChE with acetylcholine. Amiloride increased the rate of the BChE catalyzed hydrolysis of pNPB by a factor of 12 at 10 μM ligand (Fig. 1). The amiloride concentration which causes half maximal increase is 1.1 μM, which is very similar to the value of K_i for amiloride inhibition of the reaction of BChE with butyrylcholine. This suggests that both as an inhibitor and activator amiloride binds to the same site in the enzyme. Amiloride probably occupies the site to which the choline cation is bound, whereas pNPB is bound to a different site. In the ternary complex BChE-amiloride-pNPB the enzyme is much more active than in the binary complex BChE-pNPB.

Desire and Saint-Andre have studied the effect of several quaternary heterocyclic compounds on BChE catalyzed hydrolysis of alkyl acetates. The most effective ligand was *N*-methyl-7,8-quinolinium cation, which at 5 μM enhanced the hydrolysis rate of ethyl acetate by 7.5-fold [7].

Table I shows that amiloride affects the hydrolysis rate of pNPB by BChE to a greater extent than the reaction rates of other pNP esters. Probably binding of pNPB to BChE-amiloride fits best the orientation required for enhancing the enzyme activity. Binding of lower or higher homologues of pNPB to BChE-amiloride complex does not produce the optimal orientation for enzyme activity.

Inspection of Table I shows that BChE catalyzed hydrolysis of oNP esters is inhibited by amiloride in contrast to the enhanced hydrolysis rate of the pNP ester isomers. A change of the position of the nitro group in the phenyl moiety from *para* to *ortho* prevents the ester from binding to the enzyme-amiloride complex. Thus, in BChE-amiloride the enzyme 'senses' small structural changes of ligands.

Our reported results suggest that when the diuretic drug amiloride is studied in physiological systems its possible effect on cholinesterase should not be ignored.

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