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Distinct Effect of Benzalkonium Chloride on the Esterase and Aryl Acylamidase Activities of Butyrylcholinesterase

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Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) from vertebrates, other than their predominant acylcholine hydrolase (esterase) activity, display a genuine aryl acylamidase activity (AAA) capable of hydrolyzing the synthetic substrate *o*-nitroacetanilide to *o*-nitroaniline. This AAA activity is strongly inhibited by classical cholinesterase (ChE) inhibitors. In the present study, benzalkonium chloride (BAC), a cationic detergent widely used as a preservative in pharmaceutical preparations, has been shown to distinctly modulate the esterase and AAA activities of BChEs. The detergent BAC was able to inhibit the esterase activity of human serum and horse serum BChEs and AChEs from electric eel and human erythrocyte. The remarkable property of BAC was its ability to profoundly activate the AAA activity of human serum and horse serum BChEs but not the AAA activity of AChEs. Thus BAC seem to preferentially activate the AAA activity of BChEs alone. Results of the study using the ChE active site-specific inhibitor diisopropyl phosphorofluoridate indicated that BAC binds to the active site of ChEs. Furthermore, studies using a structural homolog of BAC indicated that the alkyl group of BAC is essential not only for its interaction with ChEs but also for its distinct effect on the esterase and AAA activities of BChEs. This is the first report of a compound that inhibits the esterase activity, while simultaneously activating the AAA activity, of BChEs.

INTRODUCTION

Cholinesterases (ChEs) are distinguished from nonspecific esterases by their sensitivity to the inhibitor eserine. In vertebrates, two types of ChEs exist, acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) (*1*). While the function of AChE is to terminate the signal transmission in cholinergic synapse by hydrolyzing acetylcholine (*2*), the precise physiological function of BChE has not been identified with certainty. Nevertheless, BChE is generally viewed as a backup enzyme for AChE (*3*). Since BChE has a wider substrate specificity and inhibitor sensitivity, it has also been proposed to function as a scavenger of naturally occurring poisonous compounds targeted at acetylcholine binding sites (*4*). Additional functions for AChE and BChE in development, physiology, and disease have been suggested (*5–7*). Apart from their predominant acylcholine hydrolase activity (commonly known

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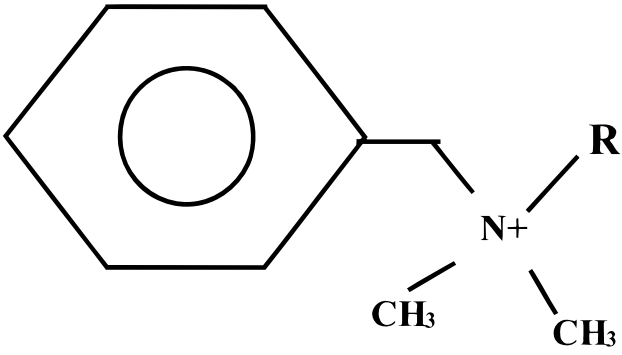
as esterase activity), ChEs also display an aryl acylamidase (AAA) activity catalyzing the cleavage of the synthetic substrate *o*-nitroacetanilide to *o*-nitroaniline and acetate (*8–10*). This AAA activity, other than being strongly inhibited by classical ChE inhibitors, is also susceptible to selective inhibition by serotonin (*11–13*). An exclusive feature of the AAA activity in human serum BChE is its several-fold activation by tyramine (*12*). A number of possible physiological functions for the AAA activity, including a role in an amine-sensitive pain mechanism, have been suggested (*9*).

The catalytic efficiency of ChEs seems to originate from the unique architecture of their active site. The X-ray structure of AChE (*14*) and site-directed mutagenesis of ChEs (*15,16*) have revealed the active site to be present within a 20 Å deep gorge. The catalytic triad is present at about 4 Å from the base of the gorge. Several functional subsites, each characterized by a defined set of amino acids, have been identified. These include the acyl pocket, the choline-binding pocket, the hydrophobic subsite, the oxyanion hole, and the peripheral anionic site. This heterogeneity of subsites explains the existence of multiple inhibitors, including competitive, noncompetitive, and irreversible ones, despite the deeply placed active site (*14,17,18*).

Alkylbenzyldimethylammonium chloride, commonly known as benzalkonium chloride (BAC), is a cationic surface active agent. It is the most widely used preservative in many ophthalmic solutions, nebulizer compounds, and nasal sprays (*19*). The charged part of the molecule interacts with many proteins with high affinity and in a very specific manner, thereby influencing their functions (*20*). In neuromuscular junctions, BAC has been shown to block transmission by acting as an acetylcholine agonist (*21*). Since BAC has a quaternary ammonium group (Fig. 1) similar to many reversible inhibitors of ChEs, it was of interest to analyze its effect on the catalytic functions of ChEs.



Benzalkonium chloride - R= $(CH_2)_{7\text{-}17}$ -CH_3

Benzyltrimethylammonium hydroxide - R= CH_3

FIG. 1. Structure of BAC and its homolog, benzyltrimethylammonium hydroxide.

In the present paper, we describe the inhibitory action of BAC on the esterase activities of ChEs from diverse sources such as human serum, horse serum, electric eel, and human erythrocytes. We further report for the first time the distinct manner in which BAC inhibits the esterase activity but activates the AAA activity of BChEs. The putative binding site for BAC on ChEs has been identified using the active site-specific ChE inhibitor, diisopropyl phosphorofluoridate (DFP). In addition the structural moiety of BAC that is responsible for its interaction with ChEs has been identified.

MATERIALS AND METHODS

Materials. Acetylthiocholine iodide (ATCI), DE-52, and procainamide were purchased from Sigma Chemical Co. (St. Louis, MO). Benzalkonium chloride was from LOBA Chemi Pvt. Ltd, (India); benzyltrimethylammonium hydroxide was from E Merck (Darmstadt, Germany). *o*-Nitroacetanilide was prepared as described elsewhere (*22*). All other chemicals used were of analytical grade and of the highest purity available.

Enzymes. Human serum BChE was purified to apparent homogeneity from outdated plasma as described earlier using DE-52 ion-exchange column and procainamide–Sepharose affinity columns (*23*). Horse serum BChE and electric eel AChE were obtained from Sigma Chemical Co.. Human erythrocyte AChE was extracted from human erythrocytes as described by Tornel *et al.* (*24*).

Effect of benzalkonium chloride. To assess the *in vitro* effect of BAC on the esterase and AAA activities of ChEs, the enzymes were preincubated with increasing concentrations of BAC for 10 min at 37°C and then assayed for activity. Control samples were devoid of BAC.

The inhibition constants K_i and αK_i were determined by using the primary Dixon plot. The values of K_s and αK_s were estimated from the secondary replots of the primary Dixon plot (*25*).

Enzyme assays. The esterase activities of ChEs were determined according to the method of Ellman *et al.* (*26*) using ATCI as substrate. The AAA activities of the ChEs were assayed as described earlier (*22*) using *o*-nitroacetanilide as substrate.

One unit of cholinesterase activity corresponds to the amount of enzyme which hydrolyses 1 μmol ATCI per min and one unit of AAA corresponds to 1 μmol of *o*-nitroaniline formed per hour, under the standard assay conditions.

Fluorescence spectroscopy. Human serum BChE at a concentration of 15 μg/ml in 0.1 M potassium phosphate buffer, pH 7.0, in the absence and presence of 10 μM BAC, was placed at 25°C in the cuvette holder of a Hitachi F-2000 fluorescence spectrophotometer. Then 8-anilino-1-naphthalene sulfonic acid (ANS) was added to the samples to a final concentration of 0.05 mM. The emission spectra were recorded with the excitation wavelength set at 380 nm. The excitation and emission bandpasses were set at 10 nm.

Statistical analysis. The graphs were plotted using the Sigma Plot program. Using the linear regression analysis of this program the value of slopes and intercepts were obtained (*28*).

RESULTS AND DISCUSSION

The inhibitory effect of BAC on the esterase activity of ChEs from different sources was concentration dependent (Fig. 2). BChEs from human and horse serum were more sensitive to inhibition by BAC than AChEs from electric eel and human erythrocyte. In all cases the inhibition was found to be reversible as the original activity was completely restored on dialysis of the BAC-inhibited enzymes against buffer devoid of BAC (data not shown). The rates of ATCI hydrolysis at variable substrate concentrations in the absence and presence of several concentrations of BAC were determined. From the pattern of lines in a Dixon plot (Figs. 3A–3D) it was determined that BAC behaved as a "mixed inhibitor" of all the ChEs. This implies that BAC can bind to the free enzyme, as well as to the enzyme–substrate complex. The slopes of the Dixon plot were linear and hence the inhibition is of the "linear-mixed" type. As the lines of the Dixon plot intersect each other above the *X*-axis, the inhibition is a mixture of the competitive and the noncompetitive type. The secondary replot of the Dixon plot, i.e., slopes versus 1/[ATCI], was linear and does not pass through the origin (inserts in Figs. 3A–3D). Hence the competitive inhibition is of the partial type. Since the replot of the intercepts of the Dixon plot versus 1/[ATCI] is linear, the noncompetitive inhibition is of the pure type. Thus, the mode of BAC inhibition of ChEs is "partial competitive–pure noncompetitive type of mixed inhibition." The equilibrium scheme for this type of mixed inhibition can be described as

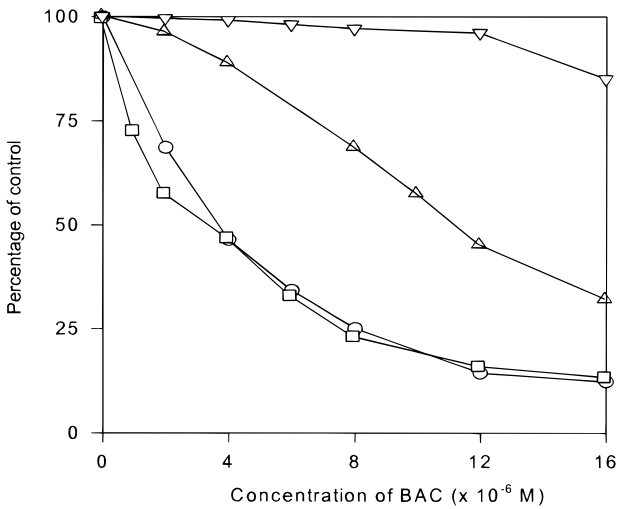


FIG. 2. Effect of BAC on the esterase activity of ChEs. Human serum BChE (○), horse serum BChE (□), electric eel AChE (△), and human erythrocyte AChE (▽) were incubated with varying concentrations of BAC for 10 min at 37°C. After incubation, the residual activities as percentages of control were determined using ATCI as substrate as outlined under Materials and Methods. The activity observed in the absence of BAC corresponds to control activity (100%). Each point is the average of three independent determinations.

$$\begin{array}{ccccc} E + S & \underset{K_S}{\rightleftharpoons} & E\,S & \rightarrow & E + P \\ + & & + & & \\ I & & I & & \\ \downarrow\uparrow K_i & & \downarrow\uparrow \alpha K_i & & \\ EI + S & \underset{\alpha K_S}{\rightleftharpoons} & ESI & & \end{array}$$

E is the enzyme, S the substrate, ES the enzyme–substrate complex, EI the enzyme–inhibitor complex, and ESI the enzyme–substrate–inhibitor complex. The inhibitor

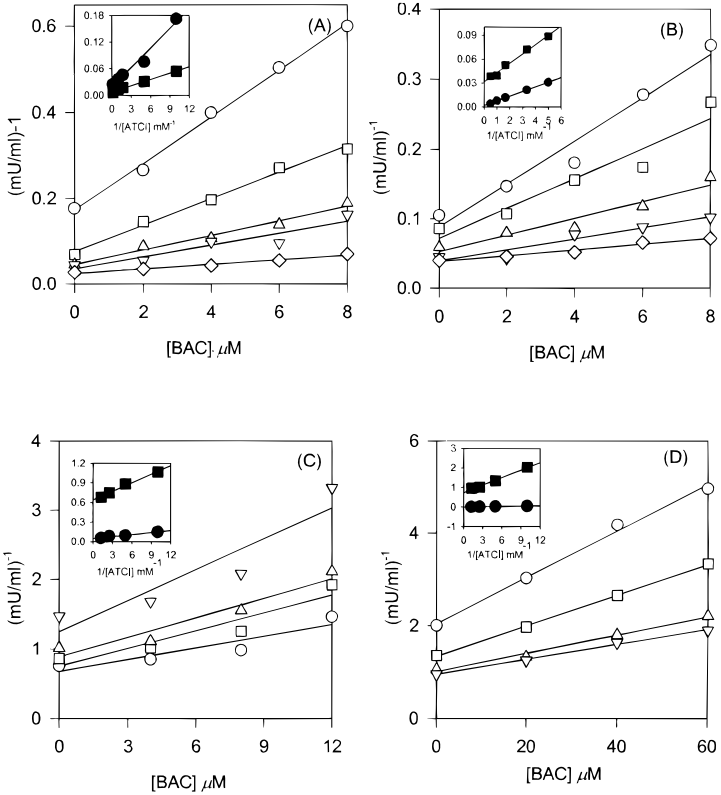


FIG. 3. Dixon plot for BAC inhibition of ChEs esterase activity: human serum BChE (A), horse serum BChE (B), electric eel AChE (C), and human erythrocyte AChE (D). The concentrations of the substrate ATCI were 5 mM (○), 1 mM (□), 0.6 mM (△), 0.2 mM (▽), and 0.1 mM (◇) for human serum BChE; 2 mM (○), 1 mM (□), 0.6 mM (△), 0.3 mM (▽) and 0.1 mM (◇) for horse serum BChE; and 0.8 mM (○), 0.4 mM (□), 0.2 mM (△), and 0.1 mM (▽) for electric eel and human erythrocyte AChE. The *X*-axis value corresponding to the point of intersection of the highest ATCI concentration line with the lowest ATCI concentration line gives the K_i, while the point of intersection of the 5 mM ATCI line with the *X*-axis gives the value of αK_i. The insert shows the secondary replot of slopes (■) and 1/*V* intercepts (●) from the Dixon plot against 1/[ATCI]. Each point represents the average of three independent determinations.