CHAPTER - III

EXCLUSIVE AND DISTINCT EFFECT OF BENZALKONIUM CHLORIDE ON ESTERASE AND ARYL ACYLAMIDASE ACTIVITIES OF HUMAN SERUM BUTYRYLCHOLINESTERASE.

1. SUMMARY

Benzalkonium chloride (BAC) reversibly inhibited human serum butyrylcholinesterase (BChE) in a concentration dependent manner. The inhibition was of the ‘linear mixed’ type with inhibition constants in the micromolar range. The characteristic property of BAC was its ability to profoundly activate the aryl acylamidase (AAA) activity associated with human serum BChE. A comparative analysis of the effect of BAC on the catalytic functions of horse serum BChE, electric eel acetylcholinesterase (AChE) and human erythrocyte AChE were carried out. The catalytic functions of horse serum BChE were modulated similarly to that of human serum BChE. Though the esterase activity of the AChEs from different sources was inhibited, BAC did not cause an activation of their AAA activity. Thus BAC seems to preferentially activate the AAA activity on BChE alone. Studies using a structural homologue of BAC indicated that the alkyl group of the detergent is essential not only for its interaction with ChEs but also to for its distinct effect on the esterase and AAA activities on
BChEs. The $IC_{50}$ values of diisopropyl phosphorofluoridate (DFP) inhibition were increased in the presence of BAC with all cholinesterases (ChEs) analysed. Also, the pseudo-first order rate constants ($k_1$) for DFP inactivation of the ChEs were decreased in the presence of the detergent. These results indicated that BAC modulates the catalytic functions of ChEs by interacting with the active site region. This is the first report of a compound that modulates the catalytic functions of BChE (esterase and aryl acylamidase) in a mutually exclusive and distinct manner.

2. INTRODUCTION

Cholinesterases (ChEs) are distinguished from non-specific 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) [4]. AChE functions in terminating neurotransmission at the cholinergic synapses by hydrolysing acetylcholine [238]. The role of BChE, beyond hydrolysing acetylcholine at concentrations that would inhibit AChE, has not been identified with certainty [239]. As BChE has wider substrate specificity and inhibitor sensitivity, it has been proposed to function as a scavenger of naturally occurring poisonous compounds targeted at acetylcholine binding sites [23]. Additional functions for AChE and BChE in development, physiology and disease have been suggested [240-242]. Apart from their predominant acylcholine hydrolase activity (commonly known as esterase activity), ChEs also display an aryl
acylamidase (AAA) activity catalysing the cleavage of the synthetic substrate o-nitroacetanilide to o-nitroaniline and acetate [53, 54, 243]. This AAA activity, in addition being strongly inhibited by classical ChE inhibitors, is also susceptible to selective inhibition by 5-hydroxytryptamine [52, 109, 244]. An exclusive feature of the AAA activity in human serum BChE is its several fold activation by tyramine [52]. A number of possible physiological functions for the AAA activity, including a role in an amine-sensitive pain mechanism have been suggested [53].

The catalytic efficiency of ChEs seems to originate from the unique architecture of the active site. The X-ray structure of AChE [113] and site-directed mutagenesis of ChEs [113, 145] have revealed the active site of the enzymes to be present within a 20Å deep gorge. The catalytic triad is present at about 4Å from the base of the gorge. Several functional subsites, each characterised by a unique set of amino acids, have been identified. These include the acyl pocket, choline binding pocket, the hydrophobic subsite, the oxyanion hole and the peripheral anionic site. This heterogeneity of potential subsites explains the existence of multiple inhibitors, including competitive, non-competitive and irreversible ones, despite the deep placed active site [113, 136, 190].

Alkylbenzylidimethylammonium 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 [245]. The charged part of the molecule interacts with many proteins with high affinity and in a very specific manner, thereby influencing their properties [246]. In neuromuscular junctions, BAC has been shown to block transmission by acting as an acetylcholine agonist [247]. Similar to many reversible inhibitors of ChEs BAC has a quaternary ammonium group (Figure 13), and hence it was of interest to analyse its effect on the catalytic activities of ChEs.

In this chapter, the inhibitory action of BAC on the esterase activities of ChEs is described. Also, the exclusive manner in which BAC activates the AAA activity on BChEs is explained. Using human serum BChE, a possible mechanism for these exclusive and distinct effects of BAC on the esterase and AAA activities on BChE is given. The structural moiety of BAC that is responsible for its interaction with ChEs has been identified. In addition, the putative binding site for BAC on ChEs has been identified using the active site specific ChE inhibitor, diisopropyl phosphorofluoridate (DFP).

3. MATERIALS AND METHODS

Materials

All chemicals were of analytical grade and purchased from Sigma Chemical Co. (St.Louis, USA) unless otherwise stated. Benzalkonium chloride was from LOBA Chemi Pvt. Ltd., (India); Benzylltrimethylammonium hydroxide was from E Merck (Darmstadt,
**Figure 13.** Structure of BAC and its homologue, benzyltrimethylammonium hydroxide

Benzalkonium chloride - $R = -(CH_2)_{8-16}-CH_3$

Benzyltrimethylammonium hydroxide - $R = -CH_3$
Germany). o-nitroacetanilide was prepared as described in the appendix IIb (page 85).

Enzymes

Human serum BChE was purified from outdated plasma as described in appendix I (page 83). Horse serum BChE and electric eel AChE were obtained from Sigma Chemical Co., St. Louis, USA. Human erythrocyte AChE was extracted from human erythrocytes as described by Tornel et al., [226].

Effect of benzalkonium chloride

To assess the in vitro effect of BAC on the esterase and AAA activities of ChEs, the enzymes were pre-incubated with increasing concentrations of BAC for 10 min at 37°C and then assayed for activities. Control samples were devoid of BAC.

The inhibition constants \( K_i \) and \( \alpha K_i \) were determined by using the primary Dixon plot. The values of \( K_s \) and \( \alpha K_s \) were estimated from the secondary replots of the primary Dixon plot [249]. The \( IC_{50} \) values and pseudo-first rate constants \( (k_r) \) for DFP inhibition of ChEs were determined as described in chapter II.

Enzyme assays

The esterase activity of ChEs was determined according to the method of Ellman et al essentially as detailed in appendix IIa (page 84),
except that 1 mM acetylthiocholine iodide (ATCI) was used as the substrate instead of BTCI. The AAA activity of the ChEs were assayed as described in appendix IIb (page 85) using o-nitroacetanilide as substrate.

One unit of esterase activity is the quantity of enzyme required to liberate 1 μmole of thiocholine per min from the substrate ATCI under the standard assay conditions. One unit of AAA activity is defined as the quantity of the enzyme required to liberate 1 μmole of o-nitroaniline in 1 hr 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 of BAC was placed at 25°C in the cuvette holder of a Hitachi F-2000 fluorescence spectrophotometer. Then 0.05 mM of 8-anilino-1-napthalene sulfonic acid (ANS) was added to the samples. The emission spectra were recorded with the excitation wavelength set at 380 nm. The excitation and emission band passes were set at 10 nm.

*Statistical analysis*

The Dixon plots were plotted using the Sigma Plot program. Using the linear regression analysis of this program the value of slopes and intercepts were obtained.
4. RESULTS AND DISCUSSION

The inhibitory effect of BAC on the esterase activity of human serum BChE was concentration dependent (Figure 14) and found to be reversible as the original activity was completely restored on dialysis of the BAC inhibited enzyme. The rates of ATCI hydrolysis at different substrate concentrations in the absence and presence of several concentrations of BAC were assayed. From the pattern of lines in Dixon plot (Figure 15) it was determined that BAC behaved as a ‘mixed inhibitor’ of human serum BChE. 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 was determined to be of the ‘linear-mixed’ type. As the lines of the Dixon plot intersected each other above the X-axis, the inhibition was a mixture of competitive and non-competitive type. The secondary replots of the Dixon plot slopes versus 1/[ATCI] was linear and does not pass through the origin (inset to Figure 15). Hence the competitive inhibition was of the partial type. Since the replots of Dixon plot intercepts versus 1/[ATCI] was linear, the non-competitive inhibition was of the pure type. Thus, the mode of BAC inhibition of human serum BChE has been deduced to be of the ‘partial competitive-pure noncompetitive type of mixed inhibition’. The equilibrium scheme for this type of mixed inhibition can be given as follows:
Figure 14. Effect of BAC on the esterase activity of BchE: The BChE enzyme was incubated with varying concentrations of BAC for 10 min at 37°C. After incubation, the residual activities as percentage of control were determined using ATCI as outlined under ‘Materials and Methods’. The activity observed in the absence of BAC corresponds to control activity (100%). Each point represents the average of three independent determinations.
Figure 15. Dixon plot for BAC inhibition of BchE: The concentration of the substrate, ATCI were 5 mM (○), 1 mM (□), 0.6 mM (△), 0.2 mM (▼) and 0.1 mM (◇). The X-axis value corresponding to the point of intersection of the 5 mM ATCI line with the 0.1 mM ATCI line gives the $K_i$, while the point of intersection of the 5 mM ATCI line with the X-axis gives the value of $\alpha K_i$. Each point represents the average of three independent determinations. The inset shows the secondary replot of slopes (■) and 1/V intercepts (●) from Dixon plot against 1/[ATCI].
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 constant $K_i$ (dissociation constant of the EI complex into free E and I) and $\alpha K_i$ (dissociation constant of the ESI complex into ES and I) were determined from the primary Dixon plot (Figure 15). The constants $K_s$ (dissociation constant of the ES complex into free E and S) and $\alpha K_s$ (dissociation constant of the ESI complex into EI and S) were determined from the secondary replots of the Dixon plot (inset to Figure 15). The estimated values of $K_i$, $\alpha K_i$, $K_s$ and $\alpha K_s$ for BAC inhibition of the esterase activity of human serum BChE were 1.03 $\mu$M, 4.06$\mu$M, 0.64 mM and 0.81 mM respectively.

An interesting observation was the activation of the AAA activity on human serum BChE by BAC (Figure 16). There was a 4-fold activation of the AAA activity at 16 $\mu$M concentration of BAC. Previous studies have shown that tyramine exclusively activated the AAA activity on human serum BChE, but to cause a 4-fold activation, a concentration as high as 1 mM of tyramine was required [109]. Thus, from the present study it is clear that BAC is a more powerful activator of human serum AAA. Further,
Figure 16. The activation of AAA activity associated with BchE: The BChE enzyme was incubated with increasing concentrations of BAC (●) or tyramine (■) for 10 min at 37°C and then they were assayed for AAA activity using o-nitroacetanilide. The activity observed without the addition of any activator corresponds to 100% activity. Each point represents the average of three independent determinations.
unlike tyramine, which has no effect on the esterase activity of human serum BChE, BAC caused a simultaneous inhibition of the esterase activity. Classical ChE inhibitors inhibit both the esterase and the AAA activities of human serum BChE and indolamines such as serotonin specifically inhibit the AAA activity. Though the active sites of both esterase and AAA activities of human serum BChE overlap [173], BAC was able to differentially modulate the two activities. This is the first report of a compound that modulates the esterase and AAA activities of human serum BChE in an exclusive and distinct manner.

A comparative analysis of the effect of BAC on the catalytic functions of horse serum BChE, electric eel AChE and human erythrocyte AChE were done. It was found that the esterase activity of the ChEs analysed were inhibited by BAC. The inhibition was also of the partial competitive- pure noncompetitive mixed type (Figure 17). The values of the various kinetic constants for BAC inhibition of horse serum BChE, electric eel AChE and human erythrocyte AChE are given in Table 7. In the case of AAA activity, BAC was able to activate the AAA activity on horse serum BChE. Surprisingly, the compound had no effect on the AAA activity of either of the AChEs analysed (Figure 18). Thus, the results indicate that BAC preferentially activates the AAA activities on BChEs only.

The difference in susceptibility to BAC activation of AAA on BChE and AChE could be attributed to the sequence difference found in these two
Figure 17. Dixon plot for inhibition of ChEs by BAC: Horse serum BChE (A), Electric eel AChE (B) and human erythrocyte AChE (C). The concentration of the substrate ATCI were 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. Each point represents the average of three independent determinations. The insets show the secondary replots of slopes (●) and 1/V intercepts (■) from Dixon plot against 1/[ATCI].
Table 7. Kinetic parameters for BAC inhibition of esterase activity of ChEs.

<table>
<thead>
<tr>
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<th>$K_i$ (μM)</th>
<th>$\alpha K_i$ (μM)</th>
<th>$K_s$ (mM)</th>
<th>$\alpha K_s$ (mM)</th>
</tr>
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<tbody>
<tr>
<td>Human serum BChE</td>
<td>1.03</td>
<td>4.66</td>
<td>0.64</td>
<td>0.81</td>
</tr>
<tr>
<td>Horse serum BChE</td>
<td>1.87</td>
<td>9.14</td>
<td>0.37</td>
<td>3.19</td>
</tr>
<tr>
<td>Electric eel AChE</td>
<td>4.16</td>
<td>12.16</td>
<td>0.068</td>
<td>0.19</td>
</tr>
<tr>
<td>Human erythrocyte AChE</td>
<td>32.28</td>
<td>58.68</td>
<td>0.18</td>
<td>0.35</td>
</tr>
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Inhibition constants $K_i$ and $\alpha K_i$ were calculated from the primary Dixon plot as described under Figure 15. The value of $K_s$ is the inverse of the X-axis intercept of the secondary replot of the intercepts from the Dixon plot versus $1/[ATCI]$ concentration, and $\alpha K_s$ is the inverse of the replot of the slopes of the Dixon plot versus $1/[ATCI]$ concentration (insets to Figure 15 &17).
Figure 18. The effect of BAC on the AAA activities of ChEs. The ChEs from horse serum BChE (○), electric eel AChE (□) and human erythrocyte AChE (△) were incubated with increasing concentrations of BAC for 10 min at 37°C and then they were assayed for AAA activity using o-nitroacetanilide. The activity observed without the addition of BAC corresponds to 100% activity. Each point represents the average of three independent determinations.
enzymes [144]. Modelling studies have revealed the absence in BChE of the six conserved aromatic amino acid residues that line the active site gorge of AChE. Also, the acyl pocket, choline binding pocket and the hydrophobic subsites are larger in BChE, whose dimensions are constrained in AChE due to the presence of bulky aromatic residues [144]. Thus, BChE but not AChE may undergo a conformational change upon binding of BAC. This conformational change could be the reason for the activation of the AAA activities on BChEs.

Evidence for a conformational change in BChE upon BAC binding comes from the ANS binding studies. The fluorescence spectrum of ANS-human serum BChE in the absence and presence of BAC is shown in Figure 19. The fluorescence of ANS upon binding to BChE shows an emission maximum of 515 nm. The emission maximum shifts to 499 nm in the presence of BAC. This shift in the emission maxima from a higher wave length to a lower wave length (blue shift) indicates that in the presence of BAC, BChE undergoes a conformational change such that its hydrophobic surface are exposed to a greater extent compared to the enzyme in the absence of BAC [269].

The structural feature of BAC (Figure 13) includes a quaternary ammonium group (analogous to cationic substrates/reversible inhibitors of ChEs), a hydrophobic region (lent by the alkyl chain linked to the quaternary ammonium group) and an aromatic group (lent by the benzyl
Figure 19. ANS fluorescence spectrum of human serum BChE in the absence (...) and presence of (—) BAC as detailed under 'Materials and Methods'. The emission spectra were recorded with the excitation wavelength set at 380 nm. The excitation and emission band passes were set at 10 nm.
Thus the ability of BAC to modulate the catalytic functions of ChEs would depend upon the relative importance of its ionic or hydrophobic group to interact with similar regions of the enzymes. The importance of hydrophobicity in BAC-ChE interaction was exemplified by studies using a structural homologue of BAC. The benzyltrimethylammonium hydroxide is a homologue of BAC where a methyl group replaces the alkyl group of BAC. This compound did not show any effect on the catalytic functions of ChEs (data not shown). Thus, BAC would possibly modulate the catalytic functions of ChEs through hydrophobic interaction (via its alkyl group) with the active site hydrophobic region of ChEs.

To test the accessibility of the active site of ChEs in the presence of BAC, the \( IC_{50} \) values and pseudo-first order rate constant for DFP inactivation of the esterase activity of ChEs in the absence and presence of BAC were calculated. In the presence of BAC, the \( IC_{50} \) values for DFP inhibition were increased for all the ChEs analysed (Table 8). This indicates that BAC by was bound to the active site of ChEs in such a way to prevent DFP from interacting with the enzymes. Further confirmation for this masking effect comes from the pseudo-first order rate constants, \( k_f \), for DFP inactivation of ChEs, which were reduced in assays performed in the presence of the detergent (Table 8). Reversible inhibitors are known to bind to choline binding subsite by a combination of columbic and van der Waal’s
Table 8. IC$_{50}$ values and pseudo-first order rate constant ($k_i$) for DFP inhibition of ChEs in the absence and presence of BAC

<table>
<thead>
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<th></th>
<th>IC$_{50}$ (M)</th>
<th>$k_i$ (min)$^{-1}$</th>
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<tbody>
<tr>
<td></td>
<td>- BAC</td>
<td>+ BAC</td>
</tr>
<tr>
<td>Human serum BChE</td>
<td>$1.75 \times 10^{-8}$</td>
<td>$6.85 \times 10^{-8}$</td>
</tr>
<tr>
<td>Horse serum BChE</td>
<td>$5.45 \times 10^{-8}$</td>
<td>$9.98 \times 10^{-8}$</td>
</tr>
<tr>
<td>Electric eel AChE</td>
<td>$7.75 \times 10^{-6}$</td>
<td>$15.81 \times 10^{-6}$</td>
</tr>
<tr>
<td>Human erythrocyte AChE</td>
<td>$14.86 \times 10^{-7}$</td>
<td>$19.34 \times 10^{-7}$</td>
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The concentration of BAC used in assays performed in the presence of the detergent were, 5 x $10^{-7}$ M with human serum BChE and horse serum BChE and 5 x $10^{-6}$ M with electric eel AChE and human erythrocyte AChE. The concentration of DFP used for the determination of $k_i$ were, 5 x $10^{-8}$ M with human serum BChE and Horse serum BChE, 8 x $10^{-6}$ M with electric eel AChE and 1.5 x $10^{-5}$ M with human erythrocyte AChE.
forces. Smaller alkyl substituents of these inhibitors bind by van der Waal’s force to adjacent hydrophobic regions and larger alkyl substituents react beyond the choline binding pocket and in fact interact with groups at the esteratic site or regions peripheral to both sites [175]. Similarly, though BAC binds to the choline binding pocket it could interact with the esteratic site through its alkyl group. This might be the reason for the observed decrease in $k$, for DFP inactivation of ChEs in the presence of BAC. Similar protection of ChEs by the non-ionic detergent Triton X-100 and the zwitterionic detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate against inactivation by organophosphates have been reported [226, 250].

Benzyltrimethylammonium hydroxide, which has only a methyl group instead of the long alkyl group of BAC, could not protect the ChEs against DFP inactivation. Thus, the action of BAC on the catalytic functions and inhibitor sensitivity (towards irreversible inhibitors) could be attributed to its alkyl group.

To summarise, it has been demonstrated that the cationic detergent BAC reversibly inhibits the esterase activity of ChEs in a mixed manner. BAC has been documented to be a neurotoxin, but its mode of action on neurons is unclear. The present investigation indicates that inhibition of ChEs could be the mechanism by which BAC exerts its neurotoxicity. Further the detergent preferentially activates the AAA activity in BChEs.
alone. This is the first report of a compound that exclusively and distinctly modulates the esterase and AAA activities of BChE in diametrically opposite way. The alkyl group of BAC and active site hydrophobic region of BChE seem to play a central role in this differential modulation. The ability of BAC to preferentially activate AAA in BChEs can also be used to differentiate human serum BChE from that of AChE. Most importantly, as BAC inhibits the esterase activity alone, it can be used as a tool to probe the possible physiological roles of AAA activity of BChEs.