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EFFECT OF TETRACAINE ON MEMBRANE-BOUND ACETYLCHOLINESTERASE ACTIVITY AND ANILINONAPHTHALENE SULPHONATE-INDUCED MEMBRANE FLUORESCENCE

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Abstract—Tetracaine (25–300 μM) reversibly inhibits (*in vitro*) AChE activity of rat brain synaptosome (4.4–100%) and erythrocyte membrane (3.9–65.2%) in a concentration dependent manner. IC_{50} values of tetracaine for AChE of synaptosome and erythrocyte membrane are 88 and 200 μM respectively. At sub-inhibitory concentrations ($\leq$10 μM) tetracaine activates (8.7–23%) AChE of synaptosome but not of erythrocyte membrane. Lineweaver–Burk plots indicate that tetracaine-induced inhibition of AChE is competitive in nature and K_i value decreases on increasing the concentration (>100 μM) of tetracaine in both synaptosome and erythrocyte membrane. Tetracaine (25–500 μM) produces a concentration dependent increase in 1-anilino-8-naphthalene sulphonate (ANS)-induced relative fluorescence (F_{470}) of both synaptosomal (6.5–102%) and erythrocyte membrane (2.4–53.3%) without shifting their emission maxima (470 nm). Further it is also noted that the quantum yield of F_{470} of both the membranes increases with the increase of tetracaine concentrations (100–500 μM). These results suggest that the interaction of tetracaine with both the enzyme and its lipid microenvironment may be the cause of inhibition of membrane-bound AChE activity.

Tetracaine [4-(butylamino)benzoic acid 2-(dimethylamino)ethyl ester] is a potent [1] and broad spectrum amine local anesthetic [2]. Local anesthetics are known to increase the lipid fluidity of biological as well as synthetic model membranes [3–8] and it has been suggested that anesthesia develops from a fluidous extension of membrane lipids [4, 9]. Recent reports from our [7, 8] and other [6, 10–12] laboratories have shown that local anesthetics affect the activities of various membrane-bound enzymes. But it is not clear whether local anesthetics affect the activities of the membrane-bound enzymes by their direct interaction with the enzyme protein or through their effect on the lipid microenvironment of the enzymes or by a combined effect on both lipid bilayer and enzyme molecule. Nachmansohn [13] has assumed that acetylcholine (ACh) is involved in the mechanism of impulse transmission along the nerve fibres and the enzyme acetylcholinesterase (AChE, EC.3.1.1.7) is believed to play a regulatory role in the electrical activities of excitable membranes by means of hydrolytic degradation of ACh.

The present investigation deals with the *in vitro* effect of tetracaine on rat brain synaptosomal and erythrocyte membrane-bound AChE activity and this effect of the drug has been monitored in relation to lipid microenvironment of AChE using 1-anilino-8-naphthalene sulphonate (ANS) as fluorescence probe. This anionic species binds noncovalently to both membrane lipids and proteins and appears to be extremely sensitive to changes in the probe environment [14].

MATERIALS AND METHODS

Adult male albino rats (Charles Foster strain) weighing 125–150 g, maintained on a standard laboratory diet and water *ad libitum* were used in the present study. Acetylcholine chloride, tetracaine hydrochloride, bovine serum albumin, ammonium salt of 1-anilino-8-naphthalene sulphonic acid were purchased from Sigma Chemical Co., U.S.A. Other chemicals were of analytical grade.

Rat brain synaptosome was prepared according to the differential centrifugation method of Gray and Whittaker [15], modified by Bradford *et al.* [16]. Erythrocyte membrane from rat blood was prepared in accordance with the method of Dodge *et al.* [17]. AChE activity in the absence and presence of tetracaine (5–400 μM) was determined following the colorimetric method of Hestrin [18], modified by Kaplay [19]. The incubation was carried out at 37° for 20 min using 0.4 mg synaptosomal protein or 0.8 mg erythrocyte membrane protein as enzyme source. Preliminary experiments were performed to ensure that the enzyme activity is linear with respect to incubation time and amount of enzyme employed. For kinetic studies the substrate concentrations were varied from 0.25 to 4.0 mM.

To study the interaction of tetracaine with the biological membranes, ANS was used as a fluorescence probe. ANS-induced relative fluorescence of synaptosomal and erythrocyte membrane was measured according to the method described by Haque *et al.* [7]. Incubation mixture containing

Table 1. *In vitro* effect of tetracaine on acetylcholinesterase (AChE) activity of rat brain synaptosome and erythrocyte membrane

Tetracaine concentration (mM)	AChE activity (μmole ACh/hr/mg protein)	
	Synaptosome	Erythrocyte membrane
0.000	9.846 ± 0.240	4.246 ± 0.105
0.005	12.123 ± 0.283[a]	4.228 ± 0.195
0.010	10.707 ± 0.228[c]	4.308 ± 0.232
0.025	8.923 ± 0.230[c]	4.080 ± 0.074
0.050	6.892 ± 0.369[a]	3.569 ± 0.154[b]
0.100	4.431 ± 0.181[a]	3.034 ± 0.062[a]
0.200	1.969 ± 0.209[a]	2.123 ± 0.080[a]
0.300	nil	1.476 ± 0.077[a]
0.400	nil	0.061 ± 0.043[a]

Each value represents the mean ± S.E.M. of four separate determinations. Significantly different from control: [a]$P < 0.001$, [b]$P < 0.02$ and [c]$P < 0.05$.

No preincubation was done in the presence of tetracaine. Other details are given in the text.

0.4 mg membrane (synaptosome or erythrocyte) protein and 100 μM ANS in the absence and presence of tetracaine (25–500 μM) in 50 mM Tris–HCl buffer, pH 7.0 was incubated at 37° for 20 min and the fluorescence was measured using excitation and emission wavelengths 385 and 470 nm respectively at 37° [20]. The quantum yield of ANS-induced relative fluorescence was measured according to the method of Azzi [21] using the above excitation and emission wavelengths.

Protein contents of membrane preparation was measured according to Lowry *et al.* [22] using bovine serum albumin as standard.

Statistical analysis was performed by using two-tailed Student's *t*-test.

RESULTS

It appears from Table 1 that tetracaine produces a significant and concentration dependent *in vitro* inhibition of AChE activity in synaptosome (4.4–100%) and erythrocyte membrane (16–100%) at concentrations 25–300 μM and 50–400 μM respectively. Tetracaine at sub-inhibitory concentrations (≤10 μM) is found to produce a significant stimulation (8.7–23%) of AChE activity in synaptosome but not in erythrocyte membrane (Table 1). The IC_{50} values of tetracaine for AChE (calculated from the results of Table 1) of synaptosomal and erythrocyte membrane are 88 and 200 μM respectively.

Repeated (thrice) washing of tetracaine-treated membrane with 20 ml of ice-cold 0.5 mM Tris–HCl buffer, pH 7.4 by means of centrifugation at 4° for 45 min at 30,000 *g* is found (Table 2) to restore AChE activity by 95.1 and 89.2% in synaptosome and erythrocyte membrane respectively.

Lineweaver–Burk plots (Fig. 1) show that tetracaine, at concentrations 50, 100 and 150 μM, increases the K_m of synaptosomal AChE by 100, 200 and 500% respectively and similarly the K_m of AChE in erythrocyte membrane is increased by 124.5, 318.9 and 546.9% in the presence of 100, 200 and 300 μM tetracaine respectively. V_{max} of AChE in both synaptosome and erythrocyte membrane remains unaltered in the presence of tetracaine at the respective concentrations mentioned above. It is also noted from Fig. 1 that the K_i value (a) remains constant in synaptosome at lower concentrations (50–100 μM) of tetracaine, (b) reduces in synaptosome (40%) and erythrocyte membrane (31%) with the increase of tetracaine concentrations from 100 to 150 μM and from 100 to 300 μM respectively and (c) at a particular concentration (100 μM) of tetracaine, is higher in erythrocyte membrane than that of synaptosome.

The nature of interaction of tetracaine with lipid bilayer of synaptosomal and erythrocyte membrane has been studied by measuring the ANS-induced relative fluorescence (F_{470}) and its quantum yield. It is apparent from the results (Table 3) that tetracaine produces a significant and concentration dependent increase in F_{470} of synaptosomal (6.5–102%) and erythrocyte membrane (5.4–53.3%) at concentrations 25–500 μM and 50–500 μM respectively. Table 3 also shows that tetracaine (100–500 μM) significantly increases the quantum yield of F_{470} of both

Table 2. Effect of repeated washing of tetracaine treated synaptosomal and erythrocyte membrane on the recovery of acetylcholinesterase (AChE) activity

Tissue	System	AChE activity (μmole ACh/hr/mg protein)	
		Before washing	After washing
Synaptosome	Control	10.154 ± 0.270 (100)	9.600 ± 0.236 (100)
	Tetracaine (0.1 mM)	5.027 ± 0.221 (49.5)	9.126 ± 0.302 (95.1)
Erythrocyte membrane	Control	4.369 ± 0.105 (100)	4.246 ± 0.197 (100)
	Tetracaine (0.2 mM)	2.422 ± 0.087 (44.6)	3.787 ± 0.125 (89.2)

Each value represents the mean ± S.E.M. of three separate determinations. Values in the parentheses represent the per cent activity of AChE.

No preincubation of the enzyme was performed in the absence and presence of tetracaine. Other details are given in the text.

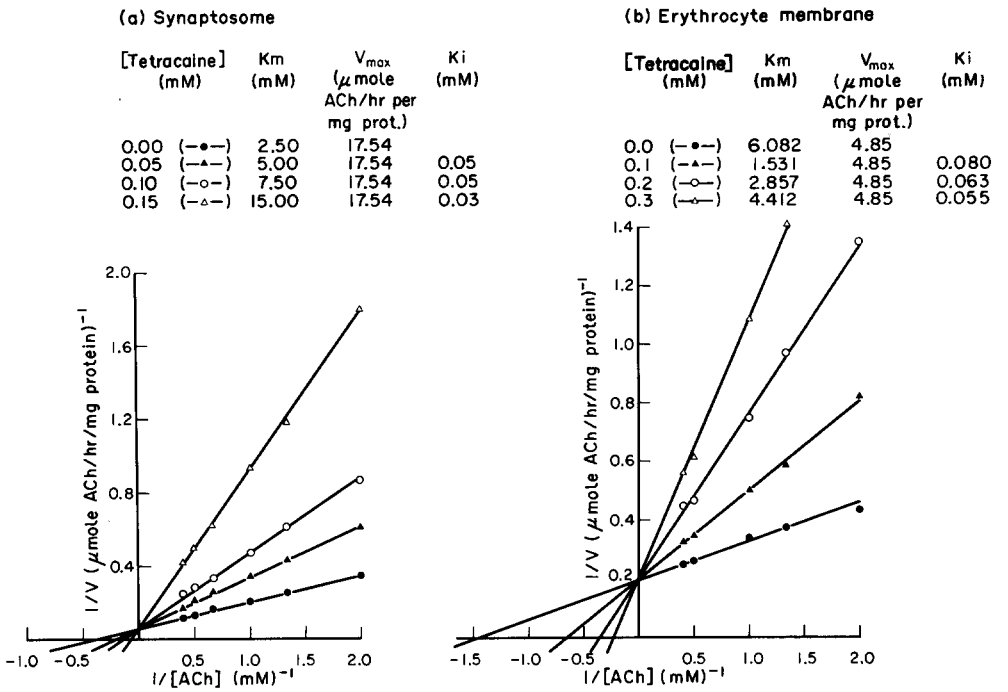

Fig. 1. Lineweaver–Burk plots of rat (a) brain synaptosome and (b) erythrocyte membrane in the absence and presence of tetracaine (50–300 μM). Other experimental details are described in the text. Each point represents the mean of four separate determinations.

synaptosomal (18.2–74.2%) and erythrocyte membrane (10.3–46.7%). Further it is noted (Fig. 2) that the emission maximum (470 nm) of ANS-induced relative fluorescence of both synaptosomal and erythrocyte membranes remains constant in the absence and presence of tetracaine (200–400 μM).

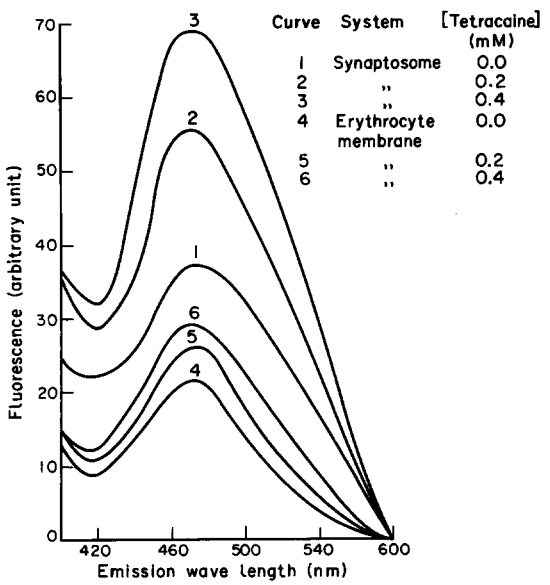

Fig. 2. Spectra of ANS-induced relative fluorescence of rat brain synaptosomal and erythrocyte membrane in the absence and presence of tetracaine. The excitation wave length is 385 nm. Other details are described in the text.

DISCUSSION

Results described in Table 1 reveal that tetracaine produces a concentration dependent *in vitro* inhibition of AChE activity in synaptosomal and erythrocyte membranes. Almost complete recovery of AChE activity by repeated washing of tetracaine-treated membranes (Table 2) indicates that the inhibition is reversible in nature. Results of Ackermann–Potter plots, dilution experiment and variation of preincubation time (not shown) also confirm the reversibility of the inhibition [8]. Tetracaine-induced decrease in substrate affinity (K_m^{-1}) without any change in the catalytic property (V_{max}) of synaptosomal and erythrocyte membrane-bound AChE activity (Fig. 1) indicates that the inhibition is competitive in nature. Amine local anesthetics, like procaine, tetracaine, are known to predominantly exist in cationic form at physiological pH [2], structurally resembling acetylcholine molecule [23]. Hence there is a possibility of the direct interaction of the cationic form of tetracaine and anionic subsite of AChE molecule.

Moreover the observed variation of K_i value at different concentrations (>100 μM) of inhibitor suggests the possibility of more than one type of interaction of tetracaine and AChE molecule. Further it appears from the comparative studies of IC_{50} and K_i values that synaptosomal AChE is more susceptible to tetracaine than that of erythrocyte membrane. A small but significant activation of synaptosomal AChE in the presence of sub-inhibitory concentrations of tetracaine (≤10 μM) possibly suggests a concentration dependent characteristic interaction

of tetracaine with the neuronal membrane rather than the non-neuronal one.

Several workers have shown that local anesthetics have very high affinity for lipids, specially phospholipids [24–26]. AChE is an extrinsic [27] phospholipoprotein of which the phospholipid part is highly involved in the regulation of its activity [28, 29]. Hence tetracaine is expected to interact with the lipids attached to or surrounding the enzyme molecules in the membranes. Studies with ANS, the well known anionic fluorescence probe, reveal that tetracaine produces a concentration dependent increase in ANS-induced relative fluorescence (F_{470}) and its quantum yield of both synaptosomal and erythrocyte membranes (Table 3). Previously it has been demonstrated that ANS molecule binds to the polar head group regions of model phospholipid membranes [14, 30] which is markedly influenced by the surface charge of the membrane [31, 32]. It has been reported that cations at 1–10 mM concentrations increase the intensity of ANS-induced fluorescence of membranes [32, 33] without changing the quantum yield [32], which is believed to be due to an increase in the number of membrane-bound ANS molecules [32, 34, 35]. Hence the observed increase in quantum yield in the presence of relatively smaller concentrations (100–500 μM) of tetracaine (Table 3) indicates that this drug-induced increase in F_{470} is not due to the increase in the number of bound ANS molecules, although it is reported to exist in the cationic form [2]. Moreover, the observed lack of shift of emission maximum (470 nm) of ANS-induced relative fluorescence of both synaptosomal and erythrocyte membrane in the presence of tetracaine (Fig. 2) indicates that this drug does not produce any change of the polarity in the vicinity of ANS binding site(s) on the membranes [24, 36]. Hence it may be suggested that the tetracaine-induced increase in F_{470} may not be due to the accumulation of positive charge on the membrane, which is further supported by the observed increase in F_{470} in the presence of benzyl alcohol [37], a neutral local anesthetic capable of increasing the lipid fluidity of biological membranes [6]. The enhancement of ANS fluorescence in biological membrane suspension has been believed to be due to the movement of the probe molecules from aqueous environment to the non-polar lipid bilayer of the membrane [14]. Tsong [14, 38] has assumed that two types of ANS binding occur in biological membrane, at relatively high concentrations, viz. type A and type B. The type A binding takes place at the deeper site, compared to the type B, in the lipid bilayer of the membrane with a relatively greater quantum yield [14, 38]. Further it has also been shown that the lower the viscosity of the lipid bilayer, the greater will be the penetration of the probe molecule into the bilayer and consequently more will be the type A binding [39]. Using chlorophyll *a* as a fluorescence probe Lee [4] has previously shown that tetracaine reduces the temperature of lipid phase transition of model phospholipid membranes which has been thought to be due to the increase in lipid fluidity of the membrane [8, 40, 41]. Thus from the present results it may be inferred that tetracaine-induced increase in F_{470} and quantum yield may be due to the greater type A binding of ANS molecules in the membrane as a result of increase in lipid fluidity. Hence it may be suggested that the change in the physical state of the lipid microenvironment is possibly associated with the inhibition of the activity of this membrane-bound enzyme [6–8, 11].

Evaluation of the changes in kinetic parameters (K_m and K_i) as well as results of fluorescence studies suggests that the tetracaine-induced inhibition of AChE activity may not be entirely attributed to either the direct binding of the drug to the enzyme molecule or the interaction of the drug and the lipid microenvironment of the enzyme. Finally it may be concluded that the tetracaine-induced inhibition of membrane-bound AChE activity is due to its combined effects on both the enzyme molecule and the lipid microenvironment.

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Table 3. *In vitro* effect of tetracaine on ANS-induced relative fluorescence (F_{470}) and quantum yield (ϕ) of rat brain synaptosomal and erythrocyte membrane

Tetracaine concentration (mM)	Synaptosome		Erythrocyte membrane	
	% increase of F_{470}	% increase of ϕ	% increase of F_{470}	% increase of ϕ
0.025	6.50 ± 0.45[b]	—	2.44 ± 0.38	—
0.050	12.32 ± 0.55[a]	—	5.42 ± 0.75[b]	—
0.100	24.80 ± 0.79[a]	18.18 ± 1.54[a]	10.30 ± 0.22[a]	10.30 ± 1.50[c]
0.200	52.00 ± 1.09[a]	32.08 ± 2.36[a]	23.80 ± 1.52[a]	18.93 ± 2.26[a]
0.400	88.70 ± 1.38[a]	—	43.00 ± 1.06[a]	—
0.500	102.00 ± 2.94[a]	74.18 ± 3.35[a]	53.30 ± 2.76[a]	46.68 ± 2.61[a]

Each value represents the mean ± S.E.M. of four separate determinations.
Significantly different from control: [a]$P < 0.001$, [b]$P < 0.005$, [c]$P < 0.01$.
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