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EVALUATION OF THE NATURE OF CAMEL RETINAL ACETYLCHOLINESTERASE: INHIBITION BY HEXAMETHONIUM

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Acetylcholinesterase (AChE, EC 3.1.1.7) has been demonstrated in retinas of several species, however, the nature of the interaction of AChE with specific inhibitors are very limited in the literature and the mode of inhibition of camel retinal AChE by hexamethonium has been studied. Hexamethonium reversibly inhibited AChE in a concentration dependent manner, the IC_{50} value being c. 2.52 mM. The K_m for the hydrolysis of acetylthiocholine iodide was found to be 0.087 mM and the V_{max} was 0.63 μmol/min/mg protein. Dixon, as well as Lineweaver–Burk, plots and their secondary replots indicated that the nature of the inhibition is of the hyperbolic (partial) mixed type, which is considered to be a partial competitive and non-competitive mixture. The values of $K_{I(slope)}$ and $K_{I(intercept)}$ from a Lineweaver–Burk plot were estimated as 0.30 mM and 0.17 mM, respectively, while K_i from a Dixon plot was estimated as 0.725 mM. The K_i was greater than K_I indicating that hexamethonium has a greater affinity of binding for the active site than the peripheral site of the camel retina AChE.

Keywords: Acetylcholinesterase; Inhibition; Hexamethonium; Retina; Kinetics

INTRODUCTION

In all mammalian retinas studied in recent years, the occurrence of acetylcholine (ACh) and the markers of the retinal cholinergic system has been fairly well documented.[1–3] Acetylcholinesterase (AChE, EC 3.1.1.7) is responsible for hydrolysis of ACh. AChE is well known to be a target for

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several drugs and a search for selective inhibitors of the enzyme has proven useful in attempts to improve current treatments against several human neurological disorders. In the present study, we described the interaction of a well known reversible inhibitor of AChE, hexamethonium, on camel retina AChE in an attempt to shed light on the effect of a specific inhibitor on the cholinergic system present in retina tissue. A survey of the literature reveals that the effect of cholinergic drugs and anticholinesterase on the function of the retina was evident.[4] The effects reported by various groups of investigators appear to vary widely. Inhibition of AChE has been reported to either hasten or reduce the process of dark adaptation.[5-7] Thus although decreases in visual activity have often been noted following exposure to anticholinesterases and muscarinic cholinergic agonist which may cause an increase in the flicker fusion frequency,[8,9] these effects have largely been ascribed to changes in accommodative ability.[10] The camel eye is of interest since it possesses features of a diurnal eye. Moreover, there is no tapetum lucidum layer between chroid and retina as found in the eyes of large nocturnal mammals.[11] Therefore, this source of AChE was selected in present study.

Hexamethonium has a wide range of application in biological system. For example, it inhibited allergic cough in the guinea pig.[12] The hexamethonium suppresses cardiac disturbances i.e. it has putative cardioprotective role in animals anesthetized by bupivacaine.[13] It was hypothesized that pre-treatment with hexamethonium would increase the threshold for cardiac dysrhythmias.[14] Nicotine significantly decreased the endurance time in rats in swimming exercises and this effect was antagonized by pre-treatment with hexamethonium.[15] A limited number of attempts have been made to investigate the kinetic parameters of AChE inhibition by hexamethonium and in the present study the kinetics of inhibition of camel retina AChE by hexamethonium was investigated.

MATERIALS AND METHODS

Materials

All reagents were of analytical grade. Acetylthiocholine (ASCh), hexamethonium and 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin and Triton X-100 were obtained from Fluka Chemika-BioChemika (Switzerland).

Methods

Enzyme Preparation

Retinas of young camels (*Camelus dromedarius*) were obtained from the local abattoir and transported to the laboratory at 4°C. The enzyme was prepared from fresh retina after homogenization and centrifugation processes as described previously.[16–18]

Enzyme Assay

AChE-catalyzed hydrolysis of the acetylthiocholine was followed by the spectrophotometric assay of Ellman *et al.*[19] To study the effect of hexamethonium, the enzyme was pre-incubated with the drug in sodium phosphate buffer (pH 7.4) for 5 min prior to addition of substrate. A blank was also run (without enzyme i.e. retina protein) to check the stability of ASCh and DTNB.

Estimation of IC_{50}

The two types of transformed data i.e. log% activity[20] and $\log v/v_0-v$ (where v_0 = velocity in the absence of hexamethonium and v in the presence of hexamethonium)[21] versus log hexamethonium concentration were plotted for the determination of IC_{50}.

Estimations of Kinetic Parameters

Michaelis–Menten parameters were estimated by means of Lineweaver–Burk plots[22] using initial velocities obtained over a substrate concentration range from 0.03125 to 0.5 mM. The assay conditions for determining the residual activities in the presence of hexamethonium were identical to the above assay procedure, except that a fixed concentration of hexamethonium was used in the assay medium.

Estimation of Protein

The protein content of the enzyme preparation was estimated according to the method of Lowry *et al.*[23] using bovine serum albumin as standard. The detergent, Triton X-100 interfered with this estimation, but this problem was overcome by centrifuging the precipitate as mentioned previously.[24,25]

Statistical Analysis

The graphs were plotted by using GraFit program.[26] The values of the correlation coefficient was obtained by the linear regression analysis of this program.

RESULTS

The kinetic parameters of camel retina AChE inhibition by hexamethonium were investigated in the present study. Two types of transformed data for the inhibition of camel retina AChE as a function of concentration are presented in Figure 1. Hexamethonium (0.50–16.0 mM) inhibited the AChE activity (18.9–66.9%) in a concentration dependent manner, the IC_{50} being c. 2.127 and 2.92 mM from log% activity and $\log v/v_0-v$ plots respectively. The mean value of IC_{50} was calculated as 2.52 ± 0.395 mM at high substrate concentration (0.50 mM).

The Michaelis–Menten constant (K_m) for the hydrolysis of acetylthiocholine iodide was determined by a Lineweaver–Burk plot and its value was found to be 0.0867 mM and the V_{max} value was 0.629 μmol/min/mg protein (Figure 2). The nature of the inhibition was of the partial mixed type in this case. This mixed type system is considered to be a mixture of partial competitive and non-competitive inhibitions. The value of K'_m calculated from the Lineweaver–Burk plots in Figure 2 and it was found to be 0.179 mM. K'_m

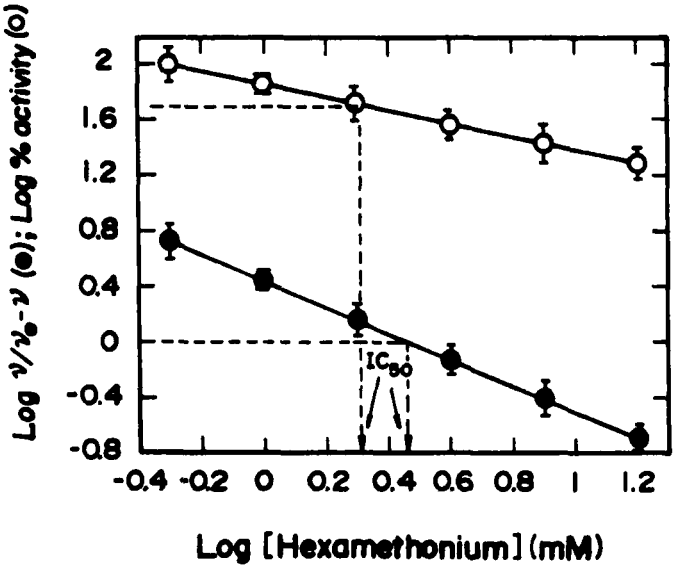

FIGURE 1 Camel retina AChE inhibition by hexamethonium. The transformed data are presented in two forms: (1) log% activity (○) and (2) $\log(v/v_0-v)$ (●) versus log concentration of hexamethonium. The correlation coefficient was 0.97 and 0.99, respectively. Each point represents the mean ± S.E.M of four determinations (each in triplicate) of the enzyme preparation.

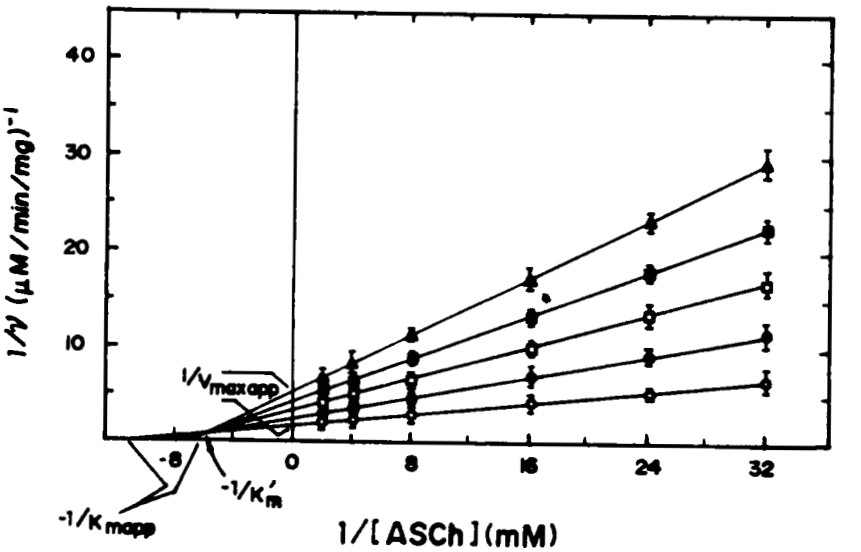

FIGURE 2 Lineweaver–Burk plot of initial enzyme velocity versus ASCh concentration in the absence (○) and presence of 1.0 mM (●), 2.0 mM (□), 4.0 mM (■) and 8.0 mM (▲) hexamethonium. The correlation coefficient was 0.982, 0.984, 0.988, 0.981 and 0.975, respectively. Each point represents the mean ± S.E.M of four determinations (each in triplicate) of the enzyme preparation.

represents the dissociation constant of AChE–ASCh–hexamethonium complex into the AChE–hexamethonium complex and ASCh.

The slope and y-axis intercept ($1/V_{max}$) of the Lineweaver–Burk plot were replotted versus the hexamethonium concentration (Figure 3(A)). The shape of both slope and $1/V_{maxapp}$ replots were hyperbolic while the reciprocal of both axis in both cases yields straight lines (Figure 3(B)). The value of β was calculated by putting the value of the y-axis intercept of V_{maxapp} replots (Figure 3(B)) in the following equation:[22] y-axis intercept $= \beta V_{max}/1-\beta$ was found to be 0.219. The value of α was estimated by putting the value of the y-axis intercept of 1/slope replot (in Figure 3(B)) in the following equation: y-axis intercept $= \beta V_{max}/K_m(\alpha-\beta)$ and it was found to be 1.680.

The inhibition constant K_i was estimated by a secondary replot (1/slope versus 1/[Hexamethonium]) of the Lineweaver–Burk plot (Figure 3(B)) as well as by a Dixon plot (Figure 4). K_I (the dissociation constant of AChE–ASCh–hexamethonium complex into AChE–ASCh complex and hexamethonium) was also estimated by a secondary replot of the reciprocal of the intercepts (V_{max}) of the Lineweaver–Burk plot plotted as a function of 1/[Hexamethonium] (Figure 3(B)).[22] The values of $K_{i(slope)}$ and $K_{I(intercept)}$

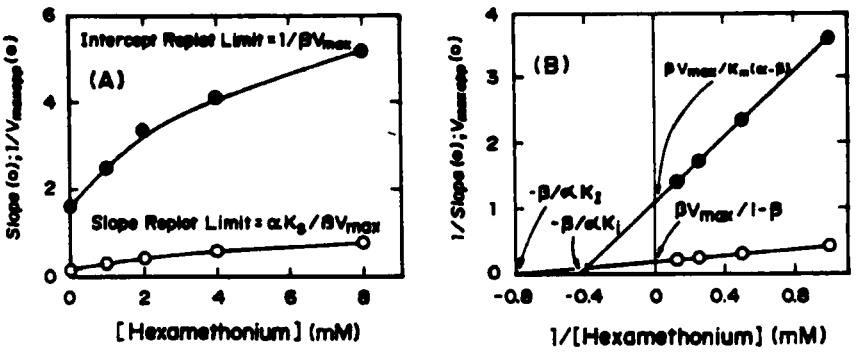

FIGURE 3 (A) Secondary replots of the Lineweaver–Burk plot: (1) slope (○) and (2) $1/V_{maxapp}$ (●) versus [hexamethonium]. (B) Secondary replots of the Lineweaver–Burk plot: (1) 1/slope (●) and (2) V_{maxapp} (○) versus 1/[hexamethonium] where the correlation coefficient was 0.997 and 0.994, respectively.

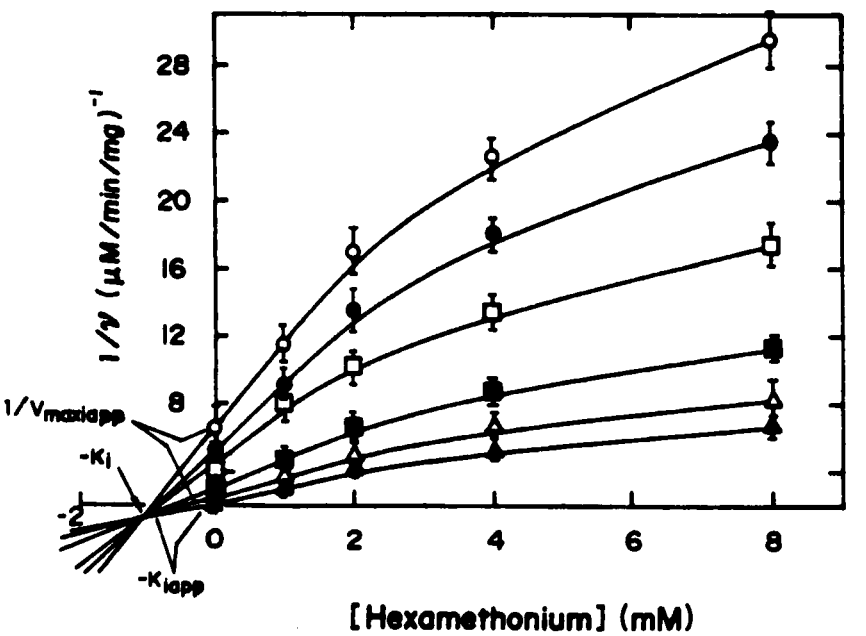

FIGURE 4 Dixon Plot for camel retina AChE at six different fixed concentrations of ASCh: (▲) 0.500 mM; (△) 0.250 mM; (■) 0.125 mM; (□) 0.063 mM; (●) 0.042 mM and (○) 0.0313 mM in the presence of hexamethonium (0–8.0 mM as a final concentration). Each point represents the mean ± S.E.M of four determinations (each in triplicate) of the enzyme preparation.

from the Lineweaver–Burk plot were estimated as 0.30 mM and 0.17 mM, respectively. The K_i value from a Dixon plot was estimated as 0.725 mM (Figure 4). The mean value of K_i was 0.513 ± 0.151 mM.

DISCUSSION

The IC_{50} for camel retina AChE was 2.52 ± 0.31 mM whereas that of bovine erythrocyte AChE was reported as 0.466 mM respectively[27] suggesting that camel AChE is 5.4 times less sensitivity to hexamethonium. The K_i values for hexamethonium with of *Torpedo* electric organ and chicken brain AChE have been reported as 0.084 and 0.242 mM, respectively,[28] whereas in this work a value of 0.513 mM was obtained for camel retina AChE.

The results of a Lineweaver–Burk plot (Figure 2) indicated that hexamethonium inhibits AChE in a mixed fashion. In case of mixed-type inhibition there are two types: partial and linear. These two types can be distinguished on the basis of the shape of Dixon plots; a curved plot means partial mixed-type inhibition is occuring. The Dixon plot was hyperbolic (Figure 4), which means that a partial mixed-type inhibition system was present.

According to the hydrolysis scheme for an acetate substrate by AChE,[29] there are several patterns by which hexamethonium could inhibit AChE i.e. either to combine (1) with free AChE, or (2) with the AChE–ASCh complex, or (3) acylated AChE. If we consider the first possibility i.e. at the free AChE and low [ASCh] stage, hexamethonium competes with ASCh for binding at the anionic substrate binding site of AChE, due to its trimethylamino group (quaternary ammonium, cationic head, analogous to the cationic quaternary nitrogen of the cholinic part of ASCh). With a high concentration of substrate and with free AChE, hexamethonium molecules cannot bind to the anionic subsite of the active center and, instead, prefer to bind to the peripheral anionic subsite. This interpretation is supported by the decrease in V_{maxapp} and increase in K_{mapp} associated with an increase in hexamethonium concentration. This means that when hexamethonium binds with the peripheral anionic subsite of the free AChE, some conformational changes take place which influences the catalytic potency of AChE in such a way that proper positioning of the esteratic and anionic pockets of the active center for acylation by ASCh are affected. This increase in K_{mapp} also indicates that hexamethonium decreases the substrate (ASCh) affinity with the anionic binding site of the

enzyme's active center.[29,45] Hence, the hexamethonium, due to a bisquaternary nature, can bridge the anionic subsite of the active center and the peripheral anionic subsite of the AChE.

Now considering the remaining two possibilities for the interaction of hexamethonium with AChE either in the AChE–ASCh complex form or acetylated AChE form (acy-AChE) to produce an AChE–ASCh–Hexamethonium or an acy-AChE–Hexamethonium complex, respectively. The first possibility is the more likely, because if hexamethonium was bound to acy-AChE, thiocholine (P_1), the first product of the reaction, would not be affected. However our results show that its production is decreased by increasing the concentration of hexamethonium which leads to the conclusion that hexamethonium does not bind with acy-AChE and instead binds with the AChE–ASCh complex. In the case of the AChE–ASCh complex, the anionic substrate binding site is occupied by the β substituent of ASCh (quaternary ammonium ligand of cholinic part of ASCh) and thus is not available to a second ligand. Therefore, one positive end of hexamethonium must bind with some other site such as the peripheral anionic subsite. The low value of the K_I/K_i ratio (0.331) also supports this suggestion.[31] These results show that the effect of hexamethonium on camel retina AChE is different from that on *Torpedo* electric organ AChE.[28]

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