MOLECULAR FORMS OF RAT BRAIN HYDROLASES AND THEIR INTERACTION WITH VARIOUS INHIBITORS

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The inhibition of molecular forms of rat brain hydrolases *in vitro* by 1,2,3,4-tetrahydro-9-aminoacridine and by 3-diethylaminophenyl-N-methylcarbamate methiodide was investigated. None of the molecular forms of nonspecific esterases was affected by these inhibitors used in concentration of 10^{-3}M and lower. The molecular forms of acetylcholinesterase were more sensitive to carbamate, the molecular forms of butyrylcholinesterase on the contrary to the acridine derivative.

Acetylcholinesterase (EC 3.1.1.7) plays an important role in the cholinergic nerve transmission even in the central nervous system[1-4]. It has been shown that this enzyme exists even in the brain in several molecular forms[4-10]. These forms differ especially in their physical chemical properties yet less in their enzymic features. Some authors report differences in the enzymic characteristics of various forms of acetylcholinesterase[4,6,10] whereas other are of the opinion that these forms are almost identical from the viewpoint of enzymic properties[5,9]. The solution of this problem calls for an adequate separation or isolation technique and a sufficient choice of substrates and inhibitors. This paper reports on the effect of two inhibitors binding to various sites of the active surface of the enzyme: of 1,2,3,4-tetrahydro-9-aminoacridine, Tacrine), which binds to the hydrophobic area of the active center of acetylcholinesterase, the so-called γ-anionic site[11,12], and of 3-diethylaminophenyl-N-methylcarbamate methiodide, whose interaction with this enzyme proceeds via carbamylation of the esteratic side of its active center[13,14]. The substrates used were selected so that both the molecular forms of acetyl- and butyrylcholinesterase (EC 3.1.1.8), whose active sites are different[4,15,16] and also the molecular forms of nonspecific esterases, where recorded data on their active sites are lacking, could be detected.

EXPERIMENTAL

Material

Female Wistar rats (farm Mezno), weighing 180—220 g, were sacrificed by bleeding, their brains were rapidly excised, washed with physiological saline, and frozen in dry ice. The thawed brains were homogenized (Ultra Turrax, Janke and Kunkel, FRG) at a 1 : 3 ratio with 0·02M Tris-HCl

buffer, pH 8·2, containing 1% of Triton X-100 (Koch Light Lab., England). The homogenate was centrifuged 30 min at 3000*g* and the supernatant obtained was recentrifuged at 105000*g*, 60 min at 4°C, in MSE (England) Super Speed 50 T.C. centrifuge. The clear supernatant was used for electrophoretic separation.

Methods

Electrophoresis. The conventional modification of polyacrylamide gel electrophoresis was used (240 V, 40 mA, 60 min).

Detection of enzymatic activities. The activity of acetyl- or butyrylcholinesterase[3] was detected after gel electrophoresis by the corresponding substrates (acetyl- or butyrylthiocholine iodide, Lachema, Brno, ČSSR) and the activity of nonspecific esterases[3] by α-naphthyl acetate (Lachema, Brno) as substrate.

Inhibition of molecular forms of enzymes. At the end of the electrophoresis run the intact gel columns were incubated with buffered (Tris-HCl buffer, pH 8·2) solutions of Tacrine or 3-diethylaminophenyl-N-methylcarbamate methiodide (concentrations 10^{-3}M—10^{-12}M). All experiments were carried out at 25°C. Following the incubation the gels were washed with distilled water and the activity of nonspecific esterases or of cholinesterases was detected in the gel as described above. Both detection methods are based on the interaction of products of the enzymatic reaction with agents which give rise to colored, insoluble products. The colored spots, whose intensity is directly proportional to the activity of the enzyme, were densitometrically evaluated in a Vitatron densitometer (Sci. Instr., Eefde, Holland). The activity of the molecular forms of the enzymes was calculated from the area under the densitometric curve and expressed in integration units. Six to eight measurements were made for each inhibitor concentration.

Statistical evaluation. The results of the determination of the activities of the individual molecular forms of the enzymes were plotted *versus* molar inhibitor concentration in probit-logarithmic transformation. The interpolation of the straight lines was made according to the corresponding programs in Type 9830 Hewlett Packard computer (USA).

TABLE I

pI_{50}-Constants Characterizing Effect of Tacrine and Carbamate on Molecular Forms of Cholinesterases

The results are means with 95% confidence intervals.

Inhibitor	1	2	3
		acetylcholinesterase	
Carbamate	5·81 (2·8— 6·8)	6·60 (4·4—8·8)	6·88 (5·9—7·9)
Tacrine	6·80 (4·8— 8·8)	5·57 (3·3—7·9)	5·88 (3·6—8·6)
		butyrylcholinesterase	
Carbamate	5·28 (3·4— 7·2)	6·16 (4·2—8·1)	absent
Tacrine	7·82 (5·4—10·3)	5·22 (2·8—7·7)	absent

RESULTS

The nonspecific rat brain esterases were resolved by polyacrylamide gel electrophoresis into 4 fractions; the activity of the fastest fraction was the lowest (12·5 ± ± 7·3%) whereas the activity of the remaining fractions was approximately the same. The last two fractions hydrolyzed thiocholine esters, even though at a considerably lower rate.

The activities of the individual enzymes investigated are affected by the two inhibitors in very different manners. The activity of nonspecific esterases was not affected by Tacrine or carbamate up to 10^{-3}M concentration. These esterases are resistent to both types of inhibitors. By contrast, the activity of acetyl- or butyrylcholinesterase decreased in the presence of Tacrine or carbamate (Fig. 1). The I_{50}-constants (inhibitor concentrations which cause a 50% inhibition of the enzyme) and their decadic logarithms, pI_{50}, respectively (Table I) were calculated from the plot of per cent of inhibition *versus* inhibitor concentration in probit-logarithmic transformation (Fig. 2). These constants characterize the effect of carbamate or Tacrine on the molecular forms of the two cholinesterases *in vitro*. It is obvious that of the molecular forms of acetylcholinesterase detected the most sensitive to carbamate are form

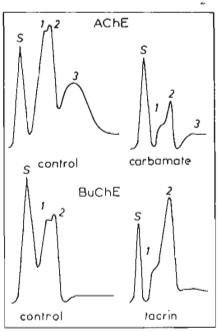

FIG. 1

Densitometric Records of Electropherograms of Rat Brain Cholinesterases: Controls, Acetylcholinesterase (AChE) Treated with 10^{-5}M Carbamate, Butyrylcholinesterase (BuChE) Treated with 10^{-8}M Tacrine

S origin, 1,2,3 denote individual fractions of cholinesterases

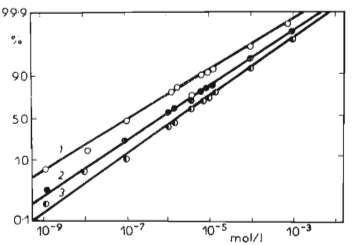

FIG. 2

Inhibition of Molecular Forms of Rat Brain Acetylcholinesterase by Tacrine in Probit-Logarithmic Transformation

The numbers at the curves denote individual forms of the enzyme. Individual points indicate inhibition values and are means of seven measurements. % per cent of inhibition, M molar Tacrine concentration.

3 and 2, respectively. The sensitivity of the electrophoretically slowest form (*1*) to this inhibitor is significantly lower. The situation with Tacrine is the opposite one: form 2 and 3 respectively are the least sensitive ones whereas the enzyme showing the smallest electrophoretic mobility is the most sensitive one. With butyrylcholinesterase form 1 again is the most sensitive one to Tacrine whereas form 2 is more sensitive to carbamate.

DISCUSSION

The detection of nonspecific esterases showed the existence of four different molecular forms. The hydrolysis of α-naphthyl acetate is not inhibited either by Tacrine or carbamate since two of the forms of nonspecific esterases also hydrolyzed thiocholine esters and could therefore be regarded as cholinesterases. The ability of acetylcholinesterase to hydrolyze α-naphthyl acetate, even through at a very low rate, has been recorded in literature[9,17].

The reported number of detectable forms of acetylcholinesterase differs with the separation technique employed. In spite of some of the results which indicate numbers higher than mean values, *e.g.* the detection of 10 active zones in rat brain homogenate[18], we can conclude that 2–3 forms of cholinesterases in rat nervous tissue by using polyacrylamide gel electrophoresis and approximately the same methods of solubilization and separation were detected[9,19,20].

A comparison of inhibitory constants is impossible since we are lacking quantitative data on the determination of the inhibition of the molecular forms of acetylcholinesterase by the two compounds. The different affinity of molecular forms of acetyl- and butyrylcholinesterase for the two inhibitors indicates fine differences in the structure of their active centers and/or its close neighborhood. These results are in agreement with the finding of different enzymatic properties of molecular forms of acetylcholinesterase[4,6,10]. Our results show, however, that nonspecific esterases which are resistent to both inhibitors do not contain an active center capable of carbamylation or an area of hydrophobic interactions binding Tacrine. On the contrary, a higher affinity of Tacrine for butyrylcholinesterase is another proof of the fact that the molecule of this enzyme contains a hydrophobic area binding this inhibitor. The finding that the affinity of acetylcholinesterase for carbamate is higher than the affinity of butyrylcholinesterase suggests that the esteratic site to which carbamate binds is not identical in acetylcholinesterase and butyrylcholinesterase.

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