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EFFECT OF REVERSIBLE INHIBITORS ON REACTIVITY OF FUNCTIONAL SITES OF THE ACTIVE CENTER OF ACETYLCHOLINESTERASE

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The effect of galanthamine, tacrine, and oxazyl* on phosphorylation of acetylcholinesterase of human erythrocytes by armin† and GD-42 (o-ethyl-S-β-ethylthioethyl ester of methylthiophosphinic acid) was studied. In the presence of galanthamine the organophosphorus inhibitors were shown to interact only with the binding site of the enzyme whose anionic region was not occupied by the reversible inhibitor. Tacrine and oxazyl lower the reactivity of the free enzyme and reduce the rate of its phosphorylation.

It is now accepted that there are two functional sites — anionic and esterase — on the active surface of acetylcholinesterase (AChE). In the presence of tetraalkylammonium ions, competitive reversible inhibitors of AChE, the molecule of an organophosphorus inhibitor (OPI) does not interact with a binding site whose anionic region is occupied by the reversible inhibitor [4]. In recent years abundant experimental data have been obtained to show the existence of noncatalytic allosteric sites and hydrophobic zones on the surface of AChE [2, 11, 12]. The effect of reversible inhibitors interacting with AChE in the noncatalytic regions on the reactivity of the binding site of the enzyme has not yet been adequately studied [10].

In the investigation described below the effect of reversible AChE inhibitors — galanthamine, tacrine, and oxazyl* — on subsequent phosphorylation of the esterase region by armin† and GD-42 (o-ethyl-S-β-ethylthioethyl ester of methylthiophosphinic acid) was investigated.

EXPERIMENTAL METHOD

The source of enzyme was a purified preparation of AChE from human erythrocytes obtained from the Perm' Institute of Vaccines and Sera, with an activity of 2.1 units/mg. Galanthamine hydrobromide, tacrine (9-amino-1,2,3,4-tetrahydroacridine), and oxazyl were used as reversible inhibitors. Inhibition constants (K_i) for AChE by these compounds were determined by the method of Lineweaver and Burk [7]. The velocity of enzymic hydrolysis of acetylcholine (ACh) was determined by continuous potentiometric titration with two burets [3] on the TTT-1c automatic titrator (Radiometer, Denmark) in the presence of 0.07 N KCl solution at pH 7.5 and 37°C.

The velocity of irreversible inhibition of activity of the enzyme by OPI was assessed under the same experimental conditions from the values of bimolecular reaction velocity constants in the presence of reversible inhibitors ($K_{II,i}$) and in their absence (K_{II}) and calculated by the equation for a pseudomonomolecular reaction:

$$K_{II} = \frac{2.3}{[I]} \cdot \lg \frac{v_0}{v_t},$$

*Bis-(o-chlorobenzyldiethylammoniumethyl)-oxamide dichloride.
†Ethyl-p-nitrophenyl ester of ethylphosphinic acid.

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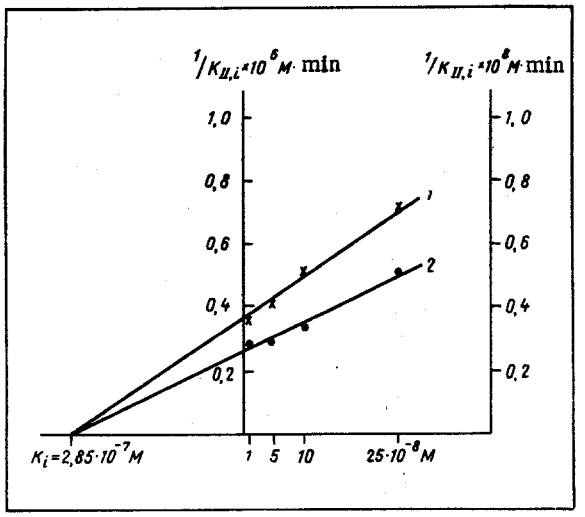

Fig. 1. $1/K_{II,i}$ plotted against galanthamine concentration for reaction of GD-42 (1) and armin (2) with AChE from human erythrocytes (mean results of six experiments). Abscissa, concentration of galanthamine (in M); ordinate: on left, $1/K_{II,i}$ for armin, on right — the same for GD-42.

where I is the concentration of the OPI; v_o and v_t the initial velocity of hydrolysis of ACh and of the substrate respectively at different time intervals after addition of the OPI. When the velocity constants of the reaction between OPI and AChE in the presence of reversible inhibitors were calculated, the velocity of hydrolysis of ACh after the addition of tacrine, galanthamine, or oxazyl was taken as v_o. Armin, a monofunctional inhibitor, reacting with the esterase site of AChE, and the compound GD-42, one of the bifunctional OPIs, reacting with both the anionic and the esterase sites of the enzyme [1], were used as OPI.

EXPERIMENTAL RESULTS

In the presence of competitive reversible inhibitors, of which galanthamine is one [6], the values of $K_{II,i}$ are apparent velocity constants of phosphorylation, not taking into account reversible inhibition or the effect of ACh on the degree of inhibition of AChE by galanthamine. The true velocity constant of the reaction between OPI and AChE (K_4) in this case is calculated by the equation [4]:

$$K_4 = K_{II,i} \cdot (1 + I/K_i).$$

The values of K_4 for the OPI tested were calculated with allowance for the measured values of $K_{II,i}$, the galanthamine concentration J, and K_i of the reversible inhibitor. The last of these values was determined by plotting a graph to show the relationship between the reciprocals of $K_{II,i}$ for armin and GD-42 and the concentration of galanthamine (Fig. 1). The value of K_i thus obtained ($2.85 \cdot 10^{-7}$ M) was practically identical with the value of K_i ($3.10 \cdot 10^{-7}$ M) calculated by the method of Lineweaver and Burk.

In the presence of galanthamine the value of K_4 for armin and GD-42 were practically identical ($P > 0.05$) with the values of K_{II} found for these OPI in the presence of the reversible inhibitor (Table 1). The results show that interaction between galanthamine and the anionic region of the binding site leads to loss of reactivity of the esterase region of this site. Under these conditions OPI, irrespective of their structure, react only with free binding sites of AChE.

Tacrine and oxazyl are noncompetitive reversible inhibitors [6], in the presence of which the value of $K_{II,i}$ is the true velocity constant for the reaction between OPI and AChE, for ACh does not affect the degree of inhibition of enzyme activity caused by these reversible inhibitors. Tacrine and oxazyl lowered the rate of inhibition of AChE activity by both OPI (Table 2). The degree of lowering of the affinity of the binding sites of AChE for the cationic OPI GD-42 was stronger than for armin with all concentrations of tacrine and oxazyl used.

TABLE 1. Kinetic Parameters of Reaction of Armin and GD-42 with AChE of Human Erythrocytes in Presence of Galanthamine (M ± m; n = 6)

Concentration of galanthamine, M	Armin			GD-42		
	$K_{II,i} \cdot 10^6$ M^{-1} min^{-1}	$K_{II}/K_{II,i}$	$K_4 \cdot 10^6$ M^{-1} min^{-1}	$K_{II,i} \cdot 10^8$ M^{-1} min^{-1}	$K_{II}/K_{II,i}$	$K_4 \cdot 10^8$ M^{-1} min^{-1}
$1 \cdot 10^{-8}$	3,79±0,08	1,02±0,02	3,92±0,08	2,7±0,03	1,08±0,04	2,79±0,03
$5 \cdot 10^{-8}$	3,38±0,09	1,15±0,05	3,95±0,05	2,54±0,08	1,15±0,08	2,97±0,05
$1 \cdot 10^{-7}$	2,98±0,12	1,30±0,03	4,02±0,03	2,03±0,05	1,44±0,05	2,74±0,08
$2,5 \cdot 10^{-7}$	1,98±0,07	1,96±0,05	3,70±0,15	1,41±0,03	2,08±0,07	2,63±0,07
$1 \cdot 10^{-6}$	0,935±0,03	4,15±0,18	4,20±0,12	0,71±0,04	4,14±0,05	3,19±0,12

Legend. K_{II} for armin $(3.88 \pm 0.08) \cdot 10^6$ min^{-1}; K_{II} for GD-42 $(2.94 \pm 0.05) \cdot 10^8$ min^{-1}; K_i for galanthamine $(3.10 \pm 0.11) \cdot 10^{-7}$ M.

TABLE 2. Kinetic Parameters of Reaction of Armin and GD-42 with AChE of Human Erythrocytes in the Presence of Tacrine and Oxazyl (M ± m; n = 6)

Concentration of galanthamine, M	Inhibition of AChE by reversible inhibitors, %	Armin		GD-42	
		$K_{II,i} \cdot 10^6$ M^{-1} min^{-1}	$K_{II}/K_{II,i}$	$K_{II,i} \cdot 10^8$ M^{-1} min^{-1}	$K_{II}/K_{II,i}$
Tacrine					
$2,5 \cdot 10^{-8}$	10,5±0,15	3,88±0,08	1,0±0,03	2,40±0,05	1,22±0,04
$5 \cdot 10^{-8}$	15,8±0,21	3,40±0,15	1,14±0,02	2,01±0,06	1,46±0,04
$1 \cdot 10^{-7}$	27,0±1,35	2,92±0,05	1,33±0,02	1,48±0,03	1,99±0,05
$2,5 \cdot 10^{-7}$	45,3±0,95	1,85±0,03	2,09±0,03	1,0±0,07	2,94±0,22
$5 \cdot 10^{-7}$	62,2±1,3	1,27±0,15	3,04±0,16	0,80±0,08	3,67±0,63
Oxazyl					
$5 \cdot 10^{-10}$	6,8±1,41	3,52±0,13	1,11±0,04	2,10±0,11	1,41±0,07
$1 \cdot 10^{-9}$	19,7±0,50	2,70±0,07	1,45±0,05	1,64±0,03	1,79±0,03
$2 \cdot 10^{-9}$	32,9±0,62	2,12±0,08	1,84±0,07	1,36±0,05	2,16±0,08
$1 \cdot 10^{-8}$	66,7±0,51	1,54±0,05	3,96±0,15	0,65±0,03	4,52±0,14

Legend. K_i for tacrine $(2.48 \pm 0.23) \cdot 10^{-7}$ M; K_i for oxazyl $(6.35 \pm 0.12) \cdot 10^{-9}$ M.

Oxazyl, like other bis-quaternary ammonium compounds, reacts with peripheral allosteric anionic sites [6, 12], whereas tacrine is adsorbed either in the region of the allosteric hydrophobic site or in the region of the hydrophobic zones of the active surface of AChE [6, 13]. The binding of tacrine and oxazyl with noncatalytic regions of AChE, unlike interaction between galanthamine and the anionic region of the binding site, not only leads to the complete loss of reactivity of the region of the binding sites which does not participate in hydrolysis of ACh, but also lowers the reactivity of the so-called free enzyme.

The data showing changes in the reactivity of the fractional regions of the binding site of AChE under the influence of reversible inhibitors can be explained from the point of view of the induced character of conformity between enzyme and ligands postulated by Koshland [9]. In the modern view, the binding site of an enzyme does not exist beforehand, but is formed only during contact with substrates or inhibitors. Cholinesterase is known to be a very flexible enzyme and, for that reason, even small changes in its conformation could influence the reactivity of the binding sites. Reversible inhibitors adsorbed on different sites of AChE lead to the creation of a more rigid secondary and tertiary structure [5, 8]. Under the conditions of limited mobility of the enzyme molecule it is much more difficult for OPI to induce changes in the conformation of the binding site which would lead to the formation of an enzyme-inhibitor complex.

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PHARMACOLOGY OF SOME INHIBITORS OF AROMATIC AMINO-ACID DECARBOXYLASE

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The sodium salts of D,L-β-(3,4-dihydroxyphenyl)lactic (I) and D,L-β-(5-hydroxyindolyl-3)lactic acids (II), and also L-α-methyldopa (AMD) are competitive inhibitors of aromatic amino-acid decarboxylase (AAAD). Unlike AMD, compounds I and II are not substrates for AAAD. Compound II selectively inhibits the decarboxylation of L-5-hydroxytryptophan. Compound I and AMD potentiate excitation induced in mice by L-dopa but do not affect excitation induced by L-5-hydroxytryptophan. Compound II weakens excitation induced by both L-dopa and L-5-hydroxytryptophan. Pyridoxin hydrochloride and pyridoxal phosphate weaken excitation induced by L-dopa and L-5-hydroxytryptophan. Compound I and AMD abolish this action of the B_6 vitamins.

KEY WORDS: D,L-β-(3,4-dihydroxyphenyl)lactic acid; D,L-β-(5-hydroxyindolyl-3)lactic acid; L-3,4-dihydroxyphenylalanine; L-5-hydroxytryptophan; L-α-methyldopa; pyridoxin.

Aromatic amino-acid decarboxylase (AAAD; EC 4.1.1.26 or 28) is a pyridoxal enzyme which forms dopamine from L-3,4-dihydroxyphenylalanine (L-dopa) or serotonin from L-5-hydroxytryptophan (L-5-HT). Known inhibitors with the action of carbonyl poisons inhibit both reactions [7]. The search for compounds capable of inhibiting one of the two reactions selectively and the study of their pharmacological properties are of theoretical interest.

In the investigation described below sodium salts of D,L-β-(3,4-dihydroxyphenyl)lactic acid (I) and D,L-β-(5-hydroxyindolyl-3)lactic acid (II) were studied as inhibitors. These substances are the hydroxy analogs of L-dopa and L-5-HT respectively (see the formulas). The hydroxyl group replacing the amino group prevents the compound from irreversibly inactivating the carbonyl group of pyridoxal. The factor of structural similarity with one of the substrates may in this case play a decisive role in the selectivity of action of the substances depending on the substrate used.

EXPERIMENTAL METHOD

The inhibitory effect of compounds I and II on AAAD was tested *in vitro* by determining the activity of the enzyme by a manometric method in Warburg's apparatus. The known substrate-like inhibitor L-α-methyldopa (AMD) [4] was used for comparison.

The decarboxylase was isolated from rabbit kidney, a tissue in which the enzyme has high specific activity [9]. The animals were decapitated and the kidneys homogenized with glass sand in the cold for 20 min in 0.067 M phosphate buffer, pH 6.8. The buffer was added in a volume of 1.5 ml per gram of kidneys. The homogenate was centrifuged for 20 min at 2000g and the supernatant was then centrifuged for 60 min at 100,000g. Samples of 1.5-2 ml of the transparent supernatant were taken. The incubation sample, in a volume of 3 ml, contained:

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