INTERACTION OF REVERSIBLE INHIBITORS WITH CATALYTIC CENTERS AND ALLOSTERIC SITES OF CHOLINESTERASES

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The kinetics of inhibition of the acetylcholinesterase (ACE) of human erythrocytes by galanthamine, tacrine, and oxazyl and the effect of these substances on enzyme activity in chicken, mouse, cat, and rat blood plasma were investigated. Galanthamine was shown to bind with ACE in the region of the anionic site of the catalytic center, oxazyl in the region of allosteric anionic sites, whereas tacrine interacts with hydrophobic regions of the enzyme.

KEY WORDS: *Cholinesterases of human erythrocytes and animal blood plasma and brain; action of reversible inhibitors; allosteric regulation.*

In 1966 Changeux [6] showed that, besides a catalytic center consisting of anionic and esterase sites, acetylcholinesterase (ACE) also contains peripheral sites which he called regulatory or allosteric sites. More recently much evidence has accumulated on the basis of which ACE can be classed among the allosteric enzymes [7, 12-14]. Since most allosteric effectors of ACE are cations [1, 3, 15], it has been postulated that the regulatory sites of this enzyme carry a negative charge. The ability of an excess of acetylcholine (AC) to inhibit ACE activity is now regarded as due to interaction between the substrate and the peripheral anionic site of the enzyme [12, 15]. The substrate inhibition effect has become widely used for the demonstration of interaction between various ligands and the allosteric sites of ACE [5, 10, 11].

The object of this investigation was to study the kinetics of hydrolysis of AC in the presence of reversible ACE inhibitors: galanthamine, tacrine, and oxazyl.

EXPERIMENTAL METHOD

The source of enzyme was a purified ACE preparation from human erythrocytes obtained from Perm' Research Institute of Vaccines and Sera. The velocity of enzymic hydrolysis of AC was determined by potentiometric titration with two burettes on the TTT-1c Autotitrator (Radiometer, Denmark) in the presence of 0.07 M KCl and at a temperature of 37°C and pH 7.5. The reversible inhibitors used were galanthamine hydrobromide, tacrine (9-amino-1,2,3,4-tetrahydroacridine), and oxazyl [bis-(o-chlorobenzyldiethylammoniumethyl)-oxamide dichloride]. To study the mechanism of the inhibitory action, the relationship was determined between the reciprocal of the reaction velocity in the presence of inhibitor and the reciprocal of the substrate concentrations in the presence of a constant concentration of inhibitor [4]. Acetylcholine iodide in concentration ranges from $2 \cdot 10^{-3}$ to $2 \cdot 10^{-4}$ M was used as the substrate. Values of the Michaelis constant (K_m) and the maximal velocities of enzymic hydrolysis with complete saturation of the active centers of the enzyme by the substrate (V) were calculated from the results. The values of $[S]_{opt}$, the AC concentration at which the optimal reaction velocity is observed, also were determined.

The effect of high AC concentrations ($5 \cdot 10^{-3}$-$5 \cdot 10^{-2}$ M), causing inhibition of ACE activity, on the anticholinesterase effect of the reversible inhibitors was studied. A graph

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TABLE 1. Kinetic Parameters of AC Hydrolysis by ACE in the Presence of Reversible Inhibitors

Inhibitor	K_M, moles	V, μmoles AC/mg protein/h	Optimal substrate concn., moles	Type of inhibition
Inhibitor absent	$0{,}93 \cdot 10^{-4}$	493	$2 \cdot 10^{-3}$	—
Galanthamine				
$2{,}5 \cdot 10^{-7}$M	$3{,}5 \cdot 10^{-4}$	470	$2 \cdot 10^{-3}$	Competitive
$5 \cdot 10^{-7}$M	$4{,}2 \cdot 10^{-4}$	450	$2 \cdot 10^{-3}$	
Tacrine				
$1 \cdot 10^{-7}$M	$0{,}90 \cdot 10^{-4}$	318	$2 \cdot 10^{-3}$	Noncompetitive
$2{,}5 \cdot 10^{-7}$M	$0{,}98 \cdot 10^{-4}$	208	$2 \cdot 10^{-3}$	
Oxazyl				
$1 \cdot 10^{-10}$M	—	—	$5 \cdot 10^{-3}$	Allosteric
$1 \cdot 10^{-9}$M	—	—	$8 \cdot 10^{-3}$	

Legend. Mean results of 6-8 experiments are given.

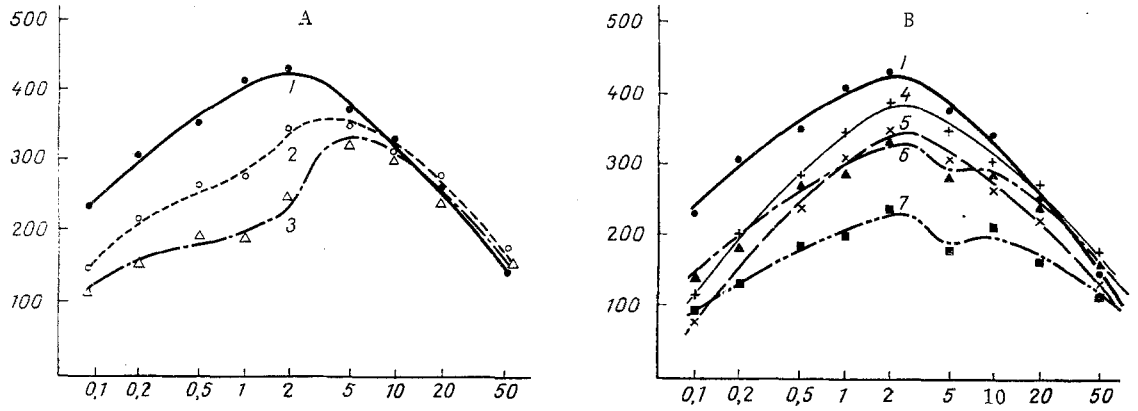

Fig. 1. Velocity of hydrolysis of AC by human erythrocyte acetylcholinesterase at different substrate concentrations in the presence of reversible inhibitors (mean results of 6-7 experiments): A) velocity of hydrolysis without addition of inhibitor (1) and in presence of oxazyl in concentrations of $1 \cdot 10^{-10}$ M (2) and $1 \cdot 10^{-9}$ M (3); B) velocity of hydrolysis in presence of galanthamine: in concentrations of $2.5 \cdot 10^{-7}$ M (4) and $5 \cdot 10^{-7}$ M (5) and of tacrine in concentrations of $1 \cdot 10^{-7}$ (6) and $2.5 \cdot 10^{-7}$ M (7). Ordinate, ACE activity (in μmoles AC/mg protein/h); abscissa, AC concentration (in mM).

to show the rate of AC hydrolysis as a function of substrate concentration in the presence and absence of inhibitors was plotted.

Values of the molar concentrations of inhibitors inhibiting cholinesterase activity by 50% (I_{50}) during incubation with blood plasma and brain homogenates from mice, rats, and cats, and also with hen blood plasma were calculated. The activity of the enzymes in these experiments was determined by Hestrin's method [8].

EXPERIMENTAL RESULTS AND DISCUSSION

Under the influence of galanthamine the affinity of the enzyme for AC was altered more than the maximal reaction velocity (Table 1). With an increase in the AC concentration up to optimal, inhibition of ACE activity by galanthamine decreased considerably, from which it follows by kinetic analysis that inhibition of competitive type was present. This inhibitor can be considered to be bound with the catalytic center of ACE rather than with its anionic site. Galanthamine, at neutral pH values, is known to be almost completely ionized, so that it can interact with negatively charged sites.

TABLE 2. Values of I_{50} (in M) for Galanthamine, Tacrine, and Oxazyl during Their Interaction with Cholinesterases of Brain and Blood Plasma of Mice, Rats, Cats, and Hens

Reversible inhibitors	Cholinesterases (BCE and PCE) of blood plasma				Brain ACE		
	mouse	rat	cat	hen	mouse	rat	cat
Galanthamine	$11{,}2\cdot10^{-5}$	$2{,}7\cdot10^{-5}$	$11{,}4\cdot10^{-5}$	Not inhibited	$6{,}7\cdot10^{-5}$	$2.2\cdot10^{-5}$	$1{,}2\cdot10^{-5}$
Tacrine	$11{,}4\cdot10^{-8}$	$2{,}0\cdot10^{-8}$	$2{,}0\cdot10^{-8}$	$8.5\cdot10^{-8}$	$65{,}0\cdot10^{-8}$	$33.3\cdot10^{-8}$	$65{,}0\cdot10^{-8}$
Oxazyl	$8{,}5\cdot10^{-6}$	$8{,}5\cdot10^{-6}$	$2{,}5\cdot10^{-6}$	$20.0\cdot10^{-6}$	$8{,}0\cdot10^{-10}$	$8{,}5\cdot10^{-10}$	$9{,}0\cdot10^{-10}$

Legend. Substrates were AC for the brain ACE of the animals, propionylcholine for rat and hen blood plasma, and butyrylcholine for mouse and cat blood plasma.

In the presence of tacrine the maximal reaction velocity was reduced without any change in the value of K_m. Kinetic analysis showed that interaction between tacrine and ACE is noncompetitive in character, i.e., that tacrine is bound in the region of noncatalytic sites.

In the absence of inhibitors the curve of ACE activity as a function of substrate concentration was typically bell-shaped. The activity reached a maximum with an AC concentration of $2\cdot10^{-3}$ M. A further increase in substrate concentration led to inhibition of ACE activity. Under the influence of galanthamine the character of this function was unchanged (Fig. 1B), further confirmation that galanthamine was bound with the catalytic center. In the presence of oxazyl the curve lost its hyperbolic shape and became sigmoid, characteristic of interaction between effectors and allosteric sites. Oxazyl protected the enzyme against substrate inhibition and shifted the maximum of ACE activity into the region of higher AC concentrations. This protection can be interpreted as competition of the effector with the substrate for the allosteric sites of the enzyme (Fig. 1A).

The character of substrate inhibition in the presence of tacrine had certain distinguishing features (Fig. 1B). In this case ACE activity reached a maximum at the same AC concentrations; substrate inhibition of ACE by 50% in the presence of tacrine was obtained at higher substrate concentrations than without the inhibitor.

Galanthamine virtually did not inhibit the activity of propionylcholinesterase (PCE) of hen blood plasma (Table 2). There is no anionic site in the catalytic center of this enzyme [2] and, for that reason, ligands which bind with these sites of ACE cannot lower the catalytic activity of PCE. This is thus firm evidence that galanthamine inhibits ACE activity through interaction with the anionic site of the catalytic center.

The affinity of tacrine for butylcholinesterase (BCE) and for PCE was higher than for ACE (Table 2). Meanwhile the value of I_{50} of tacrine for PCE of hen blood plasma was virtually the same as I_{50} for the plasma enzymes of the other species of animals. The anionic sites of ACE and BCE are known to be surrounded by hydrophobic regions adapted for adsorption of the trimethylammonium group of AC and the hydrophobic groups of other preparations. BCE has a smaller charge than ACE in the region of its anionic catalytic site, but on the other hand in BCE hydrophobic regions play a more important role in its interactions [9]. Tacrine is a substance with very strong hydrophobic properties, and that may be the reason why its affinity for BCE was stronger than for ACE. Tacrine, it may be suggested, binds with hydrophobic zones located in the region of the catalytic centers of ACE.

Oxazyl has no hydrophobic properties and, consequently, its affinity for BCE was much lower than for ACE. On the other hand, oxazyl inhibited the activity of hen plasma PCE by about the same degree as the plasma enzymes of the other species of animals. Oxazyl can be considered to be adsorbed on ACE in the region of allosteric anionic sites.

These results are further proof of the allosteric nature of ACE. They also show that hen plasma PCE can be used as a model with which to study the interaction of various ligands with noncatalytic centers of cholinesterases.

LITERATURE CITED

1. A. P. Brestkin and L. A. Ivanova, Biokhimiya, 35, 652 (1970).
2. A. P. Brestkin and T. V. Parkhomenko, Biokhimiya, 38, 467 (1973).

3. R. N. Glebov, G. M. Dolganov, and G. N. Kryzhanovskii, Dokl. Akad. Nauk SSSR, 218, 1464 (1974).
4. V. A. Yakovlev, The Kinetics of Enzymic Catalysis [in Russian], Moscow (1965), p. 41.
5. R. D. O'Brien, Biochem. J., 113, 713 (1969).
6. J. P. Changeux, Mol. Pharmacol., 2, 369 (1966).
7. H. D. Crone, J. Neurochem., 20, 225 (1973).
8. S. Hestrin, J. Biol. Chem., 180, 249 (1949).
9. M. I. Kabatchnik, A. P. Brestkin, N. N. Godovikov, et al., Pharmacol. Rev., 22, 355 (1970).
10. G. Kato, J. Biol. Chem., 247, 3186 (1972).
11. G. Kato, Mol. Pharmacol., 8, 575 (1972).
12. R. J. Kitz, L. M. Braswell, and S. Ginsburg, Mol. Pharmacol., 6, 108 (1970).
13. H. Kuhnen, Biochem. Pharmacol., 21, 1187 (1972).
14. B. D. Roufogalis and E. E. Quist, Mol. Pharmacol., 8, 41 (1972).
15. N. Wombacher and H. U. Wolf, Mol. Pharmacol., 7, 554 (1971).

ACCELERIN (FACTOR V), A CATABOLIC PRODUCT OF FIBRINOGEN

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A method of isolating a highly active and highly purified preparation of human Ac-globulin (factor V) is described. It is shown that factor V is part of the fibrinogen molecule and is an intermediate product of its conversion under the action of thrombin or of another enzyme with similar action.

KEY WORDS: *Fibrinogen; human Ac-globulin (factor V); purification and properties.*

Ac-globulin (factor V) is a plasma protein which accelerates the conversion of prothrombin in a reaction catalyzed by factor Xa, phospholipids, and Ca^{++}. Ac-globulin (accelerin), especially human, is labile and is inactivated during blood clotting. Difficulties accordingly arise in the isolation of accelerin and the study of its properties. Human factor V has so far hardly been investigated.

The object of this work was to obtain a highly active and highly purified preparation of human Ac-globulin and to study its physicochemical and immunological properties.

EXPERIMENTAL METHOD

Factor V was isolated from human plasma previously treated with $BaSO_4$ to remove the prothrombin complex. This plasma was adsorbed on DEAE-cellulose DE-52 in 0.05 M phosphate buffer, pH 7.0, the accelerin was eluted with 0.4 M phosphate buffer, pH 7.0, and the eluate was chromatographed on a column with Amberlite C G-50 [1]. The solution of factor V in 0.3 M phosphate buffer obtained after chromatography was fractionated with ammonium sulfate and the protein precipitated at 25-70% saturation with $(NH_4)_2SO_4$ was collected. The resulting precipitate was then subjected to gel filtration on a Sephadex G-200 column. The activity of the factor V was determined by a one-stage method [2].

EXPERIMENTAL RESULTS AND DISCUSSION

One of the basic conditions for obtaining a highly active preparation of Ac-globulin is that the whole procedure of purification must be carried out within 1 or 2 days. The

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