Mechanism of Anticholinesterase Activities of Cardiotoxin, Protamine and Polylysine

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Cardiotoxin, protamine and polylysine are potent inhibitors of various cholinesterases. CaCl_2 and MgCl_2 overcome the inhibition. The order of addition of the inhibitor and the protecting agent (MgCl_2) influences the final degree of the inhibition observed. These findings suggest that cardiotoxin, protamine and polylysine inhibit cholinesterases by the ionic binding of their basic groups with the anionic sites of cholinesterase molecules.

In previous papers, it was found that cobra cardiotoxin is potent in inhibiting cholinesterase of *Bungarus multicinctus* venom (Lee *et al.*, 1971; Lin Shiau *et al.*, 1976). To determine the importance of the basicity of cardiotoxin, protamine and polylysine were tested to see if they 'mimicked' the effect of cardiotoxin. To our surprise, both protamine and polylysine are even more potent than cardiotoxin in inhibiting *B. multicinctus* venom cholinesterase. The inhibition appears to be specific since not all of the basic polypeptides possessed anticholinesterase activity. Cobra neurotoxin, which is a basic polypeptide, does not inhibit *B. multicinctus* venom cholinesterase (Lin Shiau *et al.*, 1976). The purpose of the present paper is to explore the mechanism of this anticholinesterase activity of cardiotoxin as well as that of protamine and polylysine. Interaction of cations with the anticholinesterase effect of polypeptides was studied and compared with that of physostigmine.

Chang & Lee (1955) suggested that *B. multicinctus* venom cholinesterase was an acetylcholinesterase; however, the chemical structure and mechanism of action of this enzyme have not yet been elucidated. It is doubtful if only this venom cholinesterase is susceptible to the inhibitory action of these basic polypeptides, and in the present paper the purified acetylcholinesterase of *Electrophorus electricus* and pseudocholinesterase of horse serum were also used. Comparison of the susceptibility of the three cholinesterases toward cardiotoxin, protamine and polylysine and their interactions in the presence or absence of cations were carried out to elucidate the properties of *B. multicinctus* venom cholinesterase.

Materials and Methods

Cobra cardiotoxin and B. multicinctus venom

Cobra (*Naja naja atra*) and *B. multicinctus* venom were freshly collected and freeze-dried in our laboratory. Cardiotoxin was isolated from the

venom of *Naja naja atra* by the method of Lee *et al.* (1968).

Protamine and polylysine

Protamine sulphate from herring (mol.wt. 5000) and polylysine hydrobromide (mol.wt. 6000) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Miles Laboratories Inc. (Wisconsin, IL, U.S.A.) respectively.

Acetylcholinesterase and pseudocholinesterase

Acetylcholinesterase (1079 units/mg) of *Electrophorus electricus* and pseudocholinesterase (4.7 units/mg) from horse serum were purchased from Worthington Biochemical Co. (Freehold, NJ, U.S.A.).

Measurement of cholinesterase activity

Cholinesterase activity was measured by an electrometric titration method with a Radiometer titrator TTT2 and Autoburette ABU11. Cholinesterase from *B. multicinctus* venom (3.75 $\mu\text{g/ml}$) or acetylcholinesterase (0.125 $\mu\text{g/ml}$) or pseudocholinesterase (12.5 $\mu\text{g/ml}$) was used, and 3.2 mM-acetylcholine chloride was subjected to enzymic hydrolysis at pH 7.4 at 37°C for 5 min in a reaction mixture of 8 ml. The liberated acetic acid was titrated with 0.01 M-NaOH. The inhibitor (either cardiotoxin or protamine or polylysine) was added to the acetylcholine solution and the enzymic reaction was initiated by the addition of cholinesterase. The amount of 0.01 M-NaOH consumed in the absence of the inhibitor during 5 min was used as a reference (100%) and that in the presence of the inhibitor was calculated as a percentage of the reference. Spontaneous hydrolysis of acetylcholine was corrected in all cases. The number of experiments in each group was more than three. The statistical

significance of difference between the control group and the test group was verified by Student's *t* test.

Results

Dose-inhibition relationship of basic polypeptides

Fig. 1 shows the effects of cardiotoxin, protamine, polylysine and physostigmine on *B. multicinctus* venom cholinesterase, acetylcholinesterase and pseudocholinesterase. The slope of the dose-inhibition curve of cardiotoxin was about the same as that of physostigmine, but those of protamine and polylysine were steeper. The concentrations of inhibitors producing 50% inhibition (ID_{50}) on the three cholinesterases are shown in Table 1. Potency ratio was calculated from ID_{50} of physostigmine on the venom cholinesterase divided by the ID_{50} of each inhibitor. Table 1 shows that the inhibitory action of these polypeptides on the three cholinesterases was in general more potent than that of physostigmine except that cardiotoxin was weaker than physostigmine in inhibiting acetylcholinesterases. The susceptibility of the venom cholinesterase to these polypeptides was much greater than that of acetylcholinesterase and pseudocholinesterase, whereas acetylcholinesterase was much more susceptible to physostigmine than were the venom cholinesterase and pseudocholinesterase.

Effects of cations on anticholinesterase activities of basic polypeptides

Both Mg^{2+} and Ca^{2+} but neither Na^+ nor K^+ activated *B. multicinctus* venom cholinesterase (Table 2). Anticholinesterase activities of all three polypeptides were abolished by all the cations tested except that the inhibitory effect of protamine was only partially antagonized by Na^+ and K^+ . In contrast, the anticholinesterase activity of physostigmine was not affected by any of these cations.

Effect of Mg^{2+} on reversibility of anticholinesterase activities of basic polypeptides

Anticholinesterase activities of these three polypeptides were reversed by Mg^{2+} which was added to the reaction mixture before the inhibitor (Table 3). However, if Mg^{2+} was added after 5 min incubation of acetylcholinesterase with an inhibitor, the inhibition was only partially antagonized by Mg^{2+} . Anticholinesterase activity of physostigmine was not affected by Mg^{2+} at all.

Discussions

The partially purified acetylcholinesterase used in this investigation has a specific activity of 1079 units/mg. The most purified acetylcholinesterase available

has a specific activity about 10 times higher than this; thus it seems that 10% of the protein of this partial purified acetylcholinesterase is an acetylcholinesterase protein. The molecular weight of one subunit of acetylcholinesterase is reported to be 80000 (Dudai *et al.*, 1973); thus the molar concentration of acetylcholinesterase in the reaction mixture will be 0.16 nM, which is far less than the concentration of the polypeptide inhibitor (50 nM) in the reaction mixture. Accordingly, the theory of tight binding (Dixon, 1972; Henderson, 1973) of the enzyme kinetics does not apply to this study.

It has been recognized that there are two active sites (esteratic and anionic sites) on the surface of cholinesterase (Augustinsson, 1963; Augustinsson & Nachmansohn, 1949; Cohen & Oosterbaan, 1963; Koelle, 1963). The functional groups of these two sites that react directly with the substrate are probably very similar, if not identical, in acetylcholinesterase and pseudocholinesterase (Kabachnik *et al.*,

Fig. 1. Dose-inhibition curve of basic polypeptides and physostigmine on various cholinesterases

(a) *B. multicinctus* venom cholinesterase, (b) acetylcholinesterase of *E. electricus*, (c) pseudocholinesterase of horse serum. □, Cardiotoxin; △, protamine; ○, polylysine; ●, physostigmine.

Table 1. Comparison of potencies of anticholinesterase activities of polypeptides and physostigmine. Concentrations of polypeptides or physostigmine required to produce 50% inhibition (ID₅₀) on the various cholinesterases (cholinesterase of *B. multicinctus* venom, acetylcholinesterase of *E. electricus* and pseudocholinesterase of horse serum) were derived from Fig. 1. Potency ratio was calculated from the following equation:

$$\text{Potency ratio} = \frac{\text{ID}_{50} \text{ of physostigmine on venom cholinesterase (5.8 } \mu\text{M)}}{\text{ID}_{50} \text{ of inhibitor}}$$

Inhibitor	<i>B. multicinctus</i> venom cholinesterase		Acetylcholinesterase		Pseudocholinesterase	
	ID ₅₀ (μM)	Potency ratio	ID ₅₀ (μM)	Potency ratio	ID ₅₀ (μM)	Potency ratio
Cardiotoxin	0.19	33	7.4	0.8	0.52	12
Protamine	0.016	383	0.03	193	0.48	14
Polylysine	0.02	219	0.06	307	0.92	6
Physostigmine	5.8	1	1.4	4	3.1	2

Table 2. Effects of cations on anticholinesterase activities of polypeptides and physostigmine. Cholinesterase activity of *B. multicinctus* venom (3.75 $\mu\text{g/ml}$) was measured by electrometric titration with 0.01 M-NaOH in the presence or absence of cation (0.04 M) or inhibitor (0.55 μM -cardiotoxin, 0.06 μM -protamine, 0.05 μM -polylysine or 38 μM -physostigmine). The amount of 0.01 M-NaOH consumed by venom cholinesterase in the absence of cation and inhibitor is $0.28 \pm 0.05 \text{ ml}$, which is regarded as reference (100%). Data are means $\pm$ S.E. for six determinations. * $P < 0.05$, as compared with the control in the presence or absence of cation respectively.

Inhibitor	Cholinesterase (%)				
	Control	MgCl ₂	CaCl ₂	NaCl	KCl
Control	100.0 $\pm$ 5.9	159.1 $\pm$ 3.8	144.5 $\pm$ 1.6	99.1 $\pm$ 1.1	103.6 $\pm$ 5.0
Cardiotoxin	24.0 $\pm$ 1.0*	155.0 $\pm$ 4.0	159.5 $\pm$ 6.7	95.1 $\pm$ 0.8	96.2 $\pm$ 0.3
Protamine	15.8 $\pm$ 1.4*	170.0 $\pm$ 0.8*	165.1 $\pm$ 5.4*	58.3 $\pm$ 0.5*	66.5 $\pm$ 0.2*
Polylysine	16.5 $\pm$ 1.1*	183.5 $\pm$ 5.7*	182.5 $\pm$ 3.8*	93.0 $\pm$ 3.4	84.6 $\pm$ 1.4
Physostigmine	22.3 $\pm$ 0.7*	21.4 $\pm$ 0.8*	19.9 $\pm$ 0.4*	20.6 $\pm$ 0.8*	23.4 $\pm$ 0.5*

Table 3. Effect of Mg²⁺ on reversibility of acetylcholinesterase inhibitory action of polypeptides and physostigmine. Acetylcholinesterase activity of *E. electricus* was measured by the electrometric titration method. The amount of 0.01 M-NaOH consumed by acetylcholinesterase (0.125 $\mu\text{g/ml}$) in the absence of MgCl₂ for the first 5 min is $0.29 \pm 0.011 \text{ ml}$, which is regarded as reference (100 $\pm$ 3.8%). Column A shows acetylcholinesterase activity in the absence of MgCl₂ with or without the inhibitor. Column B shows acetylcholinesterase activity in the presence of 0.04 M-MgCl₂ which was added to the reactive mixture after the incubation of acetylcholinesterase with an inhibitor for 5 min. Column C shows acetylcholinesterase activity in the presence of 0.04 M-MgCl₂, which was added to the reaction mixture immediately before the addition of the inhibitor. Data are means $\pm$ S.E. for four determinations. * $P < 0.05$ as compared with the control in the presence or absence of Mg²⁺ respectively.

Inhibitor (μM)	Acetylcholinesterase activity (%)		
	A	B	C
Control	100.0 $\pm$ 3.8	237.3 $\pm$ 6.5*	237.3 $\pm$ 6.5*
Cardiotoxin (4.8)	54.4 $\pm$ 1.7*	62.6 $\pm$ 1.4*	228.8 $\pm$ 2.4
Protamine (0.12)	37.3 $\pm$ 1.4*	58.8 $\pm$ 2.4*	211.0 $\pm$ 1.7
Polylysine (0.5)	38.4 $\pm$ 0.3*	50.0 $\pm$ 0.7*	216.1 $\pm$ 4.1
Physostigmine (15.2)	13.0 $\pm$ 0.7*	4.8 $\pm$ 0.3*	19.5 $\pm$ 0.7*

1970). From the studies on the substrate and inhibitor specificity it has been suggested that cholinesterase of *B. multicinctus* venom is acetylcholinesterase rather than pseudocholinesterase (Chang & Lee, 1955). Results obtained in the present study that cardio-

toxin, protamine and polylysine inhibited not only this venom cholinesterase but also the purified acetylcholinesterase and pseudocholinesterase, were in good agreement with this contention.

There are three different types of cholinesterase

inhibitors (Myers, 1951). The first type (e.g. physostigmine) combines with both the ester grouping and the anionic groupings of the enzyme centre, the second type (e.g. di-isopropyl phosphosulfate) only with the ester grouping, and the third type (e.g. cationic inhibitors) only with the anionic grouping. The affinity of the third-type inhibitors to cholinesterase can be decreased by simple electrolytes (e.g. Na^+ , NH_4^+). Since cardiotoxin, protamine and polylysine are all strong basic polypeptides, they may belong to the third-type inhibitors, interacting with the anionic site of cholinesterase. The evidence obtained in the present study and elsewhere that cations such as Mg^{2+} , Ca^{2+} , Na^+ and K^+ antagonize anticholinesterase activity of these polypeptides but do not antagonize that of physostigmine, and that polyanions (heparin, ganglioside and RNA) abolish polypeptide effects (Lin Shiau *et al.*, 1976), suggests that these polypeptides inhibit cholinesterase by interacting their positive charges with the anionic sites of cholinesterase. Changeux (1966) found that cholinesterase possesses, in addition to anionic site in the catalytic centre, peripheral anionic sites where ligands bind and exert a regulatory role on the enzyme activity. Cations such as Ca^{2+} bind to this allosteric regulatory anionic site rather than the catalytic anionic site (Roufogalis & Quist, 1972). Since cations such as Mg^{2+} antagonized the anticholinesterase activity of cardiotoxin competitively (Lin Shiau *et al.*, 1976), these polypeptides might bind to cholinesterase on the peripheral regulatory anionic sites.

Comparative studies on the susceptibility of *B. multicinctus* venom cholinesterase, acetylcholinesterase and pseudocholinesterase to cardiotoxin revealed that the venom cholinesterase is about 40 times more sensitive to cardiotoxin than is acetylcholinesterase. By contrast, acetylcholinesterase is more sensitive to physostigmine than is the venom cholinesterase. As to protamine and polylysine, the venom cholinesterase is most sensitive, acetylcholinesterase next and pseudocholinesterase least. These results imply that the steric configuration of the anionic site of the venom cholinesterase may be different from those of acetylcholinesterase and pseudocholinesterase and is more accessible to these polypeptides. The very steep dose-inhibition curves of these polypeptides on cholinesterase also indicate that these polypeptides bind to cholinesterase specifically. Therefore it is conceivable that these polypeptides could become useful tools for research on the molecular mechanism of hydrolysis of acetylcholine and for exploration of the structure and function of the anionic site of cholinesterase. The specificity of the interaction of these polypeptides with the anionic site of cholinesterase can also be observed that a cardiotoxin-like prin-

ciple of *Bungarus fasciatus* venom (Lin Shiau *et al.*, 1975a) and melittin (possessing pharmacological actions similar to cardiotoxin) of bee venom (Mitchell *et al.*, 1971; Lin Shiau *et al.*, 1975b) also inhibited cholinesterase activity, but basic neurotoxins such as cobra neurotoxin, α -bungarotoxin and β -bungarotoxin did not possess this anticholinesterase activity (Lee *et al.*, 1971).

In conclusion, cardiotoxin, protamine and polylysine are potent inhibitors of cholinesterase (i.e. Table 1 and Fig. 1); CaCl_2 and MgCl_2 overcome the inhibition which is not simply a non-specific ionic-strength effect since NaCl and KCl did not abolish protamine inhibition (Table 2). The order of addition of polypeptide inhibitor and MgCl_2 to the reaction mixture influences the final degree of the inhibition observed (Table 3). All of these findings suggest that these polypeptides interact with the peripheral regulatory anionic sites of cholinesterase, directed by their positive charges and the specific steric configurations.

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