Does α-Bungarotoxin Inhibit Motor Endplate Acetylcholinesterase?

α-BUNGAROTOXIN, a specific and irreversible binding agent of acetylcholine receptors of the motor endplate[1,2] and of other nicotinic cholinergic receptors[3], has no inhibitory effect upon acetylcholinesterase preparation from electroplax of electric eel[3]. Štalc and Župančič[4] have, however, reported that, when a minced preparation of mouse diaphragm was used, α-bungarotoxin as well as *d*-tubocurarine markedly inhibited the membrane acetylcholinesterase when acetylcholine concentrations lower than 12 μM were used. They took this result as evidence to support their hypothesis that the anionic centre of the enzyme in the motor endplate is the acetylcholine receptor[5]. We attempted, therefore, to re-examine the effect of α-bungarotoxin by using intact acetylcholinesterase in the motor endplate in order to test the hypothesis and to establish the specificity of binding of the toxin.

α-Bungarotoxin isolated by fractionation on column chromatography[6,7] was found to be slightly contaminated with acetylcholinesterase existing in the crude venom of *Bungarus multicinctus*. The toxin solution, 1 mg ml^{-1} in acetate buffer, *p*H 4.0, was heated to 70° C for 20 min to completely decompose the enzyme activity. That α-bungarotoxin was not affected by this treatment was confirmed by its neuromuscular blocking action. The enzyme activity was measured on the intact isolated skeletal muscles according to the method proposed by Mittag *et al.*[8]. This procedure measures only the membrane enzyme located at the endplate. Two hemidiaphragms from each of several Long Evans strain rats of either sex weighing about 250 g, or four hemidiaphragms from each of several NIH strain adult mice, or six biventer cervicis muscles from each of several 7 to 15 d old male Leghorn chicks, were quickly isolated and immersed in an organ bath containing 10 ml Tyrode solution at 37° C and oxygenated with 95% O_2 and 5% CO_2. After 30 min incubation, 1 μM ^{3}H-acetylcholine (290 mCi $mmol^{-1}$, Radiochemical Centre, Amersham) was added and the enzymatic hydrolysis measured for a 10 min period of incubation. About 20% of the added acetylcholine was hydrolysed during this period. Aliquots (1 ml) of bath medium were passed through a column (6×25 mm) of Dowex 50 to remove unhydrolysed acetylcholine, and counted on a liquid scintillation counter. The enzyme activity was expressed as the rate constant according to the following formula[8]:

Rate constant (ml per min per muscle)$=[(R-S)V]/[N(T-A)]$

where R is the total increase of 'acetate' c.p.m. ml^{-1} min^{-1}; S, the spontaneous hydrolysis ml^{-1} min^{-1} in c.p.m.; V, the bath volume in ml; N, the number of muscles; T, the total c.p.m. per ml of the bath media; and A, the 'acetate' c.p.m. at zero time ml^{-1}. After the first 10 min measurement period, the test muscle preparation was incubated with α-bungarotoxin at 1 μg ml^{-1} for 70 min to completely block the neuromuscular transmission and to bind most of the acetylcholine receptors[9]. Then the enzyme activity was measured again in the presence of the toxin and expressed as a percentage of the first measurement. The preparation was then washed several times with plain Tyrode solution and, after 60 min, the enzyme activity was again measured. The results summarised in Table 1 illustrate that the enzyme of rat diaphragm was quite stable over the experimental period of about 4 h. It was found that when most of acetylcholine receptors were bound with α-bungarotoxin, the enzyme activity was slightly increased instead of markedly inhibited as reported by Štalc and Župančič. Similar results were obtained for the mouse diaphragm and chick biventer cervicis muscle. The enhancement of enzyme activity by the toxin seemed to be irreversible. No defect except a slight enhancement was observed even at ten times higher concentrations of α-bungarotoxin. *d*-Tubocurarine at a concentration of 1 μg ml^{-1}, which significantly blocked the neuromuscular transmission, also showed no inhibitory effect on the enzyme. About 20% inhibition was obtained at ten times higher concentration (Table 1). The enzyme, however, was completely inactivated by 0.01 μg ml^{-1} echothiophate, a specific inhibitor of acetylcholinesterase.

Table 1 Effect of α-bungarotoxin on endplate membrane acetylcholinesterase of rat diaphragm

Agents	*n*	Cholinesterase activity: Before treatment (1) (rate constant; ml per min per hemidiaphragm) ±s.e.	After treatment (2) (%±s.e.)	After washout (3) (%±s.e.)
Control	4	0.110±0.007	101±3.1	97±6.4
α-Bungarotoxin, 1 μg ml^{-1}	5	0.102±0.005	117±5.5*	117±7.6
α-Bungarotoxin, 10 μg ml^{-1}	4	0.123±0.005	114±3.8*	—
d-Tubocurarine, 10 μg ml^{-1}	3	0.104±0.105	81±4.8*	96±0.5

* $P<0.05$ against control.

Control hydrolysis was measured for each hemidiaphragm before treatment. The second assay was performed in the presence of agents after 70 min incubation while the third one was after subsequent washings for 60 min. The activity was expressed as a percentage of the first assay.

Our results with α-bungarotoxin and *d*-tubocurarine are diametrically opposed to those of Štalc and Župančič. With their *p*H-static titration method, the sensitivity at a substrate concentration as low as 12 μM could be unreliable. Our results, therefore, indicate that the acetylcholine receptor is not identical with the anionic site of acetylcholinesterase. Barnard *et al.*[10] have shown that binding of α-bungarotoxin to the mouse diaphragm receptor did not affect the binding of diiso-propylfluorophosphate. These two components of electric organs of electric eel[11] and torpedo[12] have been separated. The slight degree of enhancement after neuromuscular blockade by α-bungarotoxin is interesting, however. Since the enhancement was the same in extent with either 1 or 10 μg ml^{-1} of the toxin and was irreversible, it is likely to be related to the irreversible occupation of the receptor. It may be that the blockade of the association of acetylcholine with the receptor increases the opportunity of the acetylcholine molecules to get access to the enzyme.

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