EFFECT OF PRESYNAPTIC NEUROTOXINS FROM BEE AND COBRA VENOM ON SPONTANEOUS MEDIATOR SECRETION FROM MOUSE MOTOR NERVE ENDING

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Phosphorylases A_2 (PLA) from bee and cobra venoms cause triphasic changes in the frequency of miniature endplate potentials (MEPPs) recorded in the mouse diaphragm: an initial decrease of liberation of mediator followed by an increase, followed in turn by complete blocking of liberation. Removal of Ca^{++} from the perfusing solution (to concentrations below 10^{-9}M) prevented the presynaptic effect of the two PLAs. If the PLA was washed off with calcium-free solution, subsequent treatment with standard solution (2 mM Ca^{++}) caused an increase in the frequency of MEPPs. Agents increasing the Ca^{++} concentration in the axoplasm (K^+ ions, hypertonic sucrose, and the uncoupling agent 4, 5, 6, 7-tetra-2-trifluoromethylbenzimidazole) caused an increase in the MEPP frequency in muscles poisoned with PLA. Blocking the liberation of mediator evidently was not caused by exhaustion of its reserves.

KEY WORDS: presynaptic toxins; mouse diaphragm; spontaneous liberation of mediator; phospholipases; calcium.

Several neurotoxins with presynaptic action have been isolated from the venoms of animals. Many of these toxins are also type A_2 phospholipases (PLA), and the enzymic activity of the toxins is essential for manifestation of their toxic effect [3-6, 9, 11].

To study the mechanism of the blocking presynaptic action of neurotoxins of this type, it was decided to investigate the effect of PLAs isolated from the venoms of bees (BV) and the central asiatic cobra *Naja oxiana* (CV), on spontaneous mediator liberation from mammalian (mouse) motor nerve endings. BV and CV are polypeptides with molecular weights of 16,000 and 12,000, with phospholipase activity of 11 and 1.5 μmoles substrate /μg protein·min; their LD_{50} by subcutaneous injection into albino mice is 5 and 80 mg/kg respectively [1].

EXPERIMENTAL METHOD

Miniature endplate potentials (MEPPs) were recorded intracellularly in an isolated nerve–muscle preparation of the male albino mouse diaphragm. The preparation was incubated in a 2-ml bath containing physiological saline saturated with carbogen at 18°C or 30°C, pH 7.2-7.4 [3]. The MEPP frequency was recorded in the same synapse continuously for 1-3 h. At the beginning and end of the experiment the MEPP frequency also was measured in several other synapses by introducing the microelectrode successively into different muscle fibers.

EXPERIMENTAL RESULTS

BV in a concentration of 25 and 50 μg/ml and CV in a concentration of 50 μg/ml did not affect the resting potential level of the muscle fibers during incubation for 1-3 h. No changes likewise were observed in the mean amplitude of the recorded MEPPs, suggesting that the sensitivity of the postsynaptic membrane to the action of mediator likewise was unchanged.

Prolonged continuous observation on the MEPP frequency showed that three consecutive phases of the effect are characteristic of both neurotoxins: 1) a phase of initial decrease of

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TABLE 1. Effect of BV and CV on MEPP Frequency in Mouse Diaphragm ($M \pm m$)

Toxin	Concentration, μg/ml	Temperature, °C	No. of experiments	Normal state		Phase of initial block		Phase of increased frequency of MEPP		Phase of secondary block	
				potentials/sec	%	potentials/sec	%	potentials/sec	%	potentials/sec	%
BV	25	18	8	0,73±0,06	100	0,34±0,06	46,6	22,36±8,60	3630	0,29±0,06	39,7
BV	25	30	6	1,09±0,18	100	0,31±0,03	28,4	4,0,00±9,83	3670	0,23±0,07	21,1
BV	50	30	5	1,08±0,15	100	0,31±0,02	28,7	36,39±9,73	3369	0,25±0,04	23,1
CV	50	30	12	1,25±0,13	100	0,41±0,05	32,8	30,78±7,29	2462	0,34±0,04	27,2

MEPP frequency; 2) a phase of increased frequency; 3) a phase of sharp decrease in MEPP frequency up to total blocking of spontaneous release (Table 1; Fig. 1). The duration of each phase and the intensity of the effect varied in different experiments.

The initial decrease in the MEPP frequency occurred practically immediately after the appearance of BV or CV in the bath (Fig. 1) and reached a maximum after 5-30 min, after which the frequency increased by an amount which varied considerably (by 2-100 times) in different synapses, and also in character (a prolonged stable increase or separate high-frequency discharges of MEPPs). At 30°C the effect of the toxin during each phase was more clearly defined than at 18°C (Table 1). During the action of CV the first phase was usually relatively short, although the quantitative changes in MEPP frequency were identical during the action of both neurotoxins.

The enzymic activity of PLA depends on the presence of Ca^{++} in the medium. With a decrease in the Ca^{++} level to $1 \cdot 10^{-5}$ M, both CV and BV virtually lose their ability to hydrolyze the substrate. Consequently, the connection between presynaptic and enzymic activity of BV and CV could be investigated. When the preparation was added to solution lacking Ca^{++} but containing 3 mM Mg^{++} and 1 M EGTA (the level of ionized Ca^{++} in this solution must be below $1 \cdot 10^{-9}$ M), the MEPP frequency was substantially reduced (by five to seven times), for spontaneous mediator release from mammalian nerve endings is directly dependent on the extracellular Ca^{++} concentration [7]. In a calcium-free medium, BV and CV caused no changes in MEPP frequency (Fig. 2). If, however, the preparation was carefully rinsed to remove free neurotoxin with the same calcium-free solution, and the solution was then replaced by normal physiological saline (2 mM Ca^{++}), an increase in MEPP frequency was observed. In control experiments the original level only was restored, whereas in preparations treated with BV or CV (10 experiments) the increase in frequency exceeded the original level by 3.5 and 1.3 times respectively. After this the MEPP frequency fell, although in the control it remained constant (Fig. 2). These experiments show that, first, in the absence of Ca^{++} BV and CV are unable to change the MEPP frequency and, second, the firm binding of the toxins with their target is maintained. However, it must be noted that incubation of the toxin in calcium-free solution significantly weakens the subsequent presynaptic effect which arises after addition of Ca^{++}. This could be the result of partial reversibility of the binding of the neurotoxins with their targets.

To analyze the mechanism of action of the neurotoxins the effect of agents potentiating spontaneous mediator secretion on the poisoned nerve–muscle preparation was tested. In particular, it was important to determine the nature of the third phase of the effect of the neurotoxin – the blockade of spontaneous release. For this purpose the following procedures were used: 1) increasing the concentration of K^+ in the solution to 20 mM; 2) increasing the osmotic pressure of the solution by 2.5 times by the addition of sucrose; 3) addition of $5 \cdot 10^{-6}$ M 4,5,6,7-tetrahydro-2-(trifluoromethyl)benzimidazole, an uncoupler of oxidation and phosphorylation, to the solution. These procedures caused a marked increase in MEPP frequency through an increase in the ionized Ca^{++} concentration in the axoplasm of the endings. However, the mechanism of this increase differs. An increase in the K^+ concentration leads to depolarization of the endings and, consequently, to an increase in the Ca^{++} flow from the extracellular fluid into the axoplasm [8, 10]. In the two last cases, on the other hand, ionized Ca^{++} is liberated from the intracellular stores, mainly from the mitochondria [2, 7].

The effect of these procedures were compared on control preparations and at the time of action of BV and CV when the MEPP frequency was considerably reduced after the preceding increase, i.e., during the period of blockade of spontaneous release. In the presence of 20 mM

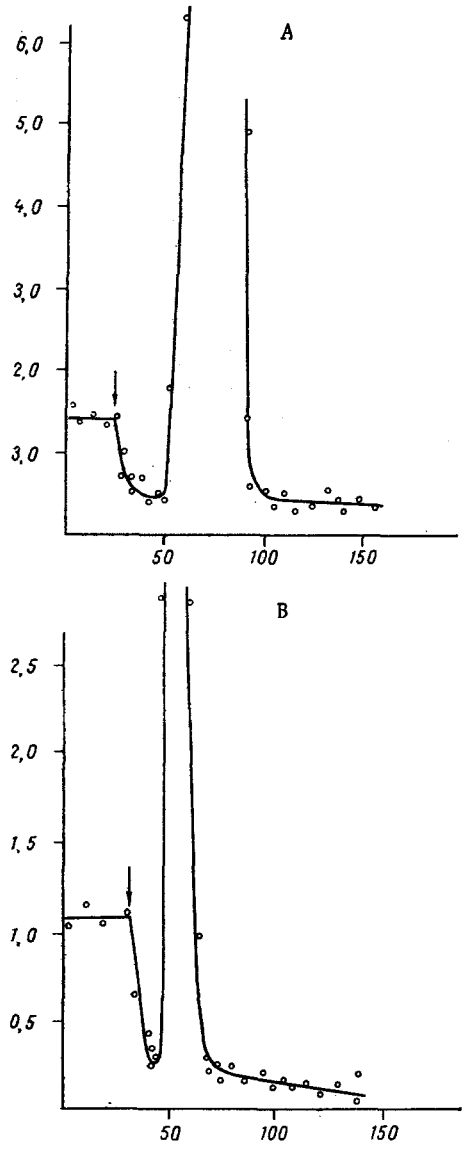

Fig. 1. Time course of effects of BV (A) and CV (B) in concentrations of 50 μg/ml at 30°C. Abscissa: time (in min); ordinate: MEPP frequency (in potentials/sec). Times of addition of toxin to solution shown by arrows.

K^+ the mean MEPP frequency was found to be the same in the control preparations and in those poisoned with BV or CV: 56.6 ± 9.0 (10), 53.8 ± 9.7 (9), and 57.7 ± 14.1 (10), respectively. A similar result also was obtained by increasing the osmotic pressure of the solution: In the control the MEPP frequency was 15.5 ± 5.5 (26), and in the poisoned preparations 18.4 ± 4.6 (34) and 21.0 ± 6.1 (7), respectively. It must be remembered that the relative increase in MEPP frequency in the poisoned preparation was actually a little greater, for the initial MEPP frequency (before treatment) was reduced in them through the action of the neurotoxins. Only in the presence of CV was the effect of an increase in K^+ concentration unequal. In some synapses, the increase in MEPP frequency was so great that it could not be accurately measured, whereas in others no increase in frequency was found whatsoever. It can tentatively be suggested that CV nevertheless causes some disturbance of the mechanism of coupling of depolarization and secretion in presynaptic endings. The effect of TTFB was expressed as a lightning increase in MEPP frequency, which could not be measured, and was qualitatively identical in the control and after treatment with the neurotoxins. These facts indicate that the increase in Ca^{++} concentration in the axoplasm of the poisoned nerve endings effectively increases the MEPP frequency, i.e., that no signs of exhaustion of the reserves of available mediator are observed.

The effects of BV and CV as revealed in these experiments on mice are thus similar in their general features to the effects of β-bungarotoxin [5, 6, 11] and notechsin [5], and also

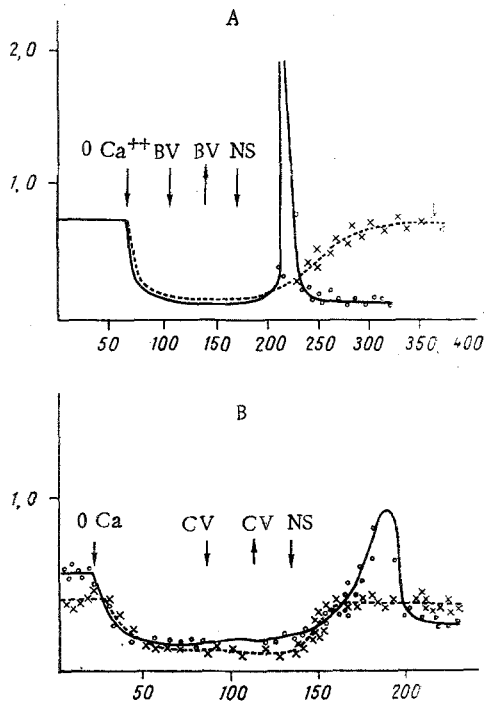

Fig. 2. Effect of BV (A) and CV (B) after incubation of preparation in calcium-free solution with EGTA. Times of replacement of normal physiological saline (NS) by calcium-free solution (0 Ca^{++}), addition of toxins, beginning of rinsing of preparation with calcium-free solution, and replacement of calcium-free solution by normal saline indicated by arrows. Continuous line — experiment, broken line — control (replacement of solutions but without addition of BV or CV). Remainder of legend as in Fig. 1.

to the effects of BV and CV [4] demonstrated previously in experiments on frogs: The same three phases of changes in MEPP frequency are observed; the action of the neurotoxin depends critically on the presence of Ca^{++} in the medium. This suggests that there is a common molecular mechanism of the toxic injury caused to nerve endings by these polypeptides. An important role in this mechanism is evidently played by their ability to act enzymically on the phospholipids of the presynaptic membrane. However, the binding of neurotoxins by nerve endings can take place in the absence of Ca^{++} and of the corresponding phospholipase activity also, suggesting the existence of some specific target on the presynaptic membrane which can selectively adsorb neurotoxins.

A detailed explanation is awaited of the causes of the phasic changes in the level of mediator liberation, but the results so far obtained suggest that the phase of block is unconnected with any exhaustion of the reserves of mediator due to the period of preceding increased activity.

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