β-BUNGAROTOXIN INHIBITION OF CALCIUM ACCUMULATION BY RAT BRAIN MITOCHONDRIA

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SUMMARY: Electrophysiological studies have shown that β-bungarotoxin modifies the release of neurotransmitter from mammalian motor-nerve terminals. In this paper we demonstrate that β-bungarotoxin can also inhibit calcium accumulation into sub-cellular fractions from rat brain at very low concentrations (2-8 pmoles toxin/mg protein). Since the calcium uptake which is inhibited has the characteristics of mitochondrial calcium accumulation (DNP-sensitivity, succinate stimulation), we conclude that the toxin affects the mitochondria. We suggest that the electrophysiological observations are consistent with direct or indirect inhibition by toxin of mitochondrial calcium uptake.

Neurotoxins have proven to be powerful tools for elucidating the mechanism of synaptic transmission. The protein neurotoxins such as cobratoxin and α-bungarotoxin have been invaluable in measuring the number and distribution of acetylcholine receptors in muscle (1,2,3). As an approach to the mechanism of neurotransmitter secretion and its regulation, we have been studying β-bungarotoxin, a pre-synaptic neurotoxin from the venom of the snake Bungarus multicinctus. This toxin, a protein of molecular weight 22,000 (4), causes failure of transmitter release in the rat phrenic nerve diaphragm preparation (4,5). Prior to failure, β-bungarotoxin induces an increase in both evoked release of transmitter in response to nerve stimulation (5), and in delayed release (4), which is the probability of observing a "spontaneous" quantal event just after evoked release (6). Since influx of calcium is needed for transmitter release, we have suggested (4) that the toxin might produce its effects on the neuromuscular junction by altering the calcium metabolism of the nerve terminal. In this paper we show by direct biochemical measurement that β-bungarotoxin is a powerful inhibitor of calcium accumulation.

Earlier studies on calcium accumulation into sub-cellular fractions from brain have shown that the major site of calcium uptake is the mitochondrion (7,8). In the experiments reported here we have measured calcium accumulation by both "crude" and "purified" mitochondrial fractions. These were prepared by homogenizing the brain of a decapitated rat in ten volumes of 0.32 M sucrose, 0.01 M Tris pH 7.4, using 35 strokes at 480 rpm of a standard Kontes glass-teflon homogenizer. The homogenate was spun at 1400 g for 10 min and the pellet resuspended in 12 ml 0.32 M sucrose. It was washed three times at 12,000 rpm and resuspended in 2 ml 0.32 M sucrose to give the crude mitochondrial fraction. To obtain the "purified" membrane fraction the "crude" preparation was layered on a discontinuous density gradient consisting of 3 ml of 2% Ficoll, 3 ml of 8% Ficoll and 3 ml 16% Ficoll, all containing 0.32 M sucrose and 0.01 M Tris pH 7.4. After centrifugation for one hour at 35,000 rpm in a Spinco SW41 rotor, the "pure" mitochondria were obtained as a brown pellet in the centrifuge tube. This procedure is a slight modification of that of Hamberger, Blomstrand and Lehninger (9). Calcium uptake was measured by incubating 200 µg of protein, or less, for 5 min at 25°C in 0.5 ml of a solution containing 20 µmoles KCl, 2.5 µmoles Tris pH 7.4, 2.5 µmoles $MgCl_2$, 0.025 µmoles $^{45}CaCl_2$ (2×10^7 cpm/µmole), 0.5 µmoles ATP and 5 µmoles potassium succinate, unless otherwise specified. The reactions were terminated by the addition of 2 ml of ice cold buffer, and immediate filtration through 0.45 µ Millipore filters. Filters were washed with 2 ml of the same buffer. Under these conditions the amount of calcium uptake is proportional to protein concentration and time of incubation. When aliquots of a crude mitochondrial fraction were exposed to purified β-bungarotoxin for 5 min at 25°C prior to measuring uptake, their ability to accumulate calcium was markedly impaired at very low toxin concentrations (Figure 1). In the experiment shown here, 50% inhibition occurred at 2.1 pmoles toxin/mg protein. Essentially identical results were obtained on mitochondria purified on Ficoll density gradients (50% inhibition at 8 pmoles toxin/mg protein). This increase in the amount of toxin required is expected since non-mitochondrial protein is removed during purification.

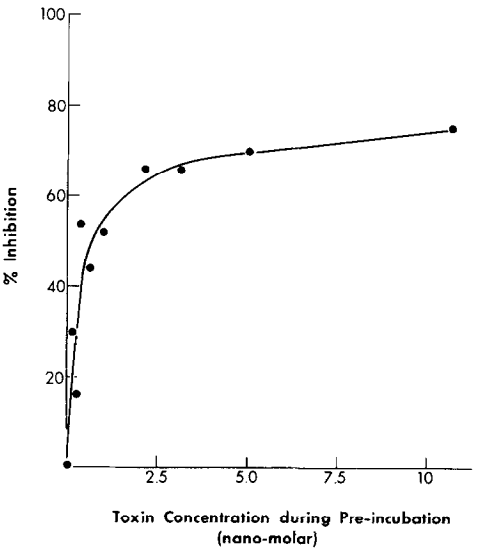

FIGURE 1. Concentration dependence of the inhibition of calcium accumulation by β-bungarotoxin. Aliquots of a "crude" mitochondrial fraction in 0.32 M sucrose, 20 mM Tris pH 7.4 were incubated at 25°C in concentrations of toxin given on the abscissa. After 5 min incubation 100 µl samples were diluted 5-fold into the reaction mix described in the text, to measure calcium uptake. Maximum uptake was the uptake after 5 min pre-incubation without toxin. Background was measured by incubating a fraction for zero time in the uptake medium. This background was substracted from all values of uptake.

The action of the toxin is not instantaneous. To show this, pure β-bungarotoxin was added to the crude mitochondrial fraction incubated at either 25°C or 0°C, aliquots removed at intervals and their ability to accumulate calcium determined (Figure 2). Some inhibition is found without pre-incubation but maximal inhibition occurs only after 10 min at 25°C and is not reached even after 15 min at 0°C. The inactivation of calcium uptake by β-bungarotoxin depends on both time and temperature.

Since the toxin is capable of reducing calcium accumulation in both crude and purified mitochondrial fractions, and since the mitochondrion is claimed to be the most active site of calcium accumulation in sub-cellular fractions from the brain (7,8), it is likely that the toxin is acting on the mitochondrion. Support for this hypothesis comes from a study of the energy requirement for calcium accumulation. Mitochondria are capable of accumulating calcium when

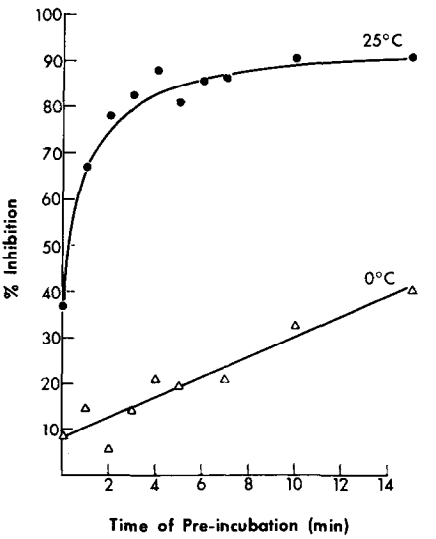

FIGURE 2. Time and temperature dependence of β-bungarotoxin inhibition. A crude mitochondrial fraction (2.3 mg/ml) was incubated in 0.32 M sucrose, 20 mM Tris pH 7.4, at 25°C or at 0°C in the presence or absence of β-bungarotoxin (0.7 pmoles toxin/mg protein). Aliquots were removed at intervals and calcium uptake measured as before. Since some inhibition can be detected at 25°C in the absence of toxin (∿5%/min) the values at 25°C have been corrected to give only toxin-specific inhibition.

provided with a metabolizable substrate such as succinate (9). In our experiments, performed at lower protein concentrations than is usual so that uptake is linear with time and protein concentration, succinate stimulation of calcium uptake is enhanced in the presence of ADP (Table 1). Both the succinate and the succinate + ADP-stimulated calcium uptake is sensitive to β-bungarotoxin. In addition, the sensitivity of calcium uptake to the uncoupling agent dinitrophenol (DNP) (9) closely parallels the sensitivity to β-bungarotoxin under several different assay conditions.

Although the toxin used in these experiments was at least 95% pure (4), the possibility remained that it was not β-bungarotoxin which blocked the calcium accumulation but some minor contaminant. Since the toxin was purified by ion-exchange chromatography, exclusion chromatography was used to try to separate the toxicity of the preparation from the inhibition of calcium accumu-

TABLE 1

Calcium Uptake in the Presence of β-Bungarotoxin and Dinitrophenol

Additions			Uptake (natoms calcium/mg prot)			% Inhibition	
Succinate*	ATP	ADP	No Inhibitor	+DNP**	+Toxin***	+DNP	+Toxin
-	-	-	3.9	2.8	2.9	28	25
+	-	-	5.3	3.2	3.8	40	29
-	+	-	9.5	3.5	3.6	63	62
-	-	+	2.7	2.4	2.2	11	20
+	+	-	71.5	5.0	8.6	93	88
+	-	+	23.1	2.8	3.7	88	84

*Conditions as in text except that, when used, the concentrations of succinate, ATP and ADP were 10 mM, 1 mM and 1 mM respectively.

**DNP was present at 0.3 μmoles/mg protein.

***Samples were exposed to β-bungarotoxin at 0.7 nmoles/mg protein for 5 min at 25°C prior to measuring calcium uptake.

lation. Chromatography of the purified toxin on G-50 Sephadex as described previously (4), showed no significant differences between the elution volumes of the protein, the toxicity and the ability to inhibit calcium accumulation (elution volume = 1.3 void volumes). If the inhibition of calcium accumulation is due to a contaminant it must have approximately the same molecular weight as the neurotoxin, and identical behavior during ion-exchange chromatography.

We conclude from these observations that β-bungarotoxin is a powerful inhibitor of calcium accumulation, and probably affects brain mitochondria directly or indirectly. Since β-bungarotoxin inhibits calcium accumulation at a much lower concentration than other inhibitors of mitochondrial calcium uptake such as ruthenium red (4 nmoles/mg protein) (11), dinitrophenol (40 nmoles/mg protein) (10), oligomycin (5 nmoles/mg protein) (10), and the calcium ionophore A23187 (0.3 nmoles/mg protein) (12), it may have a different site

of action. The precise site and whether toxin affects calcium uptake, calcium release, or the supply of energy needed to accumulate calcium against a concentration gradient, remain to be determined.

This work was initiated with the intention of finding the biochemical basis for the electrophysiological changes we and others have observed in toxin-treated preparations. The results reported here are consistent with the increased quantal content and enhanced delayed release found in vivo after β-bungarotoxin intoxication. The excitation-secretion of transmitter release (13) proposes that excitation of the nerve terminal cause an influx of calcium which, in turn, triggers a biochemical event--secretion. If this model is extended to suggest that, in analogy with the sarcoplasmic reticulum's role in relaxing muscle, intra-terminal mitochondria remove the calcium from the cytoplasm to turn off secretion, then the action of the toxin might be to inactivate partially the calcium removal system, increasing and extending the release of transmitter. A similar mechanism has been proposed by Rahamimoff and Alnaes (14) to account for the effects on the frog neuro-muscular junction of ruthenium red, an inhibitor of mitochondrial calcium accumulation. It was also proposed that botulinum toxin, another pre-synaptic neurotoxin, might affect calcium metabolism in synaptosomes, but no effect on calcium uptake was observed (15).

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