EFFECTS OF β-BUNGAROTOXIN ON CALCIUM UPTAKE BY SARCOPLASMIC RETICULUM FROM RABBIT SKELETAL MUSCLE

Y. H. Lau, T. H. Chiu, A. H. Caswell and L. T. Potter

Department of Pharmacology, University of Miami School of Medicine
Miami, Florida 33152 U.S.A.

Received September 27,1974

Summary A fluorescent chelate probe and a Millipore filtration technique have been used to study the effects of β-bungarotoxin (β-toxin) on passive and active Ca^{++} uptake and ATPase in fragmented sarcoplasmic reticulum (SR) of rabbit skeletal muscle. β-Toxin at 3 x 10^{-6} M did not affect ATPase activity. In the absence of ATP, β-toxin increased the passive uptake of Ca^{++}; in the presence of ATP, active Ca^{++} uptake was inhibited. The effect of β-toxin in SR can be detected at concentrations as low as 10^{-9} M. The results suggest that β-toxin induces Ca^{++} leakage in SR membranes.

β-Toxin, a neurotoxin isolated from *Bungarus multicinctus* venom, has been shown by Lee and co-workers (1-3) to block neuromuscular transmission at the skeletal muscle motor endplate specifically and irreversibly. They observed that the toxin initially increases and then inhibits the release of acetylcholine (ACh) from the terminals of motor nerves, while the response of postsynaptic receptors to ACh is unaffected. They also reported that low concentrations of Ca^{++} and high concentrations of Mg^{++} can delay the inhibitory effect of β-toxin (3). Ca^{++} is known to be intimately involved in transmitter release from motor nerves (4). More recently, Wagner et al. (5) have demonstrated that β-toxin inhibits the accumulation of Ca^{++} by rat brain mitochondria and have speculated that the mechanism of action of β-toxin on neuromuscular transmission may involve a direct or an indirect inhibition of Ca^{++} uptake by mitochondria inside the nerve terminals. Because β-toxin may have more generalized effects on biological membranes than was initially suspected, we have examined its effects on SR membrane which plays a major role in intracellular Ca^{++} uptake and has a relatively simple protein composition (6,7).

EXPERIMENTAL

Purification of β-toxin: β-Toxin was partially isolated according to the method described by Lee et al. (8) and was further purified on CM Sephadex C-25 to

yield a major peak of pure β-toxin. It gave a single protein band on polyacrylamide gel electrophoresis.

Preparation of SR: SR was prepared from rabbit back muscle essentially by the method of Martonosi (9) except that the microsomes were extracted only once with 0.6 M KCl. The vesicles thus prepared were suspended in 0.1 M KCl and 10 mM histidine, pH 7.3, at a protein concentration of 10-15 mg/ml.

Calcium uptake by SR: Two methods were used to measure fluxes of Ca^{++} in and out of SR. The fluorescent chelate probe with chlortetracycline as an indicator, as described by Caswell and Warren (10), was used to follow the kinetics of Ca^{++} movements. Fluorescence was determined at an excitation wavelength of 390 nm and emission of 530 nm. β-Toxin itself has no effect on the fluorescence signal. For quantitative measurements of the uptake of Ca^{++}, $^{45}Ca^{++}$ and Millipore filtration were employed as described by Meissner and Fleischer (11). The Millipore membranes were dried under a UV lamp and each was counted in 10 ml Aquasol (New England Nuclear). The exact procedure for each experiment is described under the legends of figures and table.

Assay of ATPase activity: The method of Martonosi and Feretos (12) was employed in which ATP hydrolysis was followed by recording continuously the production of H^{+}. Changes in pH were monitored with a pH meter connected to a chart recorder. The system was calibrated by adding known amounts of HCl to the incubation medium.

RESULTS AND DISCUSSION

Fig. 1 shows the initiation of Ca^{++} efflux by β-toxin from Ca^{++}-loaded SR. The increase in fluorescence of chlortetracycline on addition of ATP indicates Ca^{++} accumulation, while the fluorescence diminution which follows the addition of β-toxin indicates Ca^{++} release. Stimulation of Ca^{++} efflux occurred on addition of as little as 10^{-9} M β-toxin, i.e. with a toxin to protein ratio of 1.7×10^{-11} moles/mg protein. The rate of Ca^{++} efflux clearly increased with higher concentrations of β-toxin. Pretreatment of SR with β-toxin impaired or destroyed their ability actively to accumulate Ca^{++} upon

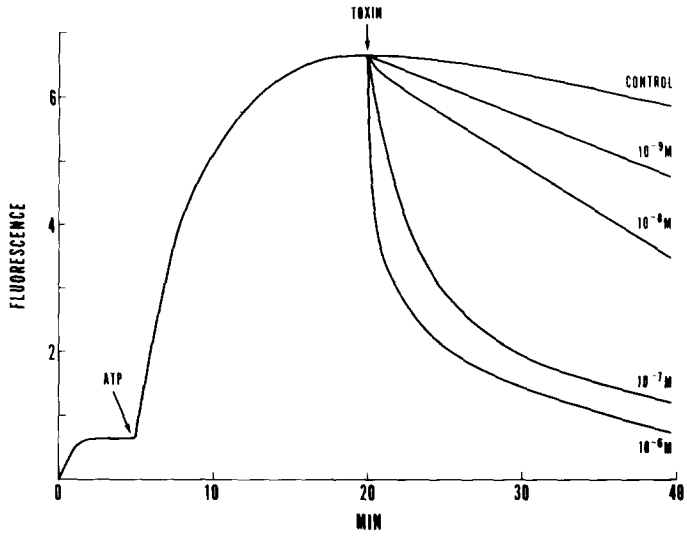

Fig. 1 Fluorescence assay of the effect of β-bungarotoxin on Ca^{++} efflux from Ca^{++}-loaded SR. 0.12 mg. SR protein was incubated in 2 ml medium A of pH 7.2 containing 0.20 M sucrose, 10 mM histidine, 10 mM KCl, 0.25 mM $MgCl_2$, 0.05 mM $CaCl_2$ and 10^{-5} M chlortetracycline as fluorescent indicator. Ca^{++} uptake was stimulated by the addition of 0.25 mM ATP. After the maximum level of fluorescence was reached, toxin (concentration as indicated) was then added.

addition of ATP. Fig. 2 shows that incubation of SR with 10^{-7} M β-toxin for 20 min caused complete abolition of active Ca^{++} uptake in the presence of ATP. At 10^{-8} M toxin there was still a rapid phase of the enhancement of fluorescence indicating rapid Ca^{++} uptake, but the maximum enhancement of fluorescence was lower than that of the control and was not maintained. This observation suggests that in the initial rapid phase of Ca^{++} uptake upon ATP stimulation, Ca^{++} influx was greater than the Ca^{++} efflux induced by 10^{-8} M β-toxin. As the rate of Ca^{++} influx began to slow down, there was net Ca^{++} efflux. Though β-toxin tended to abolish the enhancement of fluorescence due to Ca^{++} uptake in SR upon ATP addition, the level of fluorescence in the absence of ATP was higher in the presence of toxin than in the control. Fig. 2 shows that at 10^{-6} M β-toxin, the level of fluorescence was almost doubled compared to the control. The phenomenon described above suggests that β-toxin causes SR to become more permeable to Ca^{++}

In order to obtain quantitative data on the extent of Ca^{++} release in the

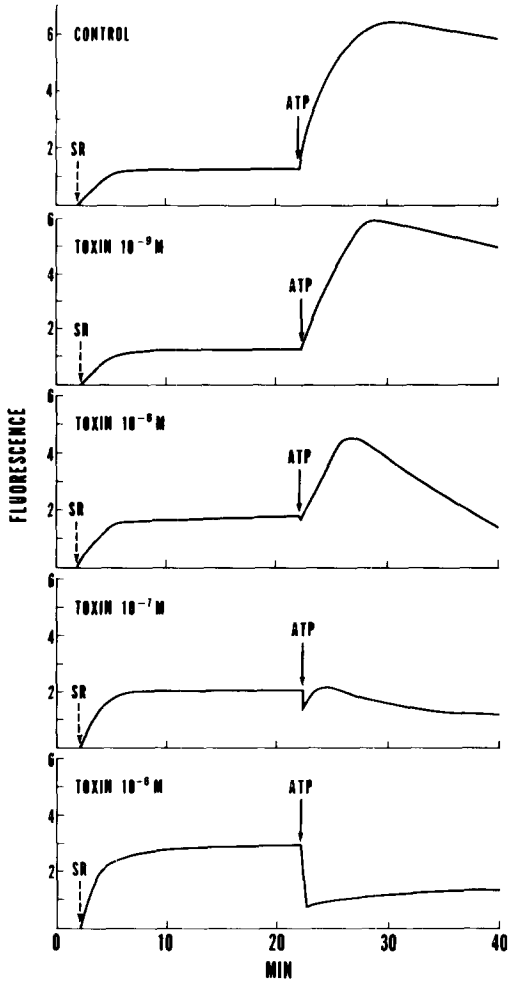

Fig. 2 Fluorescence assay of the effect of β-bungarotoxin on the accumulation of Ca^{++} by the SR. Incubation medium was the same as that described in Fig. 1.

presence of β-toxin, $^{45}Ca^{++}$ Millipore filtration was employed. Table 1 shows that this method gave results which were qualitatively similar to those obtained with the fluorescent chelate probe. It was found, however, that with the medium A (low Mg^{++} and low K^{+}) described in Table 1, Ca^{++} uptake was only 30% inhibited in the presence of 10^{-7} M β-toxin and that about 10-15% inhibition of Ca^{++} uptake was produced by 10^{-8}- 10^{-9} M β-toxin. In interpreting these experiments, it may be noted that the $^{45}Ca^{++}$ filtration technique measures the total Ca^{++} bound to SR while the fluorescent chelate probe technique measures only Ca^{++}

Table 1 Effects of β-bungarotoxin on Ca^{++} uptake by sarcoplasmic reticulum from rabbit skeletal muscle *

Conditions	Ca^{++} uptake, n moles/mg protein				
	Control	Toxin-treated (M) 10^{-6}	10^{-7}	10^{-8}	10^{-9}
Medium A					
SR	26.5 ± 2.1	47.2 ± 2.5	38.4 ± 1.8	29.8 ± 2.2	26.6 ± 2.0
SR + ATP	74.6 ± 4.1	34.9 ± 4.2	58.4 ± 4.2	71.6 ± 6.1	67.1 ± 5.7
Medium B**					
SR	6.4 ± 1.1	10.4 ± 1.1	9.3 ± 1.5		
SR + ATP	79.3 ±12.1	35.3 ± 5.4	65.6 ± 5.2		
Medium C**					
SR	5.1 ± 0.6	5.1 ± 0.6			
SR + ATP	79.5 ± 1.4	89.4 ± 2.4			

Medium A was the same as that described in Fig. 1.

* SR preparations with a protein concentration of 0.15 mg/ml were treated with β-toxin for 20 min, followed by ATP for 10 min. Aliquots (usually 1.0 ml) of reaction mixture were then filtered through Millipore membranes and the latter were counted each in 10 ml Aquasol. Means from 3-4 experiments are given ± S.E.

** Medium B and C were the same as medium A except that Mg^{++} and ATP were raised to 5 mM in the former and 0.1 M KCl replaced sucrose in the latter medium.

attached to the membrane surface, so the two techniques may not be quantitatively comparable. In addition a higher ratio of SR protein to toxin was used in the $^{45}Ca^{++}$ uptake experiments.

It has been found that the action of the toxin is inhibited by high Mg^{++} and high K^{+} in the medium (Table 1, medium B and C); these results are in agreement with the observation (3) that high Mg^{++} has a protective effect against β-toxin at the neuromuscular junction. In agreement with the fluorescence study, passive Ca^{++} uptake (i.e. Ca^{++} by SR in the absence of ATP) was almost doubled in the presence of 10^{-6} M β-toxin as compared to the control. This effect was not observed or was insignificant in the high K^{+} or high Mg^{++} medium.

We initially suspected that the effect of β-toxin on SR was due to its interaction with ATPase protein which is known to be associated with the Ca^{++} pump (13,14). However, we observed that even at 3×10^{-6} M, β-toxin neither inhibits ATPase in the presence of Ca^{++} (0.05 mM) nor enhances its activity in the presence of EGTA.

The stoichiometry between the Ca^{++} pump of SR and added toxin required to produce inhibition of Ca^{++} accumulation has been calculated. The percentage of Ca^{++} pump protein in SR has been estimated to be 70% and it has a molecular weight of approximately 100,000 (15) (confirmed by SDS gel electrophoresis on our preparation). In our $^{45}Ca^{++}$ uptake experiments, the concentration of SR protein is 0.15 mg/ml and thus Ca^{++} pump protein is present at 10^{-6} M. When toxin of 10^{-7} M is added, and even assuming that all of the toxin is bound to the Ca^{++} pump, the molar ratio of Ca^{++} pump protein to toxin is 10:1. Therefore no more than 10% of the Ca^{++} pump should be affected. However, we observed a 30% inhibition of Ca^{++} uptake at this concentration. Hence insufficient toxin is present to account for the extent of inhibition of Ca^{++} uptake under these conditions. At lower concentration of toxin the ratio is still less favorable. It therefore seems unlikely that the toxin is exerting its action through direct inhibition of the Ca^{++} pump, but rather the evidence supports the view that β-toxin increases passive permeability to Ca^{++}.

The data presented here suggest that β-toxin induces an increase in Ca^{++} ion permeability in the SR membrane. If similar effects are produced upon nerve terminal membranes as well as mitochondria, this could be the cause of the initial increase in ACh release which has been observed; the subsequent decrease in release may derive from an upset in intracellular release mechanisms due to both passive entry of Ca^{++} and a decrease in the uptake by mitochondria.

REFERENCES

1. Chang, C. C., and Lee, C. Y. (1963) Arch. Internat. Pharmacodyn. 144:241.
2. Lee, C. Y., and Chang, C. C. (1966) Mem. Inst. Butantan Simp. Internat. 33:555.

3. Chang, C. C., Chen, T. F., and Lee, C. Y. (1973) J. Pharmacol. Exp. Ther. 184:339.
4. Katz, B., and Miledi, R. (1969) J. Physiol. 203:459.
5. Wagner, G. M., Mart, P. E., and Kelly, R. B. (1974) Biochem. Biophys. Res. Commun. 58:475.
6. Martonosi, A., Halpin, R. A. (1971) Arch. Biochem. Biophys. 144:66.
7. Ikemoto, N., Bhatnagar, G. M., and Gergeley, J. (1971) Biochem. Biophys. Res. Commun. 44:1510.
8. Lee, C. Y., Chang, S. L., Kau, S. T., and Luh, S. H. (1972) J. Chromatog. 72:71
9. Martonosi, A. (1968) J. Biol. Chem. 243:71.
10. Caswell, A. H., and Warren, S. (1972) Biochem. Biophys. Res. Commun. 46:1757.
11. Meissner, G., and Fleischer, S. (1974) J. Biol. Chem. 249:302.
12. Martonosi, A., and Feretos, R., (1964) J. Biol. Chem. 239:659.
13. Warren, G. B., Penelope, A. T., Birdsall, N. J. M., Lee, A. G., and Metcalf, J. C. (1974) Proc. Nat. Acad. Sci. USA 71:622.
14. Meissner, G., and Fleischer, S. (1973) Biochem. Biophys. Res. Commun. 52:913.
15. Meissner, G., Conner, G. E., and Fleischer, S. (1973) Biochim. et Biophys. Acta 298:246