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# The Pharmacology of Pumiliotoxin-B

## I. Interaction with Calcium Sites in the Sarcoplasmic Reticulum of Skeletal Muscle

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SUMMARY

ALBUQUERQUE, E. X., J. E. WARNICK, M. A. MALEQUE, F. C. KAUFFMAN, R. TAMBURINI, Y. NIMIT, AND J. W. DALY. The pharmacology of pumiliotoxin-B. I. Interaction with calcium sites in the sarcoplasmic reticulum of skeletal muscle. Mol. Pharmacol. 19:411–424 (1981).

The actions of pumiliotoxin-B (PTX-B), a novel indolizidine alkaloid from the skin of the Panamanian frog, *Dendrobates pumilio*, have been studied in skeletal muscle of rat, frog, and crayfish with electrophysiological and biochemical techniques. PTX-B reversibly potentiates and prolongs the direct elicited muscle twitch in rat and frog skeletal muscle up to 12-fold in a concentration- and frequency-dependent manner, the potentiation being greater at the lower frequencies of stimulation. Responses of the muscle to tetanic stimulation in the presence of PTX-B are potentiated more at 10 and 20 Hz than at 50 and 100 Hz; tetanic fusion occurs earlier, and an aftercontraction is present when tetanic stimulation occurs in the presence of PTX-B. The twitch/tetanus ratio at 100 Hz is increased in the presence of PTX-B from 0.3 to more than 1.1 as a result of the increase in twitch amplitude. These effects on frog skeletal muscle are seen in the absence of any effect of PTX-B on spontaneous and evoked transmitter release, acetylcholinesterase activity, muscle action potential, delayed rectification, and cable properties of the muscle fiber. In the absence of external calcium, PTX-B prolongs but does not potentiate the twitch, while methoxyverapamil and dantrolene only partially suppress the actions of PTX-B. In crayfish skeletal muscle, PTX-B increases the rate of rise of the "calcium-dependent" action potential and shortens its duration. Biochemical studies reveal that PTX-B inhibits calcium-dependent adenosine triphosphatase from sarcoplasmic reticulum preparations of both frog and rat skeletal muscles in a concentration- and calcium-dependent manner. We suggest that PTX-B potentiates and prolongs the muscle twitch by (a) facilitating the release of calcium from storage sites within the sarcoplasmic reticulum, (b) mobilizing calcium from extracellular sites, and (c) blocking the reuptake of calcium by calcium-dependent adenosine triphosphatase.

### INTRODUCTION

PTX-B[4] is a member of a unique class of indolizidine alkaloids present in the skin of poisonous frogs of the neotropical family *Dendrobatidae* (1). PTX-B was first isolated from a Panamanian frog, *Dendrobates pumilio*, and its structure has recently been shown to be that of a 6-(6′,7′-dihydroxy-2′,5′-dimethyl-4′-octenylidene)-8-hydroxy-8-methyl-1-aza bicyclo[4.3.0]nonane (Fig. 1) (1).

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[4] The abbreviations used are: PTX-B, pumiliotoxin-B ($C_{19}H_{33}NO_3$); EPP, end plate potential; SR, sarcoplasmic reticulum; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $[Ca^{2+}]_o$, external calcium concentration; ACh, acetylcholine; PTX-A, pumiliotoxin-A.

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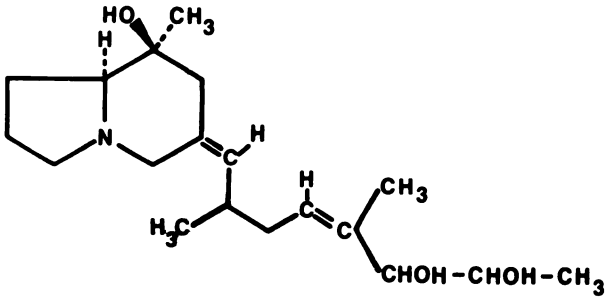


$C_{19}H_{33}NO_3$ Molec. Wt. 323.26

FIG. 1. *Structure of PTX-B*

The toxin has a remarkable effect on the contractile properties of skeletal muscle, producing a transient potentiation and then depression of the indirect evoked twitch, while the direct evoked twitch is transiently potentiated but not blocked (2). In addition, PTX-B has a rather novel effect on the nerve terminal membrane, where it induces repetitive activation of EPPs after single stimulation of the nerve (2). Both pre- and postsynaptic effects were strongly dependent on calcium ions.

Agents possessing calcium-dependent actions such as those of PTX-B would have great potential use in delineating the nature of excitation-secretion coupling in nerve and excitation-contraction coupling in muscle, processes which still remain largely unknown.

The effects of PTX-B on skeletal muscle membranes were therefore investigated with the objective of elucidating the mechanism of action of the toxin and determining whether the toxin would be useful in delineating the mechanisms whereby calcium from intracellular sites (i.e., SR in muscle) and extracellular sites mediate the processes of muscle contraction and of transmitter release from the presynaptic terminal. The effects of the toxin on the contractile properties of skeletal muscle and on Ca-ATPase from SR vesicles were examined. The data show that PTX-B caused a marked potentiation of muscle contraction in which the relaxation phase is transformed from a single decay to a multiphasic decay. It would appear that these actions may involve a facilitation of calcium influx into the muscle fiber, a release of calcium from the SR, and a partial block of Ca-ATPase by PTX-B. A preliminary report has appeared (2).

## METHODS

*Animals and preparations.* Experiments were carried out at room temperature (21–24°) on sciatic nerve-sartorius muscle preparations, on small bundles (10–20 fibers) of the semitendinosus muscle of the frog (*Rana pipiens*) and on extensor muscles of the carpopodite of the crayfish (*Procambarus clarkii*). The techniques for preparations of the muscles, stimulation, and recordings are described elsewhere (3–6). To examine the calcium-generated action potential in crayfish skeletal muscle, isotonic solutions containing $SrCl_2$ were prepared by replacing the NaCl in crayfish physiological solution with 100 mM $SrCl_2$. For twitch experiments on whole muscle or bundles, the contraction time of a single twitch (or contraction) is defined as the time required for the tension to rise from the baseline to peak; the half-relaxation time is the time required from peak to a point on the falling phase of the twitch equal to one-half the twitch amplitude (6). Approximately 1 min of rest was allowed between successive muscle stimulation at 10 Hz or greater. Contraction tension in whole muscle was recorded with a Grass force-displacement transducer (FT.03) and displayed on a Grass Model 7D polygraph and on a Tektronix oscilloscope (Model 5112). Tension in isolated bundles was recorded with the aid of a transducer tube (RCA 5734) and displayed on the oscilloscope. Photographic records were made with a Grass C4 kymograph camera and enlarged for measurement of the amplitude and time course of contraction. Acetylcholinesterase activity was assayed by a spectrophotometric method (7). All values are expressed as means ± standard error of the mean unless otherwise mentioned, and tests of significance were performed with the use of Student's *t*-test.

*Ca-ATPase activity in sarcoplasmic reticulum.* A conventional assay for Ca-ATPase activity in SR vesicles (c.f. ref. 8) was employed. SR vesicles were prepared from hind limb muscles of rat and frog essentially according to the method of Froelich and Taylor (8). These SR contain virtually no $Na^+$-$K^+$-ATPase, i.e., 0.1 mM ouabain caused no inhibition of activity (data not shown). Addition of 1 mM sodium azide did not inhibit ATPase activity of the SR preparation in the presence or absence of calcium. Isolated SR vesicles were incubated for 20 min at 30° using 0.4 μg of protein in 50 μl of medium containing 20 mM imidazole-HCl buffer, pH 7.0; 0.1 M KCl; 1 mM $MgCl_2$; 2 mM ATP; 0.1 mM EGTA; and various concentrations of added $Ca^{2+}$ or PTX-B as indicated. Under these conditions the assay was linear with time for at least 30 min and proportional with tissue ranging up to 3 μg. Reactions were stopped by placing the rack of samples in a bath of ice-cold water followed by the addition of 5 μl of 0.1 M HCl. ADP formed during the reaction was measured fluorometrically according to the method of Lowry and Passonneau (9). Protein concentration was assayed by the method of Lowry *et al.* (10).

*Solutions and drugs.* The physiological solution for frog muscle had the following composition (millimolar concentrations): NaCl, 115.5; KCl, 2.0; $Na_2HPO_4$, 1.3; $NaH_2PO_4$ 0.7; $CaCl_2$, 1.8. After bubbling with 100% $O_2$, the pH was 6.9–7.1. The physiological solution for the crayfish muscles (11) had the following composition (millimolar concentrations): NaCl, 205.0; KCl, 5.4; $CaCl_2$, 13.5; $MgCl_2$, 2.6; $NaHCO_3$, 2.3. After bubbling with 95% $O_2$–5% $CO_2$ for 20 min the pH was 7.3 to 7.4. When the $[Ca^{2+}]_o$ was altered, the osmolarity of the solution was adjusted by changing the NaCl concentration, and the preparation was equilibrated for 30–45 min prior to addition of PTX-B. In experiments in which $[Ca^{2+}]_o$ was removed and EGTA (dissolved with the aid of 0.1 N NaOH) was present, the solution contained 3.6 mM $MgCl_2$ and an appropriately reduced concentration of NaCl. In voltage clamp experiments at the end plate, sartorius muscles were treated with a hyperosmotic solution of glycerol (400–600 mM) in normal physiological solution

for 60 min and then washed with normal physiological solution for another 60 min (4). This procedure functionally disconnects the sarcotubular system from the sarcolemmal membrane, sparing excitation-secretion coupling and generation of muscle action potentials without subsequent contraction of the muscle fibers. For high potassium solutions, KCl replaced NaCl on an equimolar basis.

All drugs were stored as refrigerated stock solutions until just prior to use. Tetrodotoxin (0.3 mM) and *d*-tubocurarine (10 mM) as the chloride salt (Sigma Chemical Company, St. Louis, Mo.) were dissolved in double-distilled water. Dantrolene sodium was obtained from Eaton Laboratories, Norwich, N. Y., and methoxyverapamil from Knoll AG, Ludwigshafen, West Germany) were prepared freshly for each experiment. PTX-B was obtained as the pure toxin from the Panamanian frog *D. pumilio* (1, 12), dissolved in absolute ethanol at a concentration of 6.3 mM, and stored in the freezer.

## RESULTS

*Effect of PTX-B and caffeine on whole muscle twitch.* Isometric contractions of whole sartorius muscle elicited by direct and indirect stimulation were potentiated by PTX-B (1.5 μM). As shown in the typical tracing in Fig. 2A, the toxin's action began within seconds, and within 1 min the amplitude of the single response had increased by nearly 25%. Maximal potentiation at 1.5 μM occurred at 2 to 3 min, when both direct and indirect contractions reached 150–155% of control (Fig. 2B). Subsequently the amplitude of the indirect response decreased to 40% of control at 10 min and remained there for up to 30 min. The direct elicited response decreased gradually to control values, where it remained for the 30-min exposure period. There was no increase in the baseline tension but the muscle contractions were clearly prolonged, although the magnitude of that change cannot be ascertained from polygraph records.

The effect of varying concentrations of PTX-B (0.1–30 μM) on directly and indirectly elicited contractions of whole muscle (at 0.2 Hz) is summarized in Fig. 3. At concentrations equal to or less than 0.1 μM, the toxin neither potentiated nor depressed contractions elicited in whole muscle. The amplitude of the responses was potentiated in a concentration-dependent manner (Fig. 3, *insets*), and even at the highest concentration examined (30 μM) there was no evidence of muscle contracture although the effect of the toxin was faster and postactivation contractions could be observed. The vehicle (ethanol) had no effect on twitch characteristics at concentrations up to 100 μM, the highest used with toxin.

Sartorius muscles were also exposed to caffeine (0.1–20 mM) for comparison with the effects of PTX-B. Caffeine was ineffective at 0.1 mM but did produce a graded potentiation of both the direct and indirect evoked contractions at concentrations from 0.5 to 5 mM. For example, at 0.5 mM, caffeine potentiated the indirectly elicited twitch by 24 ± 6% and the directly elicited twitch by 14 ± 5% ($N$ = 3 muscles); at 5 mM, these values were 158 ± 10% and 92 ± 8%, respectively. Higher concentrations (i.e., 20 mM) induced muscle contracture. The potentiation occurred without apparent change in the baseline tension or in the time course of the twitch. When the concentration of caffeine was increased to 20 mM, a contracture was apparent (*vide infra*) upon which potentiated contractions were superimposed.

*Frequency-dependent actions of PTX-B on whole muscle.* Altered intervals and frequencies of stimulation appeared to influence the potentiation by the toxin. It thus seemed worthwhile to evaluate fully the effect of PTX-B at various rates of stimulation. Figure 4 illustrates the time course of potentiation by PTX-B (1.5 μM) of whole muscle contraction in the frog sartorius muscle in which stimulation was stopped just prior to the addition of PTX-B and the muscle was then stimulated indirectly and then directly (at 0.2 Hz) for several responses continuously (Fig. 4A) or at intervals of 0.5, 1 and 3 min (Fig. 4B-D, respectively). Peak potentiation occurred earlier when stimulation was more frequent, but was of greater magnitude with more infrequent stimulation. Furthermore, potentiation was immediately evident when stimulation began after a 3-min interval and depression of the response was absent (Fig. 4D). To eliminate any involvement of the transmitter on the junctional membrane, some preparations where treated with α-bungarotoxin (5 μg/ml) prior to the addition of PTX-B. In whole sartorius muscle exposed first to α-bungarotoxin (5 μg/ml) for 30 min and then to PTX-B (1.5 μM) and stimulated at 0.2 Hz continuously or at intervals of 0.5 and 1 min, the time course of potentiation was similar to that observed during direct and indirect stimulation at 0.2 Hz of control muscles (data not shown). These results demonstrate that the potentiating effects of PTX-B on muscle twitch are not dependent on prior activation of the muscle (i.e., stimulation), on membrane depolarization, or on toxin-evoked release of transmitter. However, the blockade of contraction which can occur is stimulus-dependent and difficult to reverse in whole muscle.

*Effect of PTX-B in "glycerol-shocked" muscles.* In the course of voltage-clamp experiments for examination of the effects of the toxin on end plate currents (3), excitation-contraction coupling is routinely disrupted in our

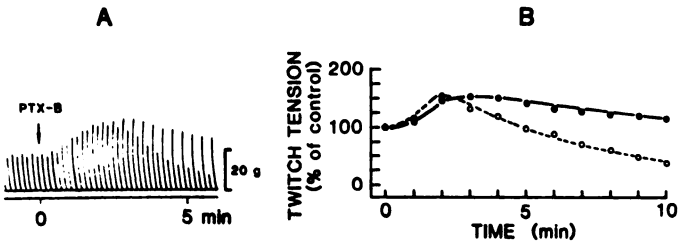


FIG. 2. *Typical response of the frog sartorius muscle to PTX-B*
The tracings in A were obtained from polygraph records of one experiment while the curves in B are the average of three to five preparations during alternating supramaximal direct (●) and indirect (○) stimulation (0.1 Hz each). Toxin (1.5 μM) was added at the *arrow* in A or at time zero in B. The indirectly elicited response was potentiated and then reduced in amplitude during the sustained potentiation of the directly elicited twitch. There are indications of a prolongation of the contraction.

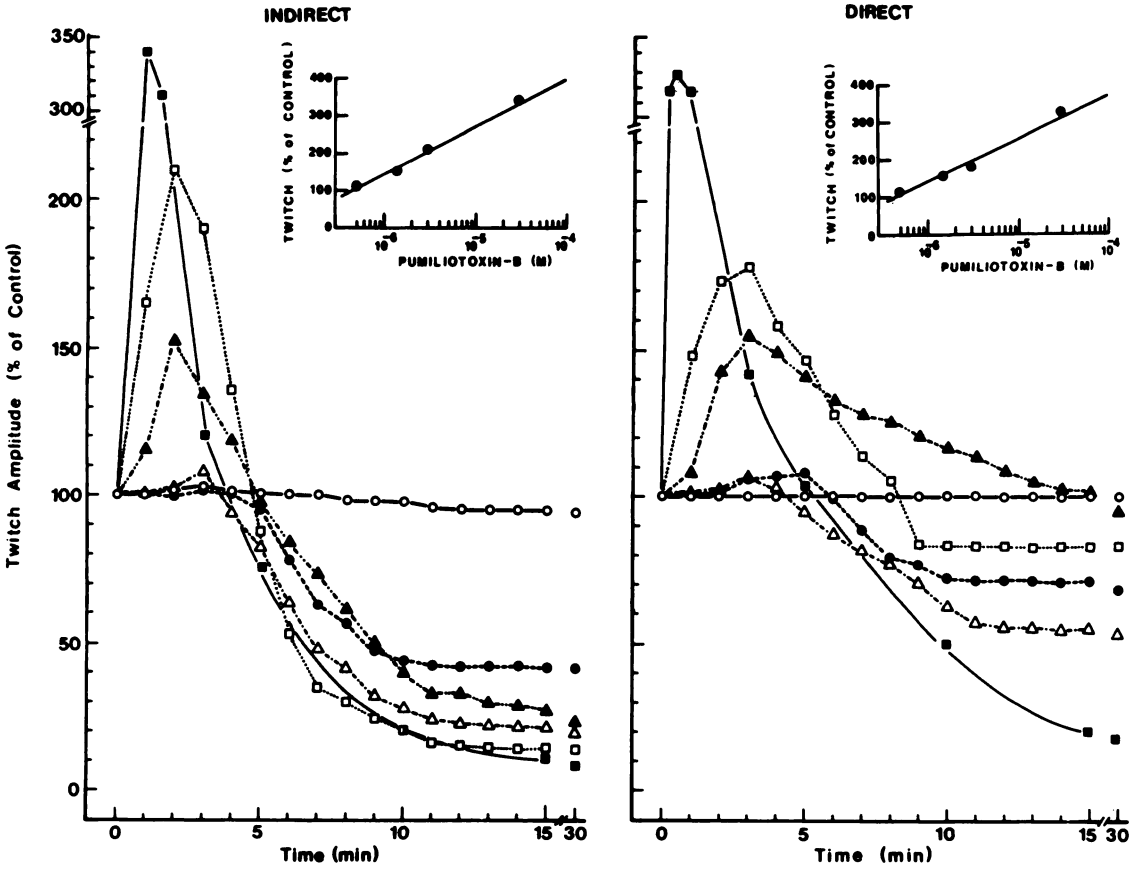


FIG. 3. *Dose-response and time-course of effect of PTX-B on the indirectly and directly elicited contractions of the frog sartorius muscle* Each point represents the mean of three to five preparations: ○, 0.1 μM; ●, 0.25 μM; △, 0.5 μM; ▲, 1.5 μM; □, 3.0 μM; ■, 30 μM. The *insets* illustrate the dose dependency of the maximal potentiation. The regression line was determined by least-squares regression analysis; $r = 0.98$. Washing for 120 min only partially reversed the effect of toxin in whole muscle. The apparent irreversibility occurs only in whole muscle and is mostly due to the highly lipophilic nature of the toxin and difficulty in its ability to diffuse away from the deep fibers of the intact preparation.

laboratory by treating the muscle with hyperosmotic glycerol-physiological solution for 1 hr and then "shocking" the muscle by washing in normal physiological solution (see Methods). This procedure effectively curtails the invasion of the action potential from the sarcolemma to some point along the T-tubule at which the alteration in ionic gradient normally signals the terminal cisternae and SR to release $Ca^{2+}$ for the contraction of the muscle (4). Immediately after the addition of PTX-B (1.5 μM) to the solution bathing the glycerol-shocked sartorius muscle (i.e., within 1 min) excitation-contraction coupling was restored and the muscles then contracted when stimulated indirectly. The muscles continued to respond mechanically to indirect stimulation even when the toxin had been washed from the experimental chamber for more than 1 hr, thus suggesting a rather slowly reversible action of the toxin.

*Effect of PTX-B on directly elicited twitch and tetanus tension of small bundles of semitendinosus muscle fibers.* Experiments using whole sartorius muscle have the drawbacks that distribution of the toxin to deep fibers may be retarded and reversibility of the effect becomes rate-limited by diffusion. To delineate more fully the action of this toxin on contractile properties of muscle and to eliminate most penetration barriers, experiments were performed with bundles of 10–20 intact fibers, and on a few occasions with 1 or 2 intact fibers, dissected from semitendinosus muscles of the frog. These preparations were placed in a small experimental chamber, stimulated at 0.2 Hz, and exposed to α-bungarotoxin (5 μg/ml) for 30 min to block the postsynaptic ACh receptors and any neural effects of PTX-B. PTX-B was then added to the bathing solution at various concentrations (0.05–30 μM) and under varying conditions of stimulation. Within seconds after the addition of PTX-B (1.5 μM) to the solution bathing a muscle stimulated at 0.2 Hz, an inflection appeared in the falling phase of the twitch (Fig. 5) such that the decay from peak tension appeared in two phases. The contraction time and relaxation to 50% of peak amplitude (half-relaxation time) occurred in a nearly normal manner at 7 sec, but the latter part of the relaxation phase was prolonged such that the time to 90% relaxation had increased from about 75 msec to more than 500 msec. By 30 sec after the addition of PTX-B with continuous stimulation (0.2 Hz), the shape of the contraction was altered: the initial contraction phase, which had a rise time equal to control, was potentiated about 30%. However, the muscle fibers did not relax completely, and after a small transient relaxation the tension again increased to 130% of control but then slowly

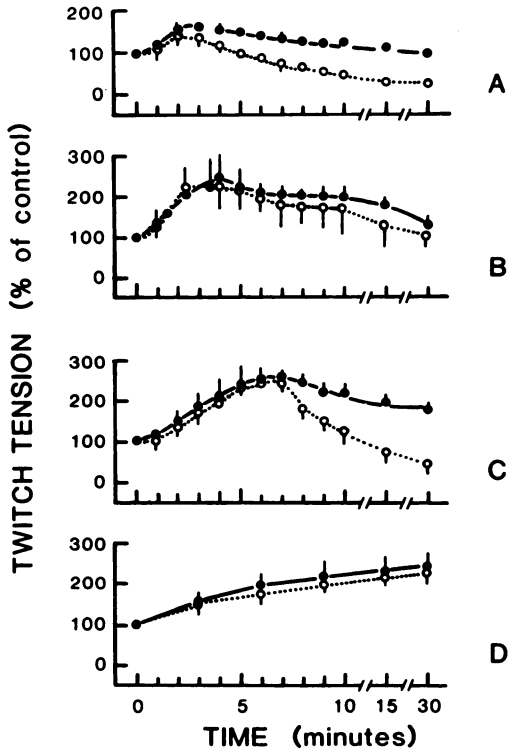


FIG. 4. *Time course of the effect of intermittent stimulation on the potentiation and depression of twitches in frog sartorius muscles by PTX-B*

Each curve is the mean of three muscles. In A, the muscle was stimulated continuously, both directly (●) and indirectly (○) at 0.2 Hz; in B, one direct and one indirect response at 0.2 Hz every 30 sec; in C, every 60 sec; in D, every 3 min.

declined toward the baseline. The half-relaxation time at 30 sec had reached nearly 500 msec as compared with the control values of less than 50 msec. By 45 sec the amplitude had increased to 185% of control (Fig. 5), but there was an inflection on the rising phase which appeared to be the potentiated peak of the initial contraction. The tension continued to rise to peak more slowly and then returned to baseline in an approximate, exponential manner with a half-relaxation time of 265 msec and 90% relaxation time of 820 msec. Subsequently the contractions became larger, reaching 230% and 330% of control at 60 and 90 sec, respectively, but then declined with continued stimulation. The contraction time simultaneously increased to between 250 and 400 msec. It appears that the two processes are contributing to the change in contraction time. First, the amplitude of the rising phase was increased; second, muscle tension continued to increase during the relaxation phase as indicated by a delay in relaxation.

Table 1 and Fig. 6 summarize the peak effects of PTX-B on fibers of the semitendinosus muscle stimulated at 0.2 Hz and exposed to concentrations of PTX-B ranging from 0.05 μM to 30 μM. At 0.05 μM, PTX-B had no effect on the contraction, half-relaxation, and 90% relaxation times or on the amplitude of the direct evoked response. As shown in Fig. 6, half-maximal potentiation of the muscle twitch occurred at 0.3 μM. There is a concentration-dependent potentiation of the muscle tension with the maximal response occurring at about 3 μM, when contraction tension reached more than 600% of control (Fig. 6). Higher concentrations of the toxin (i.e., 10 and 30 μM) produced no greater effect than at 3 μM. The

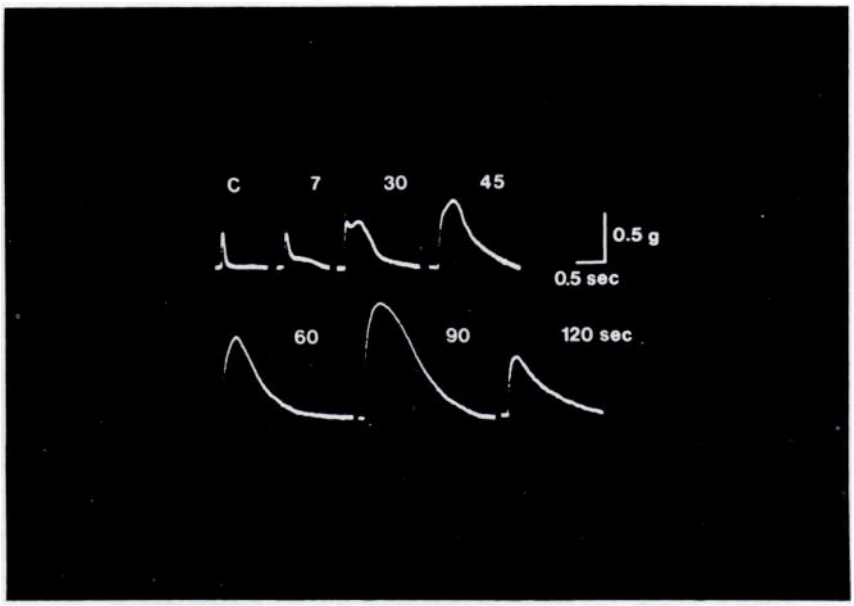


FIG. 5. *Potentiation and prolongation of the directly elicited twitch in bundles of fibers isolated from the frog semitendinosus muscles by PTX-B*

Bundles of 10–20 intact fibers were isolated from the semitendinosus muscles exposed to α-bungarotoxin (5 μg/ml) for 30 min to inactivate the ACh receptor and then exposed to PTX-B (1.5 μM). The frequency of direct stimulation was 0.2 Hz, continuously. Note the early prolongation of the relaxation phase and subsequent potentiation of both the rising phase and the relaxation phase. Each response is the result of a single direct stimulation of the muscle with a current pulse of 3-msec duration.

TABLE 1

*Effect of PTX-B on the time course of contraction in fibers of the frog (Rana pipiens) semitendinosus muscle*

The semitendinosus muscles were dissected to between 10 and 20 fibers and then exposed to α-bungarotoxin (5 μg/ml) for 30 min prior to obtaining control values. Toxin was then added to these muscles stimulated at 0.2 Hz; the peak effects shown occurred during a 30-min exposure to the toxin.

| Concentration | Contraction time | Half-relaxation time | 90% Relaxation time |
|---|---|---|---|
| *μM* | *msec* | *msec* | *msec* |
| Control | 28.0 ± 1.3 (30)[a] | 23.5 ± 1.8 | 54.9 ± 3.2 |
| 0.05 | 23.3 ± 1.7 (3) | 15.3 ± 1.4 | 44.3 ± 8.5 |
| 0.10 | 235.0 ± 50.7 (3) | 345.0 ± 10.4 | 1847 ± 55 |
| 0.20 | 217.7 ± 38.3 (3) | 778.7 ± 16.2 | 1833 ± 88 |
| 0.40 | 436.7 ± 136.7 (3) | 730.0 ± 87.2 | 1813 ± 94 |
| 0.80 | 335.0 ± 83.5 (3) | 808.3 ± 50.7 | 1360 ± 31 |
| 1.5 | 318.3 ± 78.0 (3) | 841.7 ± 172.2 | 1553 ± 158 |
| 3.0 | 344.0 ± 40.6 (5) | 956.0 ± 133.0 | 1919 ± 192 |
| 10.0 | 166.7 ± 42.6 (3) | 955.0 ± 273.6 | 2067 ± 470 |

[a] Values shown are means ± standard error of the mean; numbers of muscles examined are shown in parentheses.

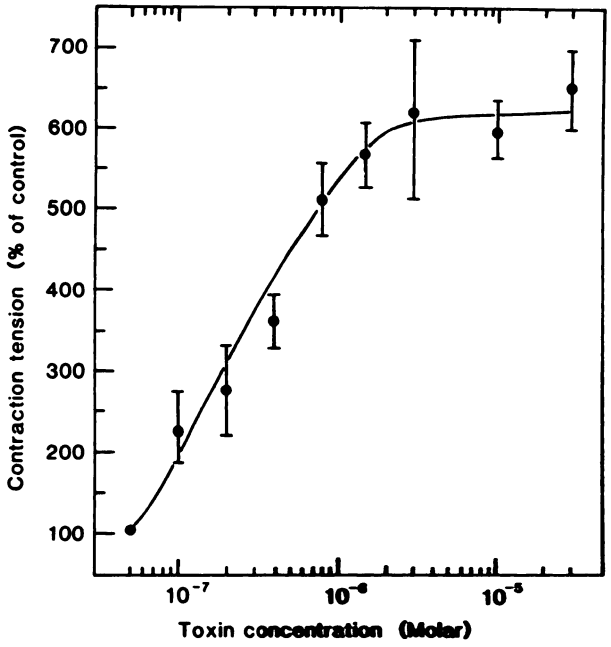


FIG. 6. *Concentration-dependent potentiation of contraction amplitude in bundles of semitendinosus fibers of the frog*

Each point is the mean (± standard error of the mean) of three to seven determinations from bundles of 10–20 intact fibers isolated from semitendinosus muscles. The bundles were first exposed to α-bungarotoxin (5 μg/ml) for 30 min to block the ACh receptor and then exposed to PTX-B (50 nM–30 μM) while stimulating continuously at 0.2 Hz. The potentiation appeared to be maximal at concentrations equal to or greater than 3 μM.

potentiation is accompanied by a marked prolongation of contraction, half-relaxation, and 90% relaxation times (Table 1). Contraction time is maximal with 0.4 μM toxin while the half-relaxation and 90% relaxation times appear to continue to increase with toxin concentration and reach nearly 1000 and 2000 msec, or 40- and 48-fold greater than control, respectively, with 3 μM toxin (Table 1).

Such potentiation and prolongation of the singly elicited response results in a contraction which resembles a tetanic response to repetitive muscle activation. However, the peak potentiation is far in excess of the normal twitch/tetanus ratio (at 100 Hz) which for any particular muscle is usually about 0.3. In fact, the twitch/tetanus ratio increased from an average of 0.32 before PTX-B to a maximum between 1.1 and 1.2, 30–60 min after exposure to toxin (*vide infra*).

Like the whole muscle, the frequency of stimulation altered greatly the response of the bundles of muscle fibers to PTX-B. That is, the potentiation and prolongation of the contraction is greater at low-frequency

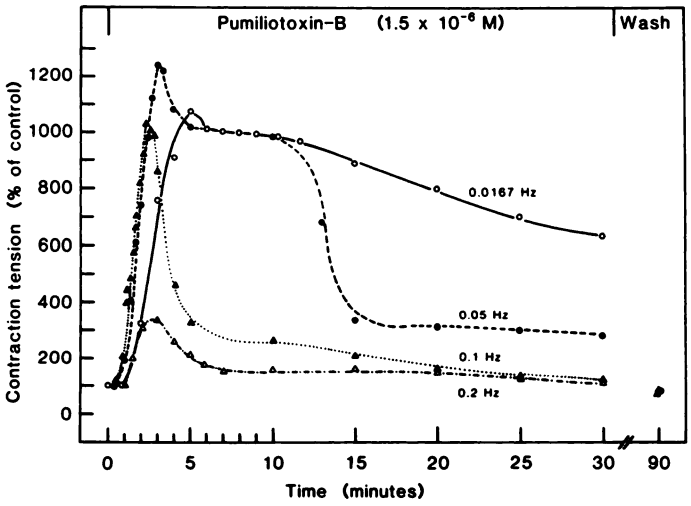


FIG. 7. *Time course and frequency dependence of the effect of PTX-B on the directly elicited twitch of bundles of semitendinosus fibers of the frog*

Each curve is a representative experiment on a bundle of 10–20 intact fibers isolated from a semitendinosus muscle first exposed to α-bungarotoxin (5 μg/ml) and then to PTX-B (1.5 μM) after 30 min. The stimulation frequency was either 0.0167 Hz (1/min), 0.05 (1/20 sec), 0.1 Hz (1/10 sec), or 0.2 Hz (1/5 sec). Note the early onset of potentiation at 0.05–0.02 Hz but the delay at the lower frequency. In addition, potentiation is prolonged to the greatest extent at the lowest frequency. Washing restored the twitch amplitude to near-normal values at all frequencies.

TABLE 2

*Frequency dependence of the effect of PTX-B on the time course and amplitude of contractions in fibers of the frog (Rana pipiens) semitendinosus muscle*

The semitendinosus muscles were dissected to between 10 and 20 fibers and then exposed to α-bungarotoxin (5 μg/ml) for 30 min prior to obtaining control values. Toxin was then added to these muscles; the peak effects shown occurred during the 30-min exposure period.

| Frequency of stimulation | Contraction time | Amplitude | Half-relaxation time | 90% Relaxation time |
|---|---|---|---|---|
| *Hz* | *msec* | *% of control* | *msec* | *msec* |
| Control | 24.3 ± 1.5[a] | 100 | 22.2 ± 2.1 | 45.5 ± 4.6 |
| 0.0167 | 272 ± 54 | 1078 ± 48 | 630 ± 95 | 3025 ± 1225 |
| 0.05 | 210 ± 40 | 1336 ± 86 | 622 ± 3 | 1312 ± 112 |
| 0.1 | 270 ± 20 | 1265 ± 235 | 610 ± 60 | 1080 ± 180 |
| 0.2 | 315 ± 78 | 260 ± 80 | 775 ± 225 | 1293 ± 183 |

[a] Values shown are means ± standard error of the mean of 12 muscles for control and 3 each at the various frequencies.

stimulation (Fig. 7; Table 2). Thus, directly elicited muscle contractions, as shown by a typical experiment in Fig. 7, are potentiated to about 325% of control at 0.2 Hz while at 0.0167 Hz peak potentiation is nearly 1100% of control and nearly 1300% at 0.05 Hz. The slower the frequency of stimulation the greater and more prolonged the potentiation of the directly elicited contraction. The peak effects of PTX-B (1.5 μM) at the four frequencies of stimulation examined on amplitude and on contraction, half-relaxation, and 90% relaxation times are shown in Table 2. Clearly, the degree of potentiation is greater at lower frequencies of stimulation, which at 0.05 Hz averages 1336% of control. The greatest prolongation in the contraction occurs at the slowest rate of stimulation (0.0167 Hz), attaining values in excess of 3000 msec.

Under control conditions and in the presence of α-bungarotoxin, these bundles of fibers responded to direct tetanic stimulation at 10 Hz and, even at 20 Hz, individual contractions were apparent. However, fusion occurred at both 50 and 100 Hz and the twitch/tetanus ratio averaged 0.30 (Fig. 8). The muscles were then exposed to PTX-B (1.5 μM), and the first recording of a response to single stimulation was obtained at 2–2.5 min and then at various intervals up to 30 min. After each response to a single pulse was recorded, the muscle was successively stimulated at 1-min intervals at 10, 20, 50, and 100 Hz. As shown in Fig. 8, the first twitch, recorded 2.5 min after toxin was added to the bath, was 2.5 times greater in amplitude than control, its contracture time had increased from 30 msec to 250 msec, the half-relaxation time was 400 msec (versus control of 46 msec), and 90% relaxation time increased from 75 to 1060 msec. Subsequent stimulation of the muscle at frequencies of 10–100 Hz revealed a remarkable effect of the toxin. Whereas small individual contractions were evident in the control muscle stimulated at 10 Hz for 1 sec, the response in the presence of PTX-B for 3 min was potentiated to the same extent as the single response, and nearly fused and approached the tension generated at 100 Hz. In addition, the relaxation phase became complex in that the tension failed to return to baseline within 100 msec after cessation of tetanic stimulation. Instead, the tension decreased abruptly at first and then with a slower time course. At 20 Hz, fusion was complete (unlike control) and the response potentiated by the presence of PTX-B approached that elicited at 100 Hz. The response at 50 Hz was 15% greater than control with a prolonged relaxation phase. Only the amplitude of the response at 100 Hz was initially unchanged, although the decay phase was prolonged. The twitch/tetanus ratio by this time had in-

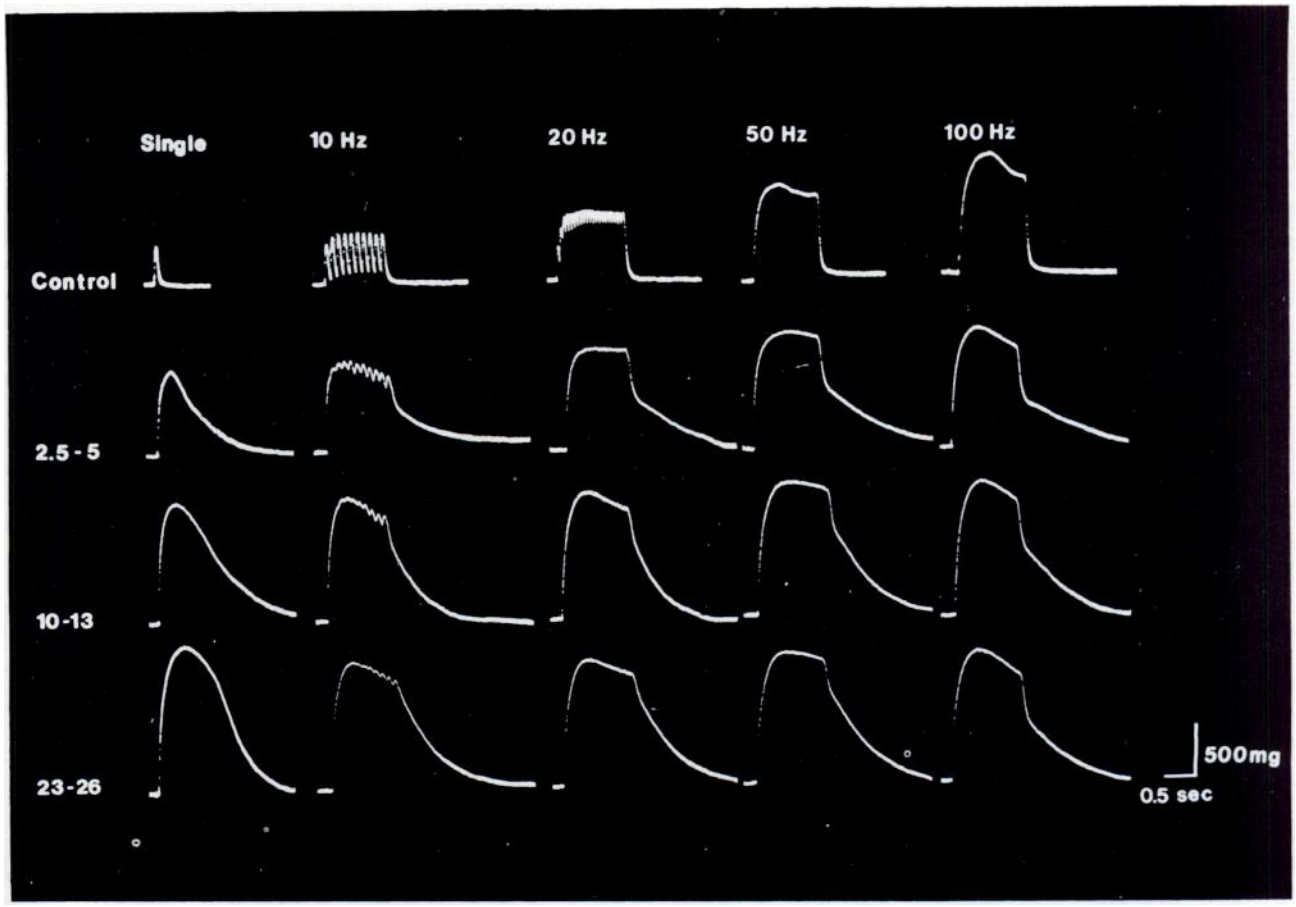


FIG. 8. *Effect of PTX-B on twitch and tetanus tension of bundles of fibers from the frog semitendinosus muscles*

The muscles were prepared as in Fig. 6 except that they were stimulated only when records were to be made. At least 1 min was allowed between successive stimulations. The twitch/tetanus ratio of the control (at 100 Hz) was 0.3 while at 30 min it was nearly 1.2, the greater proportion of the change being due to the change in contraction amplitude afforded by a single direct stimulation of the muscle. Note the prolonged aftercontraction which follows each tetanic response.

creased from 0.30 (control) to 0.7 at 5 min, 0.93 at 13 min, and 1.13 at 26 min. During this time, the maximal tetanus at 100 Hz had increased only 20% while the single contraction amplitude increased to 425% of control, exceeding the tension developed by tetanic activation of the muscle, with a half-relaxation time of 690 msec and 90% relaxation time of 1220 msec. Clearly then the change in twitch/tetanus ratio was not due mainly to a change in tetanic tension at 100 Hz but to the enormous increase in the amplitude of the response to a single stimulus. The responses at 10, 20, and 50 Hz were similarly potentiated during this time. The failure of the tension to return to baseline within 100 msec of the end of stimulation suggests that the toxin may be affecting not only the process of calcium release but also reuptake of calcium by the SR.

*Influence of $[Ca^{2+}]_o$ on toxin-induced changes in muscle twitch.* The role of $[Ca^{2+}]_o$ in mediating the effects of PTX-B was examined in both whole muscle preparations of the sartorius and small bundles of fibers from the semitendinosus muscles. The $[Ca^{2+}]_o$ was altered from $Ca^{2+}$-free + 5 mM EGTA to 9 mM $[Ca^{2+}]_o$ (Table 3). Concentrations of $[Ca^{2+}]_o$ below 0.45 mM resulted in a blockade of the indirect twitch and therefore only the directly elicited twitch was examined at this and lower concentrations. At 0.45 mM $[Ca^{2+}]_o$ the transient potentiation of the indirect response in whole muscle by PTX-B (1.5 μM) began immediately upon the addition of the toxin, peaked at 75% greater than control at 1 min, and was completely blocked in 10 min. At this lower concentration of $[Ca^{2+}]_o$ (0.45 mM), the time course for potentiation of the direct response in whole muscle by PTX-B was similar to that of control, but the maximal potentiation by 70% at 3 min was greater than in normal $[Ca^{2+}]_o$, and again there was no block of the direct response. At 0.018 mM $[Ca^{2+}]_o$, the indirect response was soon lost and, while PTX-B (1.5 μM) still potentiated and prolonged the directly elicited twitch in whole muscle, these effects were of much less magnitude than at 1.8 mM (Table 3). In the presence of a $Ca^{2+}$-free physiological solution to which EGTA (1 mM) had been added, the direct twitch in whole muscle was prolonged but not potentiated. At the highest concentration of $[Ca^{2+}]_o$, i.e., 9 mM, potentiation of both the direct and indirect twitches was delayed, beginning at 5 min and reaching a maximum of 20% greater than control at 25 min. There was no block of the twitches even when exposed for 60 min. Thus, low $[Ca^{2+}]_o$ reduced the onset of toxin action, with potentiation disappearing in $Ca^{2+}$-free solution while high $[Ca^{2+}]_o$ delayed and prolonged potentiation while

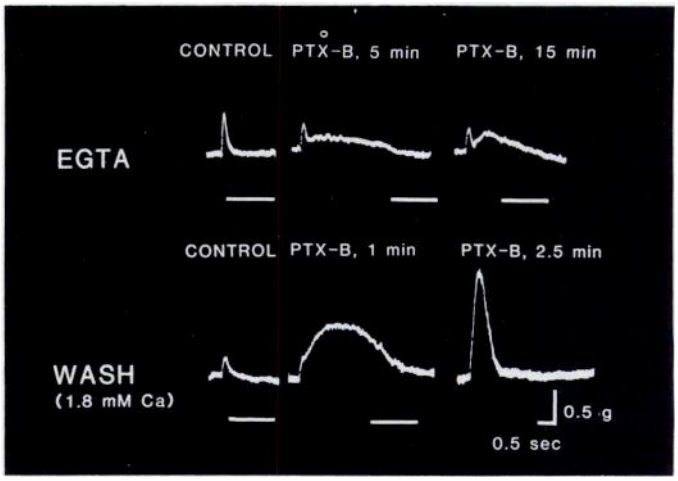


FIG. 9. *Influence of external calcium on the effects of PTX-B in bundles of fibers isolated from the frog semitendinosus muscle*

After isolation of a bundle of fibers, control recordings of muscle tension were made and the muscle then bathed in a physiological solution containing 5 mM EGTA, no $CaCl_2$, and 3.6 mM $MgCl_2$ in addition to other salts. After 30 min the EGTA control record was made and 1.5 μM PTX-B was added to the solution. Note the prolongation of the contraction without potentiation. Prolongation was maximal at about 5 min, the second peak reaching its maximal amplitude at about 15 min. Washing for 1 hr in a normal physiological solution (*WASH*, 1.8 mM $Ca^{2+}$) restored the waveform to nearly normal except that the amplitude here was reduced. Subsequent exposure to PTX-B (1.5 μM) resulted in both potentiation and prolongation of the contraction. Note the difference in time calibration of the recording for PTX-B at 2.5 min.

TABLE 3

*The effect of external calcium concentration on the action of PTX-B on directly and indirectly elicited twitches of the sartorius muscle of frog*

Values presented are means ± standard deviation of at least three muscles. The concentration of PTX-B was 1.5 μM. The muscles were exposed to each change in calcium concentration for 30–60 min before PTX-B was added; where no value appears, the response was absent.

| External calcium concentration | Potentiation | | | | | | Depression | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Onset time | | Peak time | | Peak amplitude | | Maximal block | | Time to maximal block | |
| | Indirect | Direct | Indirect | Direct | Indirect | Direct | Indirect | Direct | Indirect | Direct |
| *mM* | *min* | | *min* | | *% of control* | | *% inhibition* | | *min* | |
| None | — | — | — | 1.0 ±0.2 | — | 103.0 ±3.5 | — | 98.0 ±1.5 | — | 60.0 ±3.0 |
| 0.018 | — | Immediate | — | 0.5 ±0.2 | — | 140.0 ±5.0 | — | 85.0 ±1.8 | — | 60.0 ±4.0 |
| 0.45 | 0.05 ±0.02 | 0.06 ±0.01 | 1.0 ±0.2 | 3.0 ±0.5 | 176.7 ±7.6 | 171.0 ±6.5 | 98.0 ±5.0 | 1.0 ±1.0 | 10.0 ±1.0 | 60.0 ±4.0 |
| 1.8 | 0.18 ±0.07 | 0.20 ±0.07 | 2.10 ±0.65 | 2.8 ±0.5 | 150.5 ±4.2 | 153.7 ±6.3 | 71.7 ±7.6 | 3.0 ±2.0 | 14.0 ±1.7 | 60.0 ±5.0 |
| 9.0 | 5.7 ±2.0 | 5.8 ±2.0 | 25.0 ±5.0 | 25.0 ±3.0 | 25.3 ±3.0 | 124.3 ±4.0 | 7.0 ±2.0 | 7.0 ±2.0 | 60.0 ±3.0 | 60.0 ±2.0 |

preventing block of both the indirect and directly elicited twitches.

When the $[Ca^{2+}]_o$ was reduced to less than $10^{-9}$ M (13) by prior exposure to 5 mM EGTA, the directly elicited twitch recorded from isolated bundles of semitendinosus fibers was prolonged by PTX-B (1.5 μM) but not potentiated (Fig. 9). These results were similar to those in whole muscle. For example, pretreatment with an EGTA (5 mM)-containing physiological solution reduced the amplitude of the twitch by nearly 80% within 30–45 min while the relaxation phase was slightly prolonged. The subsequent addition of PTX-B (1.5 μM) in EGTA solution for 30 min resulted in a further reversible decrease in amplitude while the peak increase in contraction time was 40%, half-relaxation time increased from 27 msec to between 45 and 55 msec, and 90% relaxation time from 88 msec to between 600 and 1450 msec (Fig. 9). Washing for 60 min in normal physiological solution restored contraction, half-relaxation, and 90% relaxation times nearly to normal. However, if the preparation was immediately washed in a normal physiological solution (i.e., 1.8 mM $Ca^{2+}$) containing PTX-B, the contraction time remained at about 45 msec, half-relaxation time at 35–40 msec (150% of EGTA control), and 90% relaxation time at 90–100 msec (110% of EGTA control) while the amplitude returned to 97% of the EGTA control within 30 min. Subsequent washing in normal physiological solution (1.8 mM $Ca^{2+}$) restored all parameters to those of the EGTA control, but the amplitude decreased to 50%. Subsequent exposure of any preparation so treated to PTX-B (1.5 μM) in normal physiological solution resulted in responses nearly identical with those shown in Table 1 (Fig. 9).

In two additional sets of experiments, dantrolene sodium, a compound which has been proposed to reduce twitch in mammalian skeletal muscle by inhibiting calcium release from mammalian SR (14, 15) and methoxyverapamil, a compound proposed to block calcium channels (16, 17), were examined for their ability to block the action of PTX-B. Thus, small bundles of fibers were first incubated in α-bungarotoxin for 30 min, then in a solution containing either dantrolene sodium (8.3 μM) or methoxyverapamil (50 μM) for an additional 30–60 min and then exposed simultaneously to PTX-B (1.5 μM). Although dantrolene depressed the directly elicited twitch by nearly 80% after 60 min, it did not block the effect of PTX-B on the amplitude or time course of the twitch. Thus, in the presence of dantrolene plus PTX-B (1.5 μM), the directly elicited twitch reached a mean value nearly 200% of control and was prolonged to an extent similar to that seen in control preparations. Methoxyverapamil (50 μM) decreased the twitch amplitude by only 20% after 30 min without altering the time course of the contraction. Upon the addition of both PTX-B (1.5 μM) and methoxyverapamil to the solution bathing the bundle of muscle fibers, the amplitude increased to 226% of control and the time course was prolonged to between 60% and 80% of the peak values for this concentration of PTX-B. Washing with normal physiological solution restored the time course of the contraction to drug-free control values, although the twitch was only 50% of control. Subsequent exposure to PTX-B (1.5 μM) alone resulted in a nearly normal response in which values equivalent to those shown in Table 1 were attained within 30 min.

*Effect of PTX-B on caffeine-induced contractures.* The extent to which muscle contractures resulting from caffeine-elicited release of calcium might be altered by PTX-B was examined. Sartorius muscles were exposed to caffeine (20 mM) in the presence and absence of a blocking concentration of tetrodotoxin (1 μM), both before and during exposure to PTX-B. The contracture with caffeine consists of two components: a small, rapid contracture (first phase) and then a larger, sustained contracture (second phase) (Fig. 10A). The presence of tetrodotoxin had no effect on the initial (first phase) or maximal tension (second phase) developed by exposure to caffeine (20 mM), the latter being 23.5 ± 3.5 g ($n = 4$) in the absence of tetrodotoxin (1 μM) and 22.0 ± 3.2 g ($n = 4$) in its presence. However, with prior exposure to PTX-B the maximal tension developed during exposure to caffeine (29.2 ± 4.1 g; $n = 5$) was significantly greater ($p < 0.001$) than control (Fig. 10B).

*Effect of PTX-B on some electrical properties of the muscle.* The effect of PTX-B (1.5 μM) on the membrane potential, input resistance, and specific membrane resistance was also determined. Neither junctional nor extra-

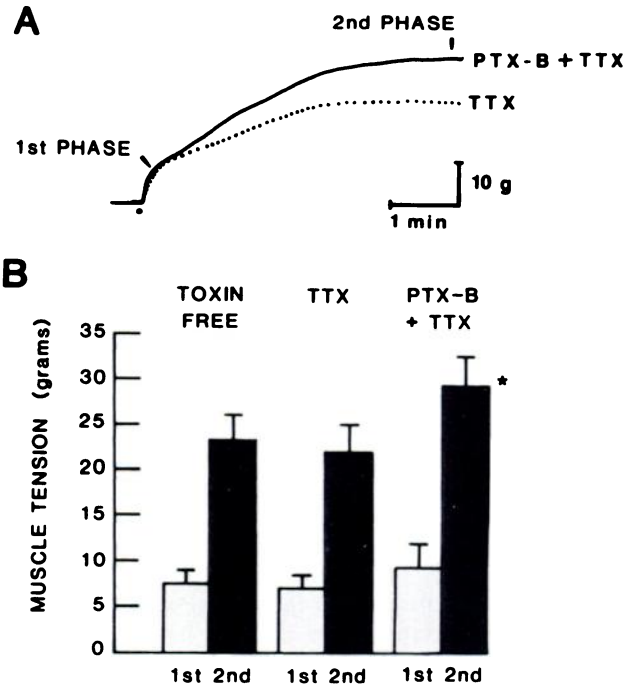


FIG. 10. *Potentiation of caffeine-induced contractures of whole sartorius muscles of the frog by PTX-B*

A. Representative tracings of individual contractures upon the addition of 20 mM caffeine (●) to the bath containing the muscle in the presence of 0.3 μM tetrodotoxin (*TTX*) or 1.5 μM PTX-B and TTX. The first and second phases of caffeine contracture are indicated.

B. Mean (± standard error of the mean) responses of three to five muscles to 20 mM caffeine in the absence and presence of TTX and 30 min after the addition of PTX-B (1.5 μM) and TTX. The *asterisk* indicates the significant difference in the second phase of caffeine contracture after exposure to PTX-B ($p < 0.001$). Note that TTX had no effect on either phase of caffeine contracture and that PTX-B potentiated only the second phase of caffeine-induced contracture.

junctional recording of the membrane potentials (−92.0 ± 0.5 mV; $n$ = 54 fibers/8 muscles) from surface fibers of the frog sartorius muscle were affected by the presence of PTX-B (1.5 μM) (−94.1 ± 1.0 mV; 51/8). Several other possibilities which might explain the changes in twitch characteristics were also examined. The input resistance and specific membrane resistance of a unit area from control fibers was 0.71 ± 0.14 MΩ ($n$ = 15 fibers/3 muscles) and 6872 ± 110 Ωcm$^2$ (15/3), respectively; in the presence of PTX-B (1.5 μM) these values were 0.73 ± 0.14 MΩ (15/3) and 8185 ± 443 Ωcm$^2$ (15/3). These values were not significantly different from control values. Similarly, the acetylcholinesterase activity of frog sartorius muscle was unaffected by PTX-B (1.5 μM), indicating that the actions of PTX-B are unrelated to inhibition of the degradation and hence prolongation of the action of ACh.

Another possibility which might explain the potentiation and prolongation of twitches by PTX-B is either a block of potassium conductance, as occurs with histrionicotoxin (18), or a delay in sodium inactivation. But there was no effect of PTX-B on either the waveform or on the threshold, amplitude, overshoot, or rate of rise of the action potential in surface fibers of the sartorius muscle elicited singly or repetitively at frequencies up to 10 Hz (Table 4). The inability to block delayed rectification was confirmed when the rectifying properties of the sartorius muscle were examined in tetrodotoxin-treated muscles in the presence of PTX-B (Fig. 11).

*Effect of PTX-B on the calcium-dependent action potential in crayfish skeletal muscle.* Since the effects of the toxin appeared to be calcium-dependent, the effect of PTX-B was further studied on a partial calcium-dependent action potential in crayfish skeletal muscle. The muscles were initially exposed to 100 mM $SrCl_2$, which partially replaced sodium chloride (see Methods for details). The resting membrane potential of the extensor carpopodite of crayfish after exposure to isotonic $SrCl_2$ for 60 min was −81.4 ± 1.1 mV (Table 5). Even when the muscle was exposed to PTX-B at concentrations up to 60 μM for 120 min, no alteration of resting membrane potential was observed (Table 5). Likewise there was no alteration of the threshold, overshoot, and the amplitude of the action potential when the muscle was exposed to PTX-B (1.5–60 μM) for 60–120 min (Table 5; Fig. 12). Although the $V_{max}$ and the time to half-amplitude of the spike were also unaffected in the presence of 1.5 to 3.0 μM PTX-B, at 15 and 30 μM PTX-B, both $V_{max}$ and time to half-amplitude of the spike were significantly increased from control ($p < 0.001$). $V_{max}$ was increased by 25% and 56% in 15 and 30 μM PTX-B, respectively, while the time to half-amplitude was reduced by 52% and 47%, respectively. There was no further increase in $V_{max}$ with the highest concentration used (60 μM); instead, $V_{max}$ decreased to 85% of control within 60 min. At these higher concentrations of toxin the muscles contracted vigorously upon stimulation, causing severe damage to muscle fibers—in some cases completely severing the bundle of

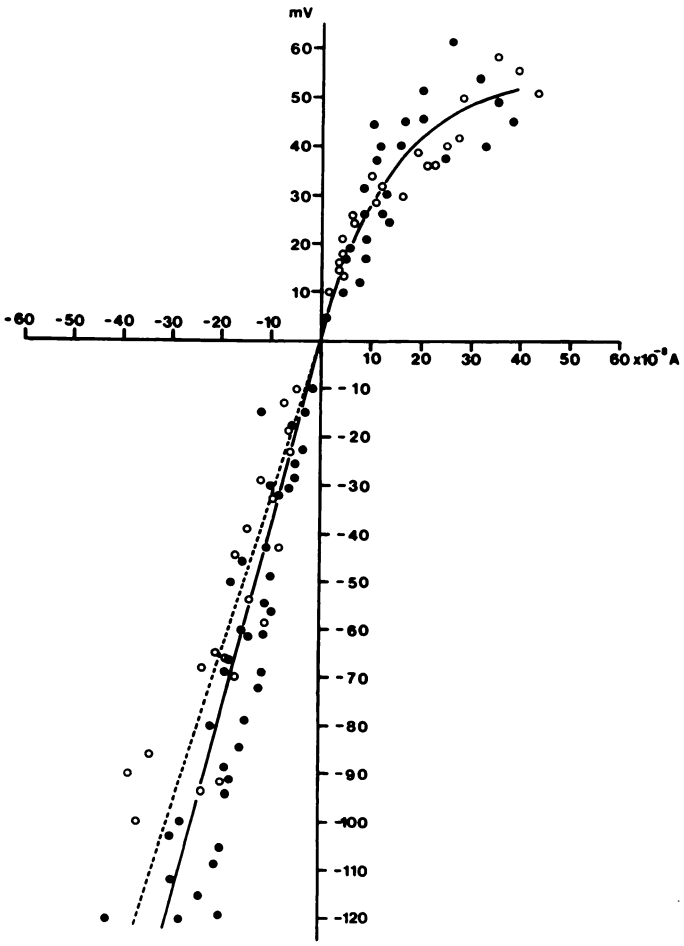


FIG. 11. *Effect of PTX-B on the voltage/current relationship in surface fibers of the frog sartorius muscle*

Recordings were obtained between 30 and 60 min after exposure to the toxin. Delayed rectification was unaffected by the toxin. ○, Control; ●, PTX-B, 1.5 mM. Each point is an individual recording from a cell at the particular current applied. Data were pooled from three muscles and represents four to six fibers/muscle for control and toxin recordings.

TABLE 4

*Effect of PTX-B on the directly elicited action potentials in surface fibers of frog sartorius muscle*

Each value represents the mean ± standard error obtained from 22 fibers of three muscles.

| Condition | Time | Threshold | Overshoot | Amplitude | $V_{max}$ |
|---|---|---|---|---|---|
| | *min* | *mV* | *mV* | *mV* | *V/sec* |
| Control | | 51.0 ± 1.8 | 40.8 ± 1.7 | 78.2 ± 1.4 | 518 ± 20 |
| PTX-B (1.5 μM) | 10 | 49.3 ± 1.0 | 41.2 ± 2.4 | 83.8 ± 2.1 | 519 ± 20 |
| | 30 | 51.0 ± 1.1 | 37.2 ± 2.9 | 78.5 ± 3.0 | 455 ± 29 |
| | 60 | 52.3 ± 0.7 | 37.2 ± 2.3 | 80.1 ± 2.5 | 512 ± 35 |
| Wash | 60 | 49.0 ± 0.8 | 36.0 ± 1.8 | 82.0 ± 1.6 | 446 ± 11 |

TABLE 5

*Effect of PTX-B on the resting membrane potential and on the threshold, overshoot, amplitude, rate of rise, and time course of the action potential in the extensor carpopodite of the crayfish (Procambarus clarkii)*

All values are expressed as means ± standard error of the mean for at least 20 fibers in 5 muscles after exposure to toxin for 60–120 min.

| Treatment | Concentration | Membrane Potential | Threshold | Overshoot | Amplitude | $V_{max}$ | Time to half-amplitude |
|---|---|---|---|---|---|---|---|
| | *μM* | *mV* | *mV* | *mV* | *mV* | *V/sec* | *msec* |
| Control | | −81.4 ± 1.1 | 68.1 ± 1.7 | 40.6 ± 2.3 | 64.2 ± 1.2 | 87.1 ± 5.2 | 96.1 ± 18.1 |
| PTX-B | 1.5 | −80.3 ± 3.5 | 65.1 ± 3.5 | 43.2 ± 1.4 | 70.1 ± 3.0 | 91.2 ± 4.2 | 88.0 ± 7.5 |
| | 3.0 | −81.0 ± 2.2 | 68.1 ± 1.8 | 40.6 ± 1.3 | 65.4 ± 2.1 | 82.0 ± 5.2 | 82.0 ± 6.0 |
| | 15.0 | −80.4 ± 3.3 | 70.9 ± 3.1 | 39.9 ± 1.1 | 63.7 ± 2.4 | 108.7 ± 1.3 | 49.8 ± 0.9[a] |
| | 30.0 | −79.5 ± 2.0 | 64.4 ± 2.3 | 41.3 ± 2.3 | 72.8 ± 1.6 | 138.8 ± 13.3[a] | 45.6 ± 12.5[a] |
| | 60.0 | −82.0 ± 4.0 | 62.5 ± 3.3 | 30.0 ± 1.6 | 66.4 ± 2.7 | 74.2 ± 6.0 | 38.7 ± 3.4[a] |
| Ethanol | 175.0 | −83.5 ± 5.0 | 65.2 ± 3.4 | 37.0 ± 1.5 | 61.6 ± 2.2 | 90.5 ± 6.8 | 85.0 ± 13.0 |

[a] Difference from control value is significant ($p < 0.001$).

fibers. Experiments carried out with ethanol at the concentration equivalent to the vehicle in 60 μM PTX-B (i.e., 175 μM) showed no effect on calcium-dependent spike in the crayfish skeletal muscle (Table 5).

*Calcium-dependent adenosine triphosphatase activity in the presence of pumiliotoxin-B.* Ca-ATPase plays an important role in maintaining high levels of calcium in storage sites of the SR and is important in restoring low cytosolic levels of calcium after depolarization-evoked release of the divalent cation from the SR (19, 20). In view of the apparent effects of PTX-B on storage and/or release of calcium during directly and indirectly elicited twitches, it was important to assess the affects of PTX-B on Ca-dependent ATPase located in the SR of skeletal muscle. The effect of various concentrations of PTX-B on Ca-ATPase from SR preparations of rat and frog hind-limb muscles was assessed (Fig. 13). Activities of SR ATPase as a function of free $Ca^{2+}$ are shown in Fig. 13. PTX-B inhibited the $Ca^{2+}$-dependent form of the enzyme from both species. At 0.2 μM free $Ca^{2+}$, a concentration approximating those estimated to exist in resting skeletal muscle and other cell types (ref. 22 and refs. therein), PTX-B inhibited the activity from rat and frog muscle by about 25%.

A comparison of the effect of various concentrations of PTX-B on SR Ca-ATPase from rat and frog is shown in Table 6. Although PTX-B inhibited the activity from both preparations, the activity in frog SR appeared more sensitive to the toxin. For example, 60 μM PTX-B inhibited the activity from frog muscle approximately 55% while the activity in SR preparations from rat was inhibited by 35% in the presence of 40 μM PTX-B. One parallel between electrophysiological and biochemical measurements is suggested by the lower activity of PTX-A against Ca-ATPase from rat hind-limb muscles: PTX-

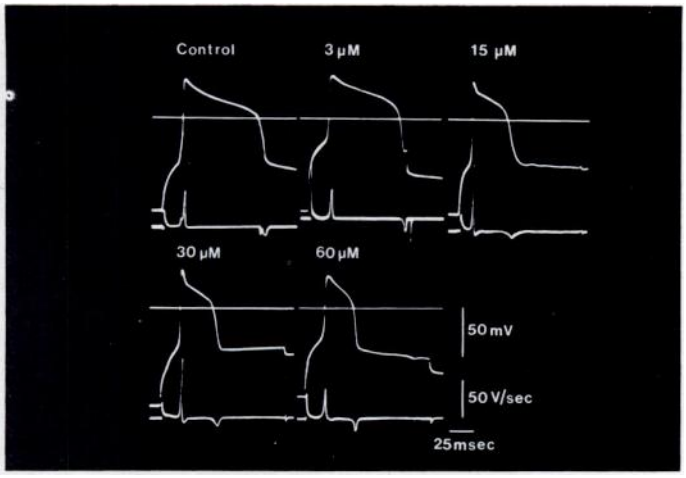


FIG. 12. *Directly elicited action potentials in surface fibers in the extensor muscle of the carpopodite of the crayfish (Procambarus clarkii) in the presence of PTX-B*

Muscles were exposed to toxin at varying concentrations from 3.0 to 60 μM. Records were obtained between 30 and 60 min after exposure to the toxin. The *upper trace* is the action potential and the *lower trace* is its first derivative (*dV/dt*); the *horizontal line* in each tracing is the zero potential. Muscles were first exposed for 30 min to a physiological solution in which $SrCl_2$ (100 mM) replaced the NaCl so that calcium-generated action potentials could be recorded (see Methods). Note the shortening of the action potential and the increased rate of rise at concentrations up to 30 μM. At 60 μM the rate of rise decreased but the action potential remained shortened.

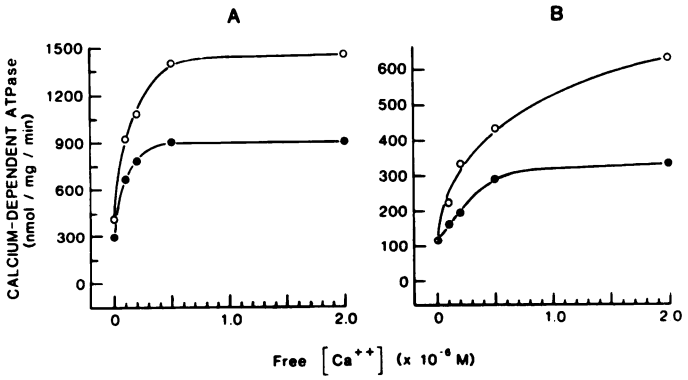


FIG. 13. *Effect of PTX-B on Ca-ATPase in sarcoplasmic reticulum preparations from rat (A) and frog (B) hind limb muscles*

SR vesicles were isolated from hind limb muscles of three adult Wistar rats and six frogs (*Rana pipiens*). Pooled fractions were incubated with 2 mM ATP, 0.1 mM EGTA and various concentrations of calcium as described under Methods. The concentration of free calcium in the presence of ATP and EGTA was calculated according to the method of Katz *et al.* (21). Each point is the mean of three replicate samples. Rat SR was incubated in the presence of 30 μM PTX-B (●) and the frog preparation was exposed to 40 μM PTX-B. ○, Activities noted in the absence of drug.

TABLE 6

*Inhibition of calcium-dependent ATPase activity in sarcoplasmic reticulum preparations from rat and frog by PTX-B*

Incubations were performed at 30° in the presence of 4.4 μM free $Ca^{2+}$. Values are means ± standard error of the mean of three replicate samples.

| Calcium-dependent ATPase | | |
|---|---|---|
| Concentration | Rat | Frog |
| μM | *nmoles/mg protein/min* | |
| 0 | 2600 ± 20 | 1813 ± 27 |
| 15 | 2362 ± 61 | 1562 ± 29[a] |
| 45 | 1900 ± 27[b] | 968 ± 10[b] |
| 60 | 1700 ± 40[b] | 823 ± 12[b] |

[a] $p < 0.01$ (0, PTX-B versus 15 μM PTX-B).
[b] $p < 0.001$ (0, PTX-B versus 45 and 60 μM PTX-B).

A was much less active than PTX-B in this regard and also in its effects on the muscle twitch (data not shown, *vide infra*).

## DISCUSSION

The present study demonstrates that PTX-B, a unique indolizidine alkaloid (Fig. 1) isolated from the Panamanian poison frog *D. pumilio*, potentiates the amplitude and prolongs the falling phase of the muscle twitch in a concentration-dependent manner. These effects were observed with both direct and indirect stimulation of the muscle. The potentiation and prolongation of the muscle twitch by PTX-B was maintained during infrequent stimulation, but at frequencies greater than one per minute the potentiation and prolongation were followed by a concentration-dependent depression of the evoked contraction. PTX-B did not appear to affect either sodium or potassium movements across the plasma membrane, since the amplitude of the action potential, its rates of rise and fall, and the membrane potential were not affected by the toxin. Likewise, neither a blockade of potassium conductance nor a delay in sodium inactivation was involved in the actions of PTX-B, since there was no effect of the toxin on the decay phase of the action potential in muscle or on delayed rectification (Fig. 11). It follows that a sodium- or potassium-dependent action by PTX-B is untenable as a basis for potentiation of the muscle twitch or the induction of repetitive firing of the nerve terminal (2). The potentiation of the twitch can be viewed as a purely postsynaptic effect, since the directly evoked twitch was potentiated and prolonged by PTX-B in the presence of a blocking concentration of α-bungarotoxin (i.e., during complete nicotinic blockade).

The effect of PTX-B on muscle twitch appears dependent in part on $[Ca^{2+}]_o$. When the $[Ca^{2+}]_o$ was decreased, there was a concomitant depression of the potentiation by PTX-B, although the effect remained very rapid in onset (Table 3). However, under such conditions the toxin still prolonged the muscle twitch. When $[Ca^{2+}]_o$ was virtually eliminated by the addition of EGTA to the bathing medium, PTX-B no longer potentiated the twitch but did significantly prolong it (Table 3; see also text). Conversely, an increase in $[Ca^{2+}]_o$ delayed the onset and time to peak of twitch potentiation with PTX-B as well as significantly depressing the magnitude of potentiation and nearly eliminating the subsequent blockade of the twitch by PTX-B. Clearly the postsynaptic effects of PTX-B, particularly the potentiation of twitch amplitude and subsequent blockade of the twitches, are influenced by $[Ca^{2+}]_o$. Dantrolene, which appears to inhibit calcium release from mammalian SR (14, 15), and methoxyverapamil, which blocks calcium channels in other preparations (16, 17), only partially prevented the action of PTX-B. Additional evidence which strongly suggests an interaction between PTX-B and calcium at sites within the SR derives from experiments with EGTA and toxin-treated muscle fibers. Washing in a physiological solution containing 1.8 mM calcium restored all parameters to near-normal, and subsequent application of PTX-B effected its usual response, indicating that sites of calcium attachment and release had been restored. However, if the preparation was first washed with a physiological solution containing 1.8 mM calcium together with PTX-B, the amplitude and waveform of the contraction was only slightly depressed and prolonged, but there were no explosive potentiation and prolongation typical of PTX-B action. Only after removal of this lipophilic toxin by further washing and its subsequent reapplication was the usual effect observed. Thus, PTX-B may have interacted with sites within the SR which normally bind the calcium ions involved in the initial potentiation of the contraction. Once PTX-B was removed from these sites, calcium was able to bind to them again and could subsequently be released by the toxin after stimulation. These effects of PTX-B are unrelated to mechanisms involving the activation or inactivation of either sodium or potassium conductances. Instead, the effects of the toxin appear to be most likely related to mobilization of calcium from both extracellular sites and from storage sites in the SR.

It appears unlikely that PTX-B serves as a calcium-chelating agent. The glycol entity in conjunction with the protonated nitrogen and 8-hydroxy group of the indolizidine ring might confer weak chelating properties. However, PTX-A, which does not have the glycol entity, is only somewhat less active than PTX-B (data not shown); a related alkaloid toxin, 251-D, which retains the indolidizine ring system but not the glycol entity, potentiates the muscle twitch, although to a much weaker extent than PTX-B, and also inhibits Ca-ATPase (data not shown). Structure-activity relationships for PTX-B and other analogues of this dendrobatid alkaloid have only just been initiated. However, it would appear that the presence of hydroxyl groups in the side chain is important for potentiation especially of the twitch. PTX-B, which has a glycol group in the side chain, is the most potent, while PTX-A, with a single hydroxyl group in the side chain, is less active; 251-D, with no hydroxyl group in the side chain, has very low activity (data not shown; see ref. 1 for structures).

The present data are consonant with at least two sites of action of PTX-B in frog skeletal muscle. First, facilitation of release of calcium from the SR by PTX-B during stimulation leading to potentiation of muscle twitch as myoplasmic levels of calcium are elevated. This facilitation might be due to effects on sodium-calcium or

calcium-calcium exchange or might be due to blockade of critical calcium binding sites within the SR; i.e., in the presence of PTX-B, action potentials invading the T-system initially might cause a greater than normal release of calcium from terminal cisternae. Potentiation by PTX-B, however, is also reduced in high $[Ca^{2+}]_o$, but under such conditions calcium perhaps competes with sites where PTX-B interacts. Second, a blockade of reuptake of calcium into the SR by PTX-B could lead to prolongation of twitch. This second site of action was demonstrated through investigation of Ca-ATPase activity where PTX-B proved to be an active inhibitor.

Several lines of evidence further support the hypothesis that PTX-B mobilizes calcium both from extracellular sites and from sites within the SR, particularly at the terminal cisternae and on SR-containing Ca-ATPase. The studies on crayfish skeletal muscle in which $SrCl_2$ has replaced much of the NaCl support an effect on mobilization of calcium from extracellular sites. There appear to be two processes affected by PTX-B in the action potential of the crayfish skeletal muscle: initially the effect on the rate of rise of the potential and subsequently a decrease in duration of the spike. It is quite clear from the increase in rate of rise that the conductance change operates more efficiently in the presence of PTX-B. The question arises as to the ionic species carrying the current during the rising phase of the spikes. Under similar conditions with isotonic $SrCl_2$ (160 mM) or by the addition of procaine to the bathing solution, similar action potentials have been observed which are apparently not due to sodium ions, i.e., not blocked by tetrodotoxin. There are of course several examples of sodium electrogenesis not responsive to tetrodotoxin or saxitoxin (23–25), but procaine did not block electrogenesis in crayfish skeletal muscle and in fact causes the graded electrogenesis to become all-or-none and of the prolonged appearance (26, 27). Additionally, PTX-B has not been found to alter normal sodium electrogenesis in frog skeletal muscle. Thus it seems more likely that divalent cations (e.g., calcium) are responsible for the spike and that the greater rate of rise with PTX-B is the result of an increased calcium conductance. The ability of PTX-B to shorten the duration of the plateau phase of the spike without initially affecting the rate of fall is more difficult to explain. It seems likely that, with an ever-increasing concentration of calcium within the muscle fiber, resulting from repetitive action potential activity, the equilibrium potential for calcium is approached faster with PTX-B and thus effectively terminates *action-potential* generation faster. Alternatively, the shortening of the duration of the action potential may be due to the massive contraction of single fibers of the extensor muscle which occurs as a consequence of the initial rising phase of the action potential. These effects, coupled with the intense contraction of the crayfish skeletal muscle exposed to $SrCl_2$ and PTX-B, support our contention that PTX-B directly affects calcium permeability. The studies on glycerol-treated sartorius muscle provide indirect evidence for effects of PTX-B on calcium release at the terminal cisternae. Thus, PTX-B will reverse the effect of disrupting excitation-contraction coupling in muscle induced by glycerol "shock," an effect ostensibly caused by sealing off of the T-tubule at its interface with the sarcolemmal membrane (4). Since the concentration of PTX-B was very low and the osmolarity of the solution well within normal limits, it seems quite unlikely that the restoration was a simple physical restitution of the continuity between the T-tubule and the sarcolemmal membrane. Although the muscle action potential might follow an alternate path in toxin-treated, glycerol-shocked muscle, avoiding the T-tubule completely and causing contraction more directly, there is no evidence available to suggest that the action potential itself is altered by PTX-B nor that any alternate pathway exists for the passage of an action potential to the terminal cisternae other than via the T-tubule (28). Another possibility must therefore be considered. If the T-tubule is not completely disrupted by treatment with glycerol, then even the smallest change in the conductance of calcium across the T-tubule:terminal cisternae interface would be sufficient to reestablish muscle contraction in toxin-treated muscle. Finally, data obtained with caffeine and tetrodotoxin indicate that PTX-B also enhances release of calcium in the absence of physiological activation, i.e., in the absence of action potentials. As shown in Fig. 10, PTX-B potentiates caffeine-induced contractures in the presence of tetrodotoxin. In this case sodium-calcium interchange would not pertain, and PTX-B must act by either facilitating caffeine-induced release from the terminal cisternae and SR and/or by blocking calcium reuptake. In toto, the results indicate that PTX-B mobilizes calcium from two sites, the extracellular space and the SR. The exact mechanism of twitch induction with PTX-B in glycerol-treated muscle is conjectural at this time and must await more extensive examination of its effect on excitation-contraction coupling.

The effects on the rising phase of the muscle twitch and its amplitude are most likely directly related to the interaction of PTX-B with the release of calcium from the SR, while the prolonged falling phases of both the single twitch and the tetanically evoked contraction are more consonant with effects of PTX-B on Ca-ATPase. The block of this enzyme by PTX-B is probably involved in the gradual block of muscle twitch during repetitive stimulation, since the inhibited enzyme can no longer maintain calcium levels in the SR.

Relatively low concentrations of PTX-B inhibit Ca-ATPase of SR of amphibian and rat skeletal muscle (Table 6), indicating that PTX-B provides a powerful tool in studying the role of Ca-ATPase in muscle function as well as defining the mechanism of calcium transport and release from SR and other calcium-storage sites. With the exception of mersalyl acid (29) and quercetin (30), no potent inhibitors of this enzyme have been described. Quinidine at 20 μM was reported to produce only a slight inhibition of Ca-ATPase from SR (31).

Differences in dose-response relationships of PTX-B on the contractile properties of the muscle and on Ca-ATPase are difficult to explain but may be related to alterations in membrane structure brought about by preparation of SR vesicles used in this study. The possibility that calcium transport and Ca-ATPase were not coupled in the preparation used in this study is suggested by the observation that ATPase activity was linear for at

least 30 min. On the basis of the observed activity of Ca-ATPase (Fig. 13) and the assumption that the maximal capacity of the vesicles is 100 nmoles/mg of protein (32) and that the $Ca^{2+}$/ATP ratio is 2 (33), one would expect the vesicles to be filled within 10 sec. Although the dose-response relationship between the effect of the toxin on Ca-ATPase in isolated SR and the effect of the toxin on electrophysiological properties in intact muscle differed, there is reason to believe that the two are related. First, concentrations required to produce effects on both the enzyme and on muscle properties are in the micromolar range. Second, there are parallels between the action of two derivatives on the enzyme activity and muscle properties, i.e., PTX-B was more potent than PTX-A in both systems.

In conclusion, PTX-B causes a significant increase in both the amplitude and duration (contraction and relaxation time) of the single evoked response which approaches the magnitude of the tetanic response in fast-twitch skeletal muscle. At stimulus frequencies of 10–100 Hz, the toxin potentiates the amplitude of the tetanic response and induces fusion at lower than normal rates of stimulation. In doing so the twitch/tetanus ratio increases from 0.3 to more than 1.1, an effect related to the more than 400% increase in twitch amplitude, and not to a smaller increase in tetanic tension (at 100 Hz). The relaxation phase after tetanus becomes markedly prolonged and complex in its decay. The effects of PTX-B on excitation-contraction coupling have been proposed to be due to displacement and/or release of calcium from extracellular sites and at sites within the SR and to an inhibition of Ca-ATPase of the SR.

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