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MECHANICAL AND ELECTROPHYSIOLOGICAL EFFECTS OF SEA ANEMONE (*Anemonia sulcata*) TOXINS ON RAT INNERVATED AND DENERVATED SKELETAL MUSCLE

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1 Some effects of the sea-anemone toxin ATX-II on mammalian nerve-muscle preparations have been described.

2 When ATX-II (10^{-8}–10^{-6} M) was applied to rat hemidiaphragm preparations, both directly and indirectly generated twitch responses were potentiated and prolonged. At the same time the resting tension of the preparations increased.

3 The increase in resting tension caused by ATX-II in innervated muscles was not prevented by curarization, but was reversed by exposure to tetrodotoxin. The increase in denervated muscles was not completely reversed by tetrodotoxin.

4 At concentrations exceeding 1×10^{-7} M, ATX-II caused a sodium-dependent depolarization of both normal and denervated muscles. The depolarization of the denervated muscles was only partially reversed by tetrodotoxin.

5 In the presence of ATX-II repetitive endplate potentials (e.p.ps) were evoked by single shocks to the motor nerves in many fibres, and in those in which a single e.p.p. was still observed, the quantum content (m) was increased. Miniature e.p.p. frequency was not increased by ATX-II, even when muscle fibres were depolarized by 30 mV.

6 The indirectly and directly elicited action potentials of normal and denervated muscle fibres were much prolonged by ATX-II. The action potentials remained sodium-dependent. The sodium-dependent tetrodotoxin-resistant action potential of the denervated muscle fibre was also prolonged by ATX-II.

7 It is concluded that ATX-II both activates, and delays inactivation of, sodium channels in mammalian skeletal muscle fibres, probably by interacting with the channel 'gate'.

Introduction

Three toxins have been isolated from extracts of the sea anemone, *Anemonia sulcata*. They are ATX-I (formula weight 4808 daltons), ATX-II (formula weight 4770 daltons) and ATX-III (formula weight 2933 daltons). The toxins are stable, basic polypeptides, and all are cross-linked in three places (Beress, Beress & Wunderer, 1975; Wunderer, Fritz, Wachter & Machleidt, 1976; Beress, Wunderer & Wachter, 1977; Wunderer & Eulitz, 1978).

All three toxins cause a spastic paralysis when injected into fishes and crustacea (Beress & Beress, 1971; Möller & Beress, 1975) and this has been shown to be due to the ability of the toxins to delay or inhibit sodium inactivation in neuronal membranes, leading to a prolongation of the action potential (Romey, Abita, Schweitz, Wunderer & Lazdunski, 1976; Bergmann, Dubois, Rojas & Rathmayer, 1976; Rathmayer & Beress, 1976).

Studies on the effects of the toxins at nerve-muscle junctions have produced equivocal results. In crustacea, the toxins cause an increase in the amplitude of evoked junctional potentials, and repetitive activity in the motor axons, but are without effect on the muscle fibre membrane (Rathmayer & Beress, 1976). The effects on amphibian preparations are broadly similar (Metezeau, Bourneau, Mambrini & Tazieff-Depierre, 1979). There appear to be no reports concerning the actions of the toxins on neuromuscular transmission in mammalian species but preliminary observations suggest that ATX-II does have a direct action on the muscle fibre membrane, causing an increase in the directly-elicited twitch response of both innervated and denervated muscle (Tesseraux & Alsen, 1978; 1979) and a depolarization of the muscle membrane (Lemeignan, Molgo & Tazieff-Depierre, 1981). Both Tesseraux &

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Alsen (1979) and Lemeignan *et al.* (1981) further suggest that the effects of ATX-II on denervated mammalian skeletal muscle are partially resistant to the actions of the inhibitor of sodium conductance, tetrodotoxin.

In this paper we describe a more detailed investigation of the effects of the *Anemonia sulcata* toxins on mamalian skeletal muscle. Some of the early results have been communicated to the German Pharmacological Society (Alsen & Tesseraux, 1979), and the International Society on Toxinology (Tesseraux & Alsen, 1978; 1979).

Methods

All experiments were carried out on preparations obtained from Sprague-Dawley or Wistar strain rats of either sex weighing between 150–200 g. The animals were killed by a blow on the head followed by exsanguination and the appropriate muscles removed and processed.

Muscle and nerve-muscle preparations

The preparations used were either the hemidiaphragm with corresponding phrenic nerve, or the soleus (SOL) muscle. Where necessary, denervation of the right hemidiaphragm was carried out by sectioning the appropriate phrenic nerve of young rats (body weight≙100 g) under hexobarbitone anaesthesia (100 mg/kg i.p.) using the technique of Lüllman & Muscholl (1954). Soleus muscles were denervated under ether anaesthesia by sectioning the appropriate branch of the sciatic nerve in the mid-thigh region. The denervated muscles were used between 3 and 20 days after surgery.

The generation and recording of mechanical responses

All mechanical responses were recorded isometrically using a Devices UF1 dynamometer with a 3559 amplifier (H.F. cut 150 Hz) and MX2 pen recorder. For the recording of responses to indirect stimulation the appropriate nerve was stimulated with a pulse width of 0.02–0.05 ms at frequencies ranging between 0.1 and 2 Hz. Direct stimulation of the muscles was with pulses of 5 ms duration delivered at supramaximal intensity and at frequencies ranging between 0.1 and 2 Hz.

Electrophysiological recording methods

The electrical properties of muscle fibres were recorded with intracellular glass microelectrodes filled with 3 M KCL. They had tip potentials of < 5 mV and d.c. resistances of 5–15 MΩ. When it was necessary to cause local changes in membrane potential, or to generate an action potential by direct stimulation, a current-passing electrode filled with 3 M KCL was inserted into the same fibre as the recording electrode, at a distance of 50–100 μm. The muscle fibre membrane was locally hyperpolarized to between −90 and −95 mV before passing superimposed depolarizing currents of 10 ms duration (cf. Redfern & Thesleff, 1971). Indirect action potentials were generated by stimulating the appropriate nerve stump and recording the intracellular action potential. The recording and current generating circuits were homebuilt and have been described by Allan, Gascoigne, Ludlow & Smith (1977).

Miniature endplate potentials were recorded from putative endplate regions by the described intracellular techniques. The noise level of the recording system was approximately 100 μV peak to peak. Endplate potentials were recorded in response to indirect stimulation in the presence of 10 mM magnesium.

Drugs and chemicals

ATX-I and ATX-II were isolated and purified according to the method described by Beress *et al.* (1975). Tetrodotoxin was obtained from Sankyo Co. Ltd, Tokyo. All other drugs and chemicals used were obtained from the normal commercial sources and were routinely of the highest grade available.

Bathing solutions

The isolated tissues were usually maintained in a bathing solution with the composition (mM): K^+ 5.0, Na^+ 150, Ca^{2+} 2.0, Mg^{2+} 1.0 Cl^- 148, $H_2PO_4^-$ 1.0, HCO^-_3 12.0 and D-glucose 11.0.

The bathing fluid was aerated with 95% O_2/5% CO_2 and maintained at a temperature of 27–32°C. When Mg^{2+} was used to prevent neuromuscular transmission, $MgCl_2$ (9 mM) was added to the solution, to give a final Mg^{2+} concentration of 10 mM.

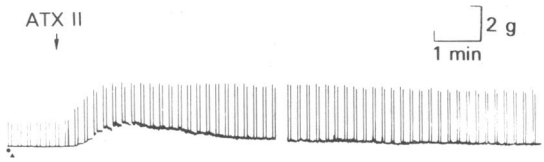

Figure 1 Mechanical responses of a rat innervated hemidiaphragm preparation to direct (●) and indirect (▲) stimulation. At the arrow, sea-anemone toxin ATX-II (2×10^{-7} M) was introduced to the preparation. Note the long-lasting potentiation of the response to both forms of stimulation, and the transient increase in resting tension. The interval between the two panels represents a time of 2 min during which the preparation was continuously stimulated.

No further adjustment was made. The 'choline bathing fluid' was prepared by substituting the NaCl (137 mM) in the original solution with choline chloride. Similarly, a low chloride solution was prepared by replacing NaCl with sodium isethionate.

Statistical analysis

Where two means were to be compared, Student's *t* test was applied. A difference between the means was considered statistically significant if $P < 0.05$.

Results

Investigation on innervated muscles

Effects on mechanical responses ATX-II evoked a dose-dependent increase in the contractile force of the muscle after both direct and indirect stimulation, and a transient increase in the resting tension (Figures 1 and 2). The dose-dependent increase of both parameters was not influenced by changes in stimulus frequency over the range 0.1–2 Hz. The threshold concentration of ATX-II needed to demonstrate the change in twitch tension was approximately 10^{-8} M.

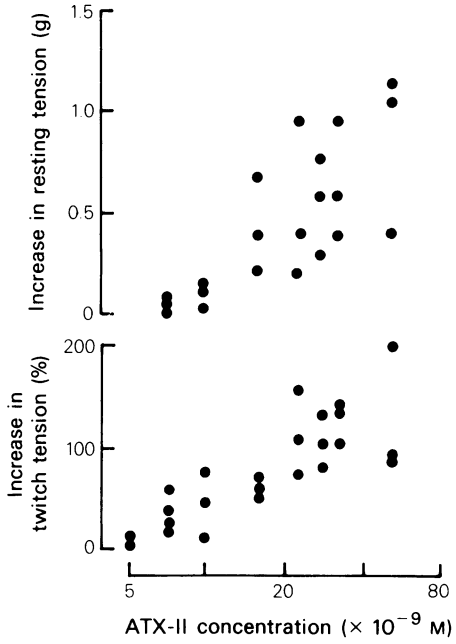

Figure 2 Dose-related effects of sea-anemone toxin, ATX-II, on contractile force and resting tension of a directly stimulated rat innervated hemidiaphragm preparation. Each point represents an observation on a single preparation, and in each case relates to the maximal change observed in the measured parameter.

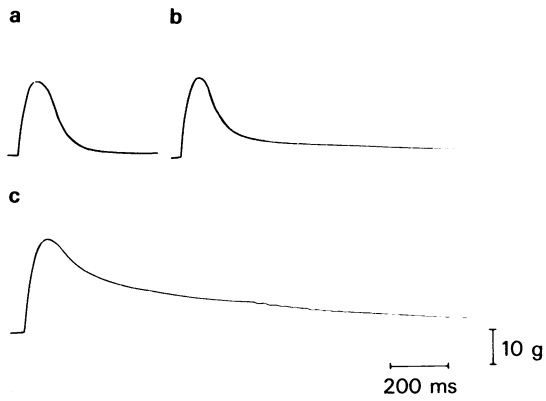

Figure 3 Indirectly elicited twitch responses of a rat innervated hemidiaphragm preparation recorded at various times after exposure to sea-anemone toxin, ATX-II (2×10^{-7} M). (a) Control response; (b) 1 min after ATX-II; (c) 15 min after ATX-II.

At this concentration, the increase in twitch tension was variable. Routinely, however, a prolongation of the twitch was observed. At higher concentrations (between 1 and 2×10^{-7} M) the initial response was an increase in both duration and amplitude of the twitch after 2–3 min followed by a marked slowing of the response, due primarily to a pronounced 'veratrine-like' after contracture (Krayer & Acheson, 1946) at about 15 min (Figure 3). By 35 min exposure to the toxin, the twitch response became irregular in amplitude though still slow, and by one hour the mechanical response had ceased altogether. Once the preparation had ceased to respond to stimulation, prolonged washing did not restore mechanical activity.

When the twitches produced by indirect stimulation were abolished by treatment with (+)-tubocurarine (1.2×10^{-6} M) the effects of ATX-II on the directly stimulated muscles were unchanged. The response to indirect excitation was not restored by the addition of ATX-II to the bathing fluid.

Because of the comparatively small amount of ATX-I available, few experiments could be performed with this toxin. However, the effects of ATX-I on the muscles were qualitatively similar to those of ATX-II, but ATX-I was approximately 200 times less active.

The change in resting tension precipitated by exposure to ATX-II (10^{-8}–10^{-7} M) was transient but could be converted into a long-lasting increase in tension by pretreatment of the muscle with caffeine at a concentration of 4–5 mM.

If the directly-elicited twitch response of the muscle was blocked by the application of tetrodotoxin (TTX, 10^{-7}–10^{-6} M), ATX-II was incapable of restoring the mechanical response of the preparation.

Effects on resting membrane potential ATX-II caused a depolarization of skeletal muscle fibres. The depolarization was small at concentrations below 10^{-7} M but substantial at concentrations in excess of 10^{-7} M. For example, at a concentration of 2×10^{-7} M the resting membrane potential of isolated SOL muscle fibres declined from a normal value of about −73 mV to about −50 mV within 15 min. The depolarization was critically dependent upon the presence of sodium ions. Thus, if choline was used to replace the sodium ions, or if the preparation was bathed in the presence of TTX (10^{-6} M), the toxin did not elicit a depolarization at all. Moreover, an ATX-II-induced depolarization could be reversed by the addition of TTX (10^{-6} M) to the bathing medium. The results of a typical experiment illustrating some of these points are presented in Table 1.

Neither the replacement of potassium ions by TEA nor of chloride ions by isethionate prevented the ATX-II-generated depolarization of the muscle fibres.

Table 1 also shows that the absence of sodium ions did not appear to affect the binding of the toxin to the appropriate part of the muscle fibre membrane. Thus in the experiments in which choline was used to replace the sodium normally present in the bathing fluid, ATX-II caused no depolarization; as soon as the bathing fluid was changed from one containing choline plus ATX-II to one containing sodium alone (i.e. without replacing ATX-II) the depolarization occurred within 30 s.

Effects on action potentials Action potentials generated in SOL by stimulating the motor nerve were studied in the presence of dantrolene sodium (2.5×10^{-5} M). This concentration of dantrolene had no effect on the resting membrane potential or the measured parameters of the action potential generated in normal SOL muscle fibres (Table 2). ATX-II, 2.0×10^{-8} M, had no effect on either the resting membrane potential or on the indirect action potential. Increasing the concentration of ATX-II to 10^{-7} M resulted in a small but significant fall in the resting membrane potential, no significant change in the maximum rate of rise or the amplitude of the overshoot of the action potential, and a significant sixfold increase in the duration of the action potential at −50 mV (Table 2). The increase in the duration of the action potential was due to a pronounced delay in the falling phase. Although the mean duration of the action potential at −50 mV was increased from 2 to 12 ms, the variation in duration was considerable and some typical examples of action potentials are illustrated in Figure 4. The mean resting membrane potential at which the 'hump' in the falling phase of the action potential appeared was −26 mV.

Increasing the concentration of ATX-II to 2×10^{-7} M caused such a large depolarization of the muscle fibres that it was not possible to generate useful action potentials by indirect stimulation. At these concentrations, therefore, action potentials were generated by the double micro-electrode technique (see Methods) from a resting potential pre-set to −90 mV. The amplitude of the overshoot of the action potentials was reduced, as was the maximum rate of rise (Table 3). The action potential duration was prolonged, sometimes exceeding 1 s in duration and the most prolonged potentials often exhibited oscillations in the plateau phase of the action potential (Figure 5).

Table 1 Effect of sea-anemone toxin, ATX-II (2.0×10^{-7} M), on the resting membrane potential of isolated normal and denervated soleus muscles

	Resting potential (mV)	
	Normal muscle	*Denervated muscle*
'Normal' bathing fluid	−72.5 ± 0.9 (22)	−59.5 ± 1.2 (13)
'Choline' fluid	−69.8 ± 0.7 (22)	−52.4 ± 1.4 (11)
'Choline' fluid + ATX-II 2.0×10^{-7} M	−70.0 ± 1.0 (18)	−52.7 ± 1.2 (15)
Replace 'choline' + ATX-II with 'normal' fluid	−54.9 ± 1.3 (20)	−30.7 ± 1.4 (18)
Add TTX 10^{-6} M	−72.3 ± 1.4 (13)	—

Note that ATX-II does not cause a depolarization in the absence of Na^+, and that the Na^+-dependent depolarization is reversed by the administration of tetrodotoxin (TTX 10^{-6} M). The results are quoted as mean ± s.e. The figures in parentheses represent number of observations.

Table 2 Effects of sea-anemone toxin, ATX-II (2.0×10^{-8} M–2.0×10^{-7} M), on the resting membrane potential and on indirectly elicited action potentials in rat isolated soleus muscles in the presence of dantrolene sodium (2.5×10^{-5} M)

	Resting potential (mV)	*Action potential*		
		Vmax (Vs^{-1})	*Overshoot (mV)*	*Duration at −50 mV (ms)*
Normal bathing fluid	−73.0 ± 0.7 (10)	219 ± 6 (10)	24.4 ± 0.7 (10)	2.1 ± 0.1 (10)
+ Dantrolene	−72.2 ± 1.1 (24)	213 ± 12 (23)	23.0 ± 1.5 (23)	2.4 ± 0.7 (23)
Dantrolene + ATX-II 2.0×10^{-8} M	−72.3 ± 1.3 (8)	199 ± 8 (8)	19.4 ± 1.6 (8)	2.3 ± 0.3 (8)
Dantrolene + ATX-II 1.0×10^{-7} M	−67.6 ± 0.4 (16)	177 ± 3 (16)	15.8 ± 0.5 (16)	12.7 ± 3.0 (15)
Dantrolene + ATX-II 2.0×10^{-7} M	−49.6 ± 2.1 (12)	—	—	—

The results are quoted as mean ± s.e. The figures in parentheses represent the number of fibres studied.

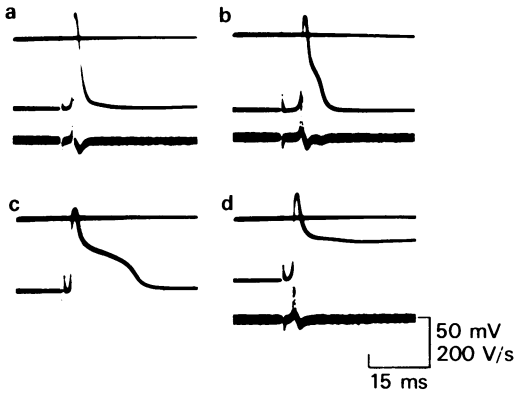

Figure 4 Indirectly elicited action potentials recorded intracellularly from rat muscle fibres before (a) and 15 min after (b–d) exposure to sea-anemone toxin, ATX-II (1×10^{-7} M). In each panel, the upper trace represents zero potential, the middle trace the voltage record, and the lower trace the first differential of the voltage record. Each record was obtained from a different muscle fibre. Dantrolene sodium (2.5×10^{-5} M) was used to inhibit mechanical activity.

The ionic basis of the action potential in ATX-II The action potentials generated in the presence of ATX-II were remarkably similar in general form to those generated in dorsal root ganglion cells (Dichter & Fischbach, 1977). In these cells, the initial rapid Na^+-dependent phase of the action potential is followed by a Ca^{2+}-dependent phase that gives rise to a plateau with a duration of about 10 ms. It is also well known that the plateau phase of the cardiac action potential is Ca^{2+}-dependent. It was considered possible, therefore, that the plateau on the ATX-II action potential in soleus muscle fibres might be due to the appearance of an inward Ca^{2+} current. Indirect evidence suggests that this was not the case. Thus, it was not possible to separate the fast phase from the plateau phase of the action potential by the use of TTX, and the development of plateau phase was unaffected by the presence of Co^{2+} 10 mM (Figure 5). It seems probable, therefore, that it is a 'simple' Na^+-dependent action potential.

Effects on junctional potentials At a concentration of $1–2 \times 10^{-7}$ M, the toxin had no effect on miniature endplate potential frequency (see Table 4), indicating that at this concentration it caused no significant

Table 3 Effect of sea-anemone toxin, ATX-II (2.0×10^{-7} M), on some properties of action potentials elicited from a standard resting potential (−90 mV) using a double microelectrode technique

	Action potential	
	Vmax (Vs^{-1})	*Overshoot (mV)*
Before ATX-II	252 ± 7 (13)	35.7 ± 1.4 (13)
ATX-II 2.0×10^{-7} M	108 ± 8 (3)	9.3 × 2.7 (10)

The results are quoted as mean ± s.e. The figures in parentheses represent the number of fibres studied.

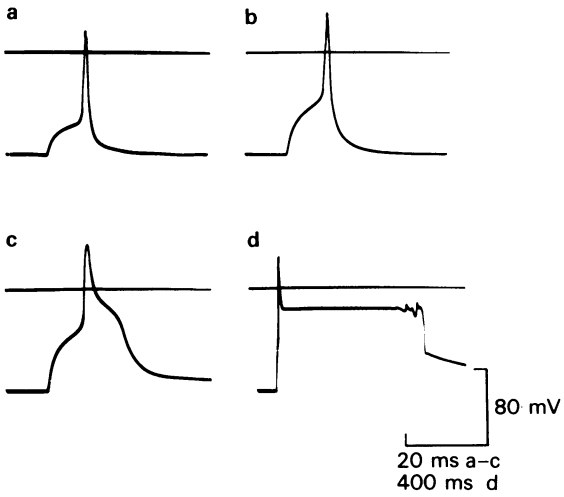

Figure 5 Action potentials generated using the double microelectrode technique (see Methods) in innervated rat soleus muscles. Each action potential was generated during the last 1 ms of a 10 ms pulse from a membrane potential preset to −90 mV. (a) Represents a 'normal' action potential; (b) an action potential generated 15 min after exposure to Co^{2+} (10 mM) and (c) and (d) action potentials generated 20 min after exposure to sea-anemone toxin, ATX-II (2×10^{-7} M), in the presence of Co^{2+}. In each panel, upper trace represents zero potential, lower trace the voltage record.

depolarization of the nerve terminal. Neither the rise-times nor the time to 50% decay of evoked endplate potentials were changed (Table 4), but endplate potential amplitude was increased in the presence of ATX-II, 1×10^{-7} M. This was shown to be due to an increase in the quantum content of the endplate potential (Table 4). However, these data relate only to observations made on fibres that responded to a single stimulus by generating a typical endplate potential. In the majority of cases (13 out of 20), the endplate potentials were complex and highly varied in profile. They ranged from 'pairs' of endplate potentials separated by a delay of 10–16 ms, to highly complex potentials containing up to 7 profiles (Figure 6).

Investigations on denervated muscles

Effects on mechanical responses In denervated muscles ATX-II evoked a persistent contracture of the muscle. The degree of contracture varied extensively between ATX-II concentrations of 10^{-8} to 10^{-7} M

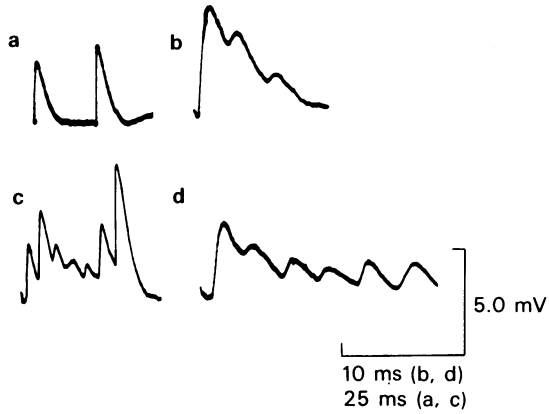

Figure 6 Junctional potentials recorded intracellularly from an indirectly stimulated rat soleus muscle 15 min after exposure to sea-anemone toxin, ATX-II (1×10^{-7} M). The preparation was maintained in the presence of 10 mM Mg^{2+} and was excited by single stimuli of 0.02 ms duration.

Table 4 Effects of sea-anemone toxin ATX-II on miniature endplate potential (m.e.p.p.) frequency and some characteristics of endplate potentials (e.p.ps) in Mg^{2+} (10 mM)-blocked soleus fibres

	Control	*ATX-II* 1.0×10^{-7} M	*ATX-II* 2.0×10^{-7} M
m.e.p.p. frequency (s^{-1})	1.3 ± 0.15 (16)	1.3 ± 1.6 (19)	1.2 ± 0.13* (10)
e.p.p. rise time (ms)	1.3 ± 0.03 (5)	1.2 ± 0.03 (6)	—
e.p.p. time to ½ decay (ms)	1.3 ± 0.09 (5)	1.3 ± 0.13 (6)	—
e.p.p. quantum content	7.4 ± 1.48 (6)	12.3 ± 2.79 (7)	—

The complex endplate potentials commonly seen in the presence of ATX-II (see Results) were not included in the calculations of the various parameters of e.p.ps presented above.

*These data were obtained from a muscle in which mean membrane potential had fallen from −73.2 ± 1.1 mV ($n = 10$) to −46.8 ± 1.1 ($n = 20$) as a result of exposure to ATX-II. They indicate that the depolarization of the muscle fibre is not mirrored by a depolarization of the nerve terminal.

The results are quoted as mean ± s.e. The figures in parentheses represent the number of fibres studied.

but no definite dose-response curve was obtained. The effect persisted even after washing the preparation for 1–2 h.

Pre-treatment of the preparations with (+)-tubocurarine (1.2×10^{-6} M) did not prevent the generation of contractures by ATX-II, thereby excluding the interaction of ATX-II with the nicotinic receptor. Moreover, TTX (10^{-7} to 10^{-6} M) exerted only a partial antagonistic effect against the ATX-II contracture (Figure 7).

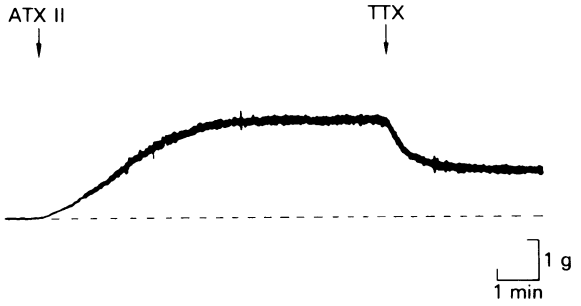

Figure 7 The well maintained contracture generated in a rat denervated hemidiaphragm by exposure to sea-anemone toxin, ATX-II (2×10^{-7} M), was only partially inhibited by tetrodotoxin (TTX, 0.8×10^{-7} M).

The directly elicited twitch of the denervated skeletal muscle responded to ATX-II in a similar manner to innervated muscle, in that the twitch response was potentiated and prolonged. When the twitch response was blocked by exposure to TTX ($10^{-7} - 10^{-6}$ M) the administration of ATX-II caused a partial reversal of the block (Figure 8).

Effects on resting membrane potential The effect of the toxin on the resting membrane potential of denervated muscle fibres was almost identical to that on innervated muscle fibres (Table 1). As with innervated muscle fibres, depolarization was not seen to occur in the presence of choline (Table 1), but the presence of TTX did not altogether prevent the onset of depolarization; neither did the administration of TTX during the action of ATX-II completely reverse the depolarization.

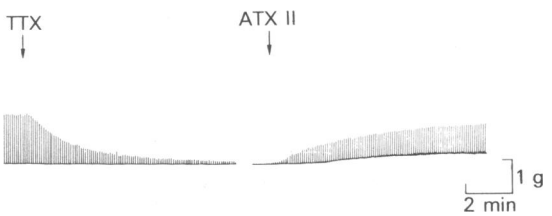

Figure 8 Mechanical responses of a rat denervated hemidiaphragm preparation elicited by direct stimulation (0.1 Hz, 2 ms duration). The responses were abolished by tetrodotoxin (TTX, 0.8×10^{-7} M) but exposure to sea-anemone toxin, ATX-II (2×10^{-7} M), in the continued presence of TTX resulted in the appearance of a contracture, and the partial restoration of the mechanical response.

Effects on action potentials The actions of ATX-II on action potentials generated in denervated skeletal muscle fibres either by direct stimulation utilizing outward-going current or anode-break excitation (see Marshall & Ward, 1974) were similar. Thus in all cases the falling phase of the action potential was much prolonged and some typical examples of action potentials of various types and the effects of ATX-II are shown in Figures 9 and 10.

In view of the observation that ATX-II could restore, to some degree, the excitability of denervated muscles poisoned by TTX (see above), the effects of ATX-II on action potential generation in the presence of TTX were studied in some detail in denervated muscles. Denervated muscle fibres generate TTX-resistant action potentials provided the resting membrane potential of the fibre is pre-set to a local level of −80 mV or more (Redfern & Thesleff, 1971) by the passage of inward current; TTX-resistant action potentials cannot usually be generated at the resting membrane potential of a denervated fibre (typically −60 to −65 mV; see Table 1).

In skeletal muscle fibres, the maximum rate of rise of the action potential is very much dependent upon muscle fibre membrane potential. The relationship between membrane potential and maximum rate of rise for denervated muscle fibres is shown in Figure 11. Figure 11 also shows the relationship for the TTX-resistant action potential and the TTX-resistant action potential in the presence of ATX-II.

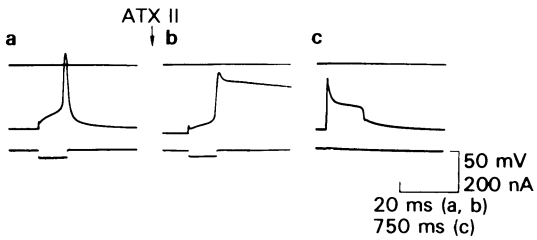

Figure 9 Action potentials generated using a double microelectrode technique (see Methods) in rat denervated soleus muscle fibres. Each action potential was generated during the last 1 ms of a 10 ms pulse, from a membrane potential preset to −90 mV. (a) Represents a 'normal' action potential, and (b) and (c) action potentials generated 15 min after exposure to sea-anemone toxin, ATX-II (1×10^{-7} M). The upper trace represents zero potential, the middle trace the voltage record and the lower trace the current passed. Dantrolene sodium (2.5×10^{-5} M) was used to inhibit mechanical activity.

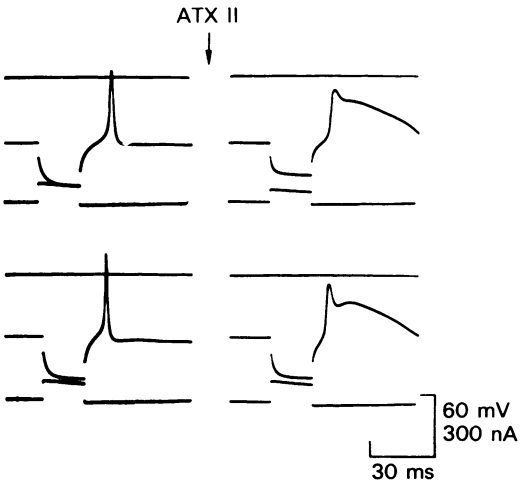

Figure 10 Action potentials generated using anode-break excitation in rat denervated soleus muscle fibres. The records on the left represent 'normal' action potentials; those on the right were generated 15 min after exposure to sea-anemone toxin, ATX-II (1×10^{-7} M). The upper trace represents zero potential, the middle trace the voltage record and the lower trace the current passed. Dantrolene sodium (2.5×10^{-5} M) was used to inhibit mechanical activity.

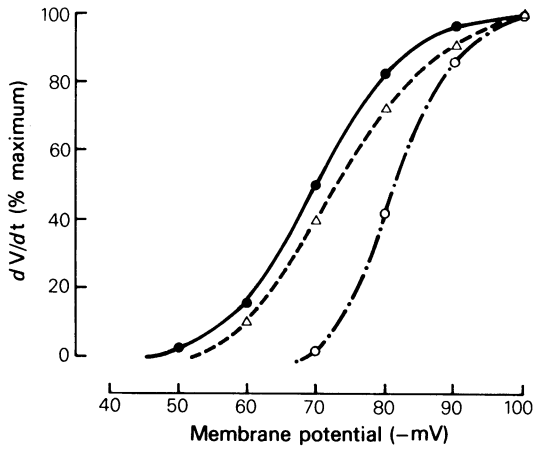

Figure 11 The relationship between the maximum rate of rise (dV/dt) of the action potential and resting membrane potential in rat denervated soleus muscle fibres. Each curve was obtained following experiments on at least 10 fibres. The 'normal' curve (●) was generated first, followed by the curve obtained in the presence of tetrodotoxin, 10^{-6} M (○) which was followed by that generated in the presence of both tetrodotoxin and ATX-II, 1×10^{-7} M (△). All action potentials were generated using the double microelectrode technique. The value of dV/dt obtained at a membrane potential of −100 mV was used as the reference point.

If it is accepted that maximum rate of rise is a function of sodium current (see Hansen-Bay & Stricharz, 1980) then these curves represent a form of 'activation' curve. The membrane potential at which '50% activation' is achieved is −70 mV in denervated muscle fibres, −80 mV in TTX-poisoned muscle fibres and −73 mV in TTX-poisoned fibres in the presence of ATX-II 1×10^{-7} M.

The falling phase of the TTX-resistant action potentials of the denervated muscles was also prolonged by exposure to ATX-II, but the degree of prolongation was not measured.

Discussion

The experiments described in this paper indicate quite clearly that the *Anemonia sulcata* toxins ATX-I and ATX-II have a direct effect on mammalian skeletal muscle fibre membranes.

In the first series of experiments, on innervated muscles, it was shown that the toxins caused an increase in the amplitude and duration of the indirectly and directly elicited twitch response. This phase of activity was often associated with a dose-dependent transient increase in the resting tension of the muscle. Neither the change in twitch response nor the change in resting tension were prevented by pretreatment of the nerve-muscle preparations with (+)-tubocurarine, thus excluding the possibility that the effects were mediated either directly or indirectly by nicotinic cholinoceptors. However, the change in resting tension was prevented by tetrodotoxin. These results suggest that the actions of the toxins ATX-I and ATX-II are on the muscle fibre directly, and that at least some of the effects involve the movement of sodium ions across the muscle fibre membrane.

The effects of ATX-II on the mechanical responses of the denervated muscles were similar to those seen in innervated muscles. That is, a potentiation and prolongation of the muscle twitch, and an increase in the resting tension. In the case of the latter response, however, the tension developed in denervated muscle was well maintained, whereas that in the innervated muscle was transient.

ATX-II also provoked a depolarization of both the innervated and denervated muscle membrane. The depolarization was prevented by the removal of sodium ions from the bathing fluid but not by the removal of either potassium or chloride ions. Thus it seems highly probable that the cause of the depolarization was a selective increase in permeability to sodium ions. This suggestion is further supported by the observation that in innervated muscles, the depolarization was prevented by prior exposure of the muscle to TTX, and could be reversed by subsequent

exposure to TTX. In denervated muscles, exposure to TTX did not wholly prevent the depolarizing caused by ATX-II, presumably because of the presence of TTX-resistant sodium channels in denervated mammalian skeletal muscle fibres (Redfern & Thesleff, 1971).

Since the toxin-induced increase in resting tension in the innervated muscle was also blocked by tetrodotoxin, it seems reasonable to draw the inference that the depolarization gave rise to the contracture. The transient nature of the contractures in the innervated muscle is consistent with many earlier observations; so too is the observation that in the presence of caffeine the contractures are potentiated in both amplitude and duration (Lüllman, Preuner & Sunano, 1974). Lüllman *et al.* (1974) suggest that this is because it is the rate of change in resting potential rather than the absolute level of the depolarization that acts as the stimulus for shortening. The contraction occurs because the depolarization initiates the liberation of Ca^{2+}, probably from an internal store, the muscle relaxing as the released Ca^{2+} is re-accumulated by the sarcoplasmic reticulum. In the presence of caffeine, the re-accumulation of Ca^{2+} by the sarcoplasmic reticulum is blocked (Weber & Herz, 1968) and so the tension is maintained.

The contracture in the denervated muscle was only partially prevented by the application of TTX, probably because of the presence of TTX-resistant sodium channels in denervated muscles. However, the observation that the contracture in the denervated muscle is maintained is more difficult to explain. There is no evidence that the sarcoplasmic reticulum of denervated muscle is unable to accumulate Ca^{2+}; in fact, accumulation may be more avid than in normal muscle (Howell, Fairhurst & Jenden, 1966). In view of the basic nature of ATX-II and its relatively large size, it is difficult to believe that it has a direct effect on an internal membrane system. It seems possible, however, that in some circumstances the toxin is capable of increasing Ca^{2+} fluxes across the membrane, in addition to causing a depolarization, and this may act to keep the internal level of Ca^{2+} above that required for mechanical activation.

Although voltage-clamping would be necessary to determine the precise mode of action of ATX-II, it seems probable that the action potential remains specifically Na^+-dependent (see Results, p. 9). The prolongation of the falling phase of the action potential is, therefore, most likely due to a delay in sodium inactivation. This is a well-defined feature of the action of the toxin on action potential generation in neuronal membranes (see Introduction). Preliminary observations (Harris, unpublished) suggest that the current/voltage relationship of the muscle fibre membrane in the presence of TTX is unchanged by exposure to ATX-II, implying that the delayed increase in potassium conductance and its inactivation is unaltered.

The prolongation of the action potential was seen in directly and indirectly stimulated normal muscle fibres and the effect on the action potential was probably the cause of the potentiation of both amplitude and duration of the muscle twitch, since it would result in an increase in the duration of the active state of the muscle (Sandow, 1965).

The prolongation of action potentials seen in neuronal membranes (see Introduction) was probably responsible for the increase in the quantal content of endplate potentials. However, the multiple endplate potentials commonly seen (see Figure 7) imply that repetitive firing of the axonal membrane occurs in the presence of ATX-II. This repetitive firing of the neuronal membrane is almost certainly the cause of the veratrine-like after-contracture seen in the muscle. It should be noted that very similar responses are seen at excitatory nerve/muscle junctions in 'opener' muscles of the 1st and 2nd walking legs of the crayfish, *Astacus leptodactylus* (Rathmayer & Beress, 1976).

It is commonly suggested that the toxins of *Anemonia sulcata* are representative of a class of compounds that have little capacity to activate voltage-sensitive sodium channels but are capable of enhancing the activity of those compounds that do exhibit such an ability (see Catterall, 1980, for example). This view is based on the observation that the toxins of *Anemonia sulcata* neither depolarize intact axons nor increase sodium conductance in neuroblastoma cells (Rathmayer & Beress, 1976; Catterall & Beress, 1978). The results presented in Table 4 also suggest that mammalian motor nerve terminals are not depolarized by ATX-II. However, ATX-II is able to provoke a persistent and large depolarization of SOL skeletal muscle fibres, specifically by increasing sodium conductance, which suggests that the toxin is able to activate sodium channels in mammalian skeletal muscle.

It was a consistent finding that the maximum rate of rise and the amplitude of the overshoot of the action potential were reduced in the presence of ATX-II. This was a rather surprising finding in view of the evidence that the toxin enforced sodium channel opening and at the same time delayed channel closing. It may be that ATX-II also reduces the total sodium current flowing during the initiation of the action potential. Only further examination of the pharmacology of ATX-II using voltage-clamp techniques will allow the derivation of definitive data on these points.

In the absence of voltage-clamp data, it is also difficult to discuss the effects of ATX-II on the TTX-resistant action potential of denervated skeletal

muscle. The curves relating dV/dt to resting membrane potential (Figure 11) suggest that under the influence of ATX-II, the voltage dependence of the TTX-resistant sodium channel is changed so that 'activation' is achieved at a less negative membrane potential. It seems clear that this phenomenon is responsible for the partial restoration of the mechanical responsiveness of denervated muscle in the presence of TTX. Thus, the TTX-resistant action potential is normally generated only when the membrane potential of the muscle fibre exceeds -80 mV; this can only be achieved experimentally since the resting potential of a typical denervated fibre is only -60 to -65 mV. In the presence of ATX-II, an action potential may be generated at membrane potentials similar to the resting potential (-60 to -65 mV). It should be noted, of course, that the term 'activation' as used in the context of these experiments is rather imprecise. This is because its definition is based upon figures pertaining to the maximum rate of rise (dV/dt) of the action potential rather than to sodium conductance (G_{Na}). In fact, at a first approximation $G_{Na} \propto (dV/dt)^2$ (see Colquohoun, Rang & Ritchie, 1974; Hansen-Bay & Strichartz, 1980).

In conclusion, the experiments outlined in this paper demonstrate that ATX-II is capable of depolarizing skeletal muscle fibre membranes, and of prolonging the duration of the muscle fibre action potential. Both phenomena appear to be mediated via the fast sodium channel. The data cannot be fully analysed without voltage-clamp facilities but they suggest that the toxin activates sodium channels by initiating the opening of the sodium channel 'gate', that it inhibits the closure of the sodium 'gate' and that it changes the voltage dependence of 'gating' in the TTX-resistant sodium channel of denervated muscle fibres.

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