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The effects of *Anemonia sulcata* toxin II on vertebrate skeletal muscle

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1 Some effects of the sea anemone toxin, ATX-II, on vertebrate skeletal muscle have been described.

2 At a concentration of 1×10^{-7}–1×10^{-6}M, ATX-II caused a sodium-dependent depolarizaton of the muscle fibres of the rat soleus and extensor digitorum longus, of the mouse soleus and extensor digitorum longus and of the chicken posterior latissimus dorsi. The muscle fibres of the frog sartorius were insensitive to the toxin.

3 Action potentials generated by direct stimulation were prolonged by ATX-II, but the degree of prolongation was variable. Chicken posterior latissimus dorsi muscle fibres were most sensitive in this regard, and mouse extensor digitorum longus were least sensitive.

4 Both denervated and immature muscle fibres were more sensitive to ATX-II than mature innervated muscle fibres. The sensitivity to ATX-II declined rapidly as muscle fibres matured.

5 In some muscles, the prolongation of the action potential was enhanced by repetitive stimulation, but not by the passive depolarization or hyperpolarization of the muscle fibres.

6 The actions of ATX-II could be reversed by washing in all but the innervated soleus of the mature rat.

Introduction

The toxin, ATX-II, is isolated from crude extracts of the sea anemone *Anemonia sulcata (Anemonia viridis)*. The toxin causes a depolarization and a prolongation of the action potential of the muscle fibres of the rat soleus (SOL) and of the diaphragm of both rat and mouse (Alsen *et al.*, 1981; Chang *et al.*, 1983).

Data concerning the effects of ATX-II on the limb muscles of mice are less consistent. Erxleben & Rathmayer (1984) have reported that ATX-II depolarizes muscle fibres of the mouse slow-twitch SOL and also causes a prolongation of the muscle fibre action potential, whereas the muscle fibres of the mouse fast-twitch extensor digitorum longus (EDL) exhibit the changes in action potential profile, but are not depolarized. By contrast, preliminary results obtained by Harris & Pollard (1982) suggested that action potentials were not prolonged by ATX-II in mouse EDL muscles.

Similar inconsistencies exist in consideration of the effects of ATX-II on frog skeletal muscle. Thus Metezeau *et al.* (1979) reported that ATX-II had no effect on frog skeletal muscle fibres, while Erxleben & Rathmayer (1984) found that although ATX-II caused no depolarization, the action potential was prolonged. These latter data are consistent with preliminary observations reported by Harris & Pollard (1982).

There is general agreement that ATX-II depolarizes denervated muscle fibres and also causes prolongation of the action potentials (Alsen *et al.*, 1981; Ravens & Schöllhorn, 1983; Erxleben & Rathmayer, 1984). Preliminary observations by Harris & Pollard (1982) and Tesseraux & Harris (1983) suggest that denervation may actually increase the sensitivity of skeletal muscle to ATX-II.

Since ATX-II probably prolongs action potential duration by inhibiting the inactivation of the fast Na^+ current, it seems possible that the toxin has a major role to play in the study of the biophysics of excitable membranes. For this reason, many of the inconsistencies documented above require clarification.

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Methods

The experiments were carried out on muscles obtained from female Wistar rats weighing 100–150 g, from Swiss white mice of either sex weighing 25–35 g, from adult specimens of the frog *Rana temporaria* and from young chickens, approximately 6 weeks post hatching, of undetermined strain. The animals were routinely killed by a blow to the head followed by exsanguination, and the appropriate muscles were removed.

The muscles used were the soleus (SOL) and the extensor digitorum longus (EDL) muscles of rats and mice, the sartorius muscle of the frog and the posterior latissimus dorsi muscle (PLD) of the chicken. Some rat muscles were denervated by sectioning the sciatic nerve of one hind limb in the mid-thigh region under ether anaesthesia. The denervated muscles were used between 1 and 7 days after surgery. Immature SOL muscles were created in the rat by making a subcutaneous injection of the snake venom toxin, notexin into the dorsilateral aspect of one hind limb and allowing 4 days or more for the underlying soleus muscle to regenerate (Harris *et al.*, 1975; Harris & Johnson, 1978). The electrical properties of the muscle fibres were recorded with intracellular glass microelectrodes filled with 3 M KCl. They had tip potentials < 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 membrane was locally hyperpolarized to between −90 and −95 mV before passing superimposed depolarizing current of 1–2 ms duration. Action potentials were always generated in the presence of dantrolene sodium (2.5×10^{-5} M). This concentration of dantrolene has no measurable effect on the parameters that define the action potential (Alsen *et al.*, 1981) but allows the action potential to be studied in the absence of any mechanical artefact. The duration of the action potential was measured at −50 mV. The stimulating and recording circuits were home-built and have been described in detail by Allan *et al.* (1977). The noise level of the recording system was approximately 100 μV peak to peak.

ATX-II was a gift from Dr Béress of the University of Kiel; notexin was a gift from Dr D. Eaker of the University of Uppsala; tetrodotoxin was obtained from Sankyo Co., Tokyo. All other drugs and chemicals used were obtained form the usual commercial sources and were routinely of the highest grade available.

The mammalian and avian preparations were maintained in a bathing solution with the following 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 frog muscles were maintained in a bathing solution of the composition (mM): K^+ 2.0, Na^+ 114.4, Ca^{2+} 1.8, Cl^- 117.6, HCO_3^- 2.4 and D-glucose 2.24. The bathing fluids were maintained at room temperature (20–21°C) and aerated with 95% O_2/5% CO_2. Results are presented as the mean ± 1 standard error of the mean (s.e.mean). Where two means are

Table 1 Effect of ATX-II on the resting membrane potential (mV) of several skeletal muscles

Muscle	*Before ATX-II*	*After ATX-II*		
		10^{-7} M	2×10^{-7} M	10^{-6} M
Rat SOL	−73 ± 0.4 (72)	−63 ± 0.7* (60)	−47 ± 1.0* (20)	ND
Rat EDL	−77 ± 0.4 (71)	−72 ± 0.8* (35)	−69 ± 1.6* (15)	−47 ± 4.6* (13)
Mouse SOL	−72 ± 0.4 (76)	−60 ± 0.7* (76)	ND	−49 ± 0.7* (44)
Mouse EDL	−75 ± 0.4 (99)	72 ± 0.6* (48)	ND	−63 ± 1.4* (60)
Chicken PLD	−69 ± 0.8* (26)	−47 ± 0.5* (25)	ND	ND
Frog sartorius	−89 ± 1.3 (60)	−84 ± 1.0* (19)	ND	−86 ± 1.7 (60)

Values expressed as mean ± 1 s.e.mean. Figures in parentheses represent number of fibres. ND = not done.
* = significantly different from data before ATX-II.

compared Student's t test has been applied. A difference between the means was considered statistically significant if P was less than 0.05.

Results

Investigations on innervated muscle

Effects on resting membrane potential ATX-II (1×10^{-7}–1×10^{-6} M) caused a dose-dependent depolarization of all the skeletal muscles used, with the possible exception of frog sartorius (Table 1). There were, however, large variations in the sensitivity of the different muscles. At a concentration of 10^{-7} M for example, the mean muscle fibre membrane potential of PLD fell by 31%, that of mouse and rat SOL by 17 and 14% respectively, and that of mouse and rat EDL by less than 10%. The insensitivity of the rat and mouse EDL muscles was relative rather than absolute, since exposure to ATX-II, 10^{-6} M resulted in a large depolarization in both muscles.

As previously described for rat SOL (Alsen *et al.*, 1981), the depolarization caused by ATX-II was sodium-dependent and could be reversed by exposure to tetrodotoxin (TTX) in all muscles examined. For example, in one experiment on a chicken PLD muscle, the mean membrane potential was reduced from -71 ± 0.9 mv ($n = 12$), to -49 ± 1.0 mv ($n = 13$) following the application of ATX-II (10^{-7} M). Twenty minutes after exposure to TTX (10^{-6} M) and in the continued presence of ATX-II, the membrane potential was restored to -69 ± 1.0 mV ($n = 12$).

Effects on action potentials ATX-II (10^{-7} M) caused a prolongation of the action potential of muscle fibres in most muscles, but the sensitivity of different muscles was highly variable. By far the most sensitive muscle was chicken PLD in which the average duration of the muscle fibre action potential at -50 mV was increased from approximately 2 ms to more than 500 ms, individual action potentials occasionally exceeding 5 s in duration. As previously described (Alsen *et al.*, 1981), rat SOL was also sensitive to the toxin at this concentration, mean action potential duration being increased from 2.5 to 12 ms. The sensitivity of rat and mouse EDL and frog sartorius muscle fibres to ATX-II was relatively less. Mouse EDL appeared to be particularly insensitive to ATX-II. At a concentration of 10^{-7} M, for example, action potentials were prolonged by only 10%, and increasing the concentration of ATX-II from 10^{-7} M to 10^{-6} M resulted in no further change.

The amplitude of the overshoot of the action potentials was reduced in all muscle fibres with the exception of frog sartorius muscle, in which overshoot amplitude was unchanged. The results are illustrated in Figure 1 and summarised in Table 2.

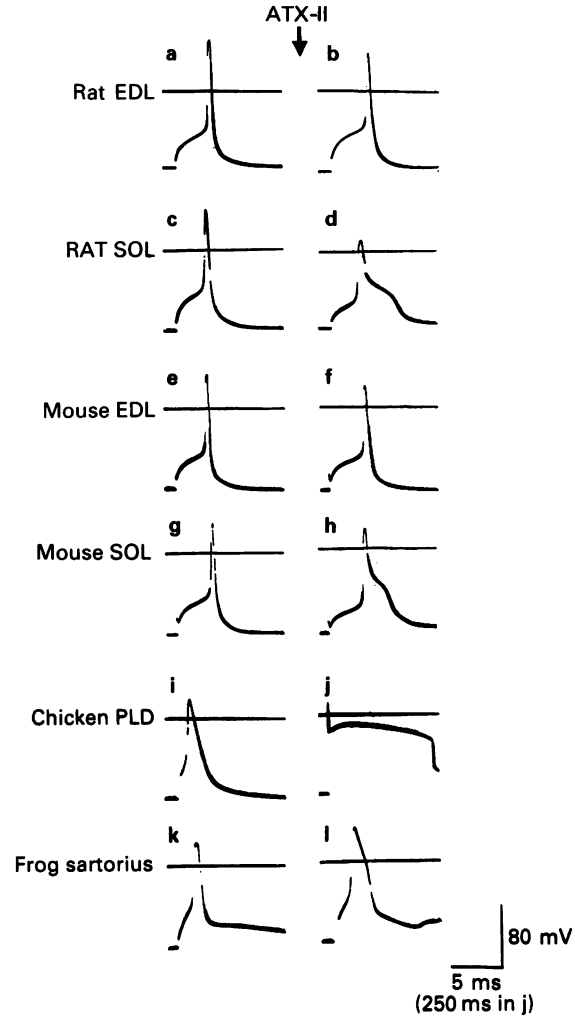

Figure 1 Action potentials generated by direct intracellular stimulation (see Methods) in muscle fibres of a variety of skeletal muscles. Each action potential was generated using a current pulse of 1–2 ms duration. Action potentials to the left were generated before exposure to ATX-II, those to the right after exposure to ATX-II (10^{-7} M). In each panel, the upper trace represents zero potential, the lower trace represents the voltage records. Figure retouched.

In the frog sartorius, even increasing the concentration of ATX-II to 10^{-6} M had no significant effect on the amplitude of the action potential (29.7 ± 1.9 mV, $n = 38$).

In a limited number of experiments, the effect of ATX-II on the maximum rate of rise (dV/dt) of action potentials was assessed. In rat and mouse muscle, dV/dt was suppressed by ATX-II at concentrations of

Table 2 Effect of ATX-II on muscle fibre action potentials in various skeletal muscles

Muscle	*Before ATX-II* Duration (ms)	Overshoot (mV)	*After ATX-II* (10^{-7} M) Duration (ms)	Overshoot (mV)
Rat SOL	2.5 ± 0.06 (59)	26.9 ± 1.03 (55)	11.9 ± 1.04* (59)	16.8 ± 1.04* (59)
Rat EDL	1.9 ± 0.10 (27)	37.3 ± 2.16 (27)	2.7 ± 0.14* (30)	27.8 ± 1.8* (30)
Mouse SOL	1.8 ± 0.03 (64)	30.1 ± 1.52 (64)	3.2 ± 0.29* (74)	21.9 ± 1.3* (74)
Mouse EDL	1.8 ± 0.04 (54)	40.3 ± 1.39 (54)	2.0 ± 0.05* (50)	31.0 ± 1.58* (50)
Chicken PLD	2.1 ± 0.11 (26)	20.0 ± 1.93 (26)	503 ± 261* (24)	12.2 ± 2.11* (24)
Frog sartorius	1.1 ± 0.06 (12)	32.0 ± 1.7 (46)	2.7 ± 0.16* (10)	32.4 ± 2.38 (10)

Values expressed as mean ± 1 s.e.mean. Figures in parentheses represent number of fibres. * = significantly different from data before ATX-II.

10^{-7} M to 10^{-6} M. In the frog sartorius muscle, there was no suppression of dV/dt (Table 3).

Typically, prolonged action potentials generated a more or less well-defined plateau (see Figure 1 and also Alsen *et al.*, 1981). The plateau usually came to an end at a distinct point when the membrane potential returned promptly to normal. Occasionally there were oscillations in the plateau phase which sometimes preceded a relatively slow return to normal in the action potential (Figure 2a,b). In other cases, repetitive firing was initiated by a single stimulus (Figure 2c). This latter feature was seen especially in frog sartorius and mouse SOL muscles, and has been previously reported in mouse muscles by Chang *et al.* (1983) and in frog muscles by Erxleben & Rathmayer (1984).

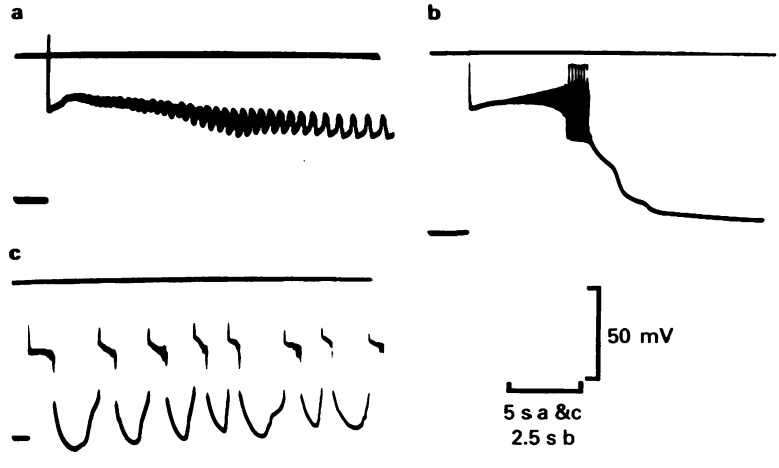

Figure 2 Various forms of repetitive firing induced in muscle fibres following exposure to ATX-II, 10^{-7} M. Records (a) and (b) were obtained from a mouse SOL in which action potentials were generated by direct intracellular stimulation. Record (c) was obtained in an indirectly stimulated mouse SOL. In each panel, the upper trace represents zero potential and the lower trace the voltage record.

Table 3 Effect of ATX-II on maximum rate of rise of action potentials (Vs^{-1})

Muscle	*Before ATX-II*	*After ATX-II*	
		10^{-7} M	10^{-6} M
Rat SOL	284 ± 12 (43)	169 ± 13* (8)	ND
Rat EDL	326 ± 18 (10)	179 ± 6* (8)	ND
Mouse SOL	301 ± 14 (27)	ND	234 ± 14* (28)
Mouse EDL	403 ± 23 (35)	ND	269 ± 12* (53)
Frog sartorius	371 ± 27 (35)	ND	373 ± 24 (39)

Values expressed as mean ± 1 s.e.mean. Figures in parentheses represent number of fibres. ND = not done. * = significantly different from data before ATX-II.

In rat SOL muscles the duration of a second and subsequent action potential generated in response to a train of stimuli was often greater than the duration of the first action potential. It seemed possible that repetitive activity sensitized the muscle fibres to the toxin. This was investigated by generating and recording a single action potential by direct (intracellular) stimulation in the absence or presence of ATX-II, then stimulating that same muscle fibre using intracellular stimulation for 5–10 s at 10 Hz, before recording the last action potential of the series. In the absence of ATX-II, such repetitive activity had no effect on the duration of the action potentials of muscle fibres in rat and mouse SOL and EDL muscles. In the presence of ATX-II (10^{-7} M) the duration of rat SOL muscle fibre action potentials was increased by a further 50% (from 10 ± 1.3 ms, $n = 10$ to 15 ± 1.8 ms, $n = 10$). There was no significant increase in duration in any of the other muscles tested.

In another series of experiments the effects of passive changes of the resting membrane potential were examined. In these experiments the muscles were maintained in the presence of ATX-II (10^{-7} M). A current passing electrode was used to generate a single action potential in a given muscle fibre. The same stimulating electrode was then used to hyperpolarize or depolarize the muscle fibre to − 120 mV or to − 50 mV respectively for 30 s. Thereafter,the membrane potential was reset to − 90 mV for the generation of another action potential. Neither manipulation had any effect on the duration of the action potential in either rat or mouse SOL or EDL.

Spontaneous repetitive activity A few muscle fibres, especially in mouse SOL muscles, generated spontaneous action potentials when exposed to ATX-II. This occurred without depolarization and could take one of three forms. The fibre might generate trains of action potentials in a highly regular fashion, short runs of action potentials preceded and followed by periods of quiescence, or small, irregular runs of action potentials interspersed with oscillations in the membrane potential (Figure 3). In every case, the action potentials were

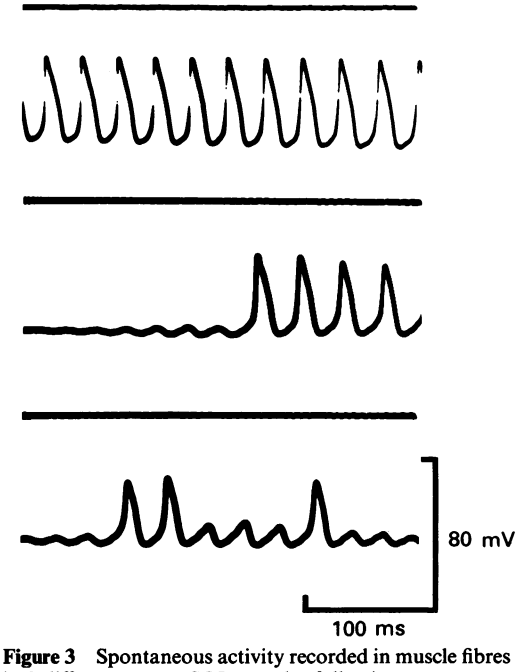

Figure 3 Spontaneous activity recorded in muscle fibres in 3 different mouse SOL muscles following exposure to ATX-II, 10^{-7} M. In each panel, the upper trace represents zero potential and the lower trace the voltage record.

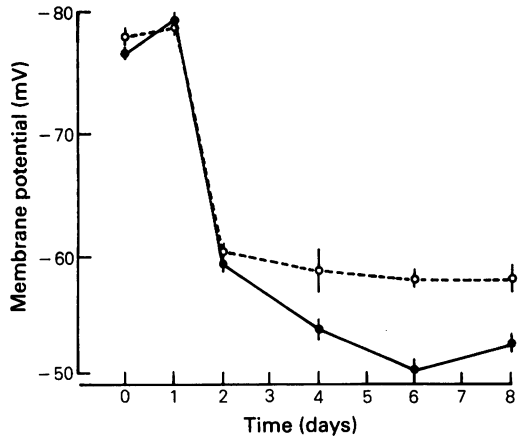

Figure 4 The resting membrane potential of rat SOL muscle fibres at various times after denervation: (○) data obtained on muscles before exposure to ATX-II, 10^{-8} M. Exposure to ATX-II, 10^{-8} M had no effect on resting membrane potential during the first 2 days following denervation (●). From 3 days onwards, the denervated muscle became sensitive and was depolarized by the toxin. Rat innervated SOL muscles are insensitive to ATX-II at concentrations $< 10^{-7}$ M.

of low amplitude, and rarely overshot zero membrane potential.

Investigations on rat denervated muscle

Effects on resting membrane potential Muscle fibre membrane potentials fell by between 15 and 20 mV in both EDL and SOL muscles during the first 3 days after denervation and remained stable thereafter. The denervated muscles were very sensitive to ATX-II, a concentration of 10^{-8} M inducing a depolarization in both SOL and EDL of 5–10 mV (see Figure 4). With reference to rat innervated SOL and EDL muscles (see Alsen *et al.*, 1981 and Table 1) it may be seen that denervation resulted in a shift in the threshold concentration of ATX-II needed to cause a depolarization from 10^{-7} M to 10^{-8} M in SOL and from 10^{-6} M to 10^{-8} M in EDL.

Effects on action potentials Denervation resulted in an increased sensitivity to ATX-II in both SOL and EDL muscles of the rat. In EDL muscles denervated for 6 days, for example, ATX-II induced a prolongation of the action potential at a concentration of 10^{-8} M; muscles denervated for 3 days showed no such increase in sensitivity (Figure 5).

The rate of increase in sensitivity was assessed by determining the proportion of muscle fibres generating prolonged action potentials in rat EDL muscles as a function of time after denervation. In this context, an

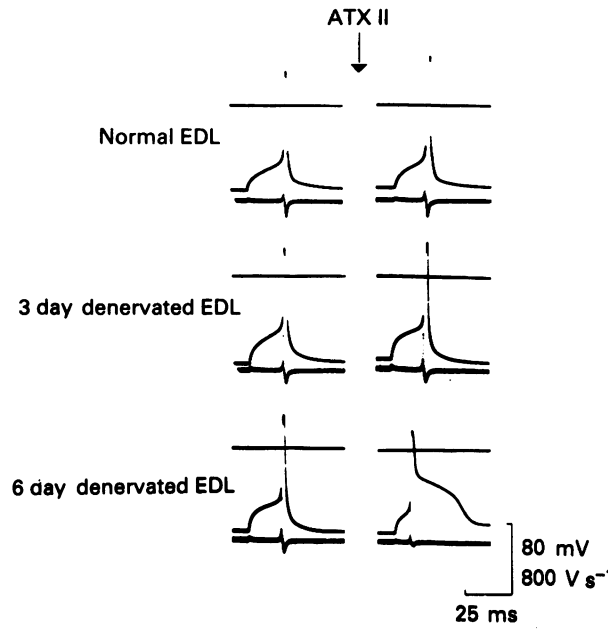

Figure 5 Action potentials generated by direct intracellular stimulation in rat EDL muscle fibres before and after exposure to ATX-II, 10^{-8} M. Note that normal and 3-day denervated muscles were insensitive to this concentration of toxin. In each panel the upper trace represents zero potential, the middle trace represents the voltage record and the lower trace represents the first derivative of the voltage change (i.e. dV/dt).

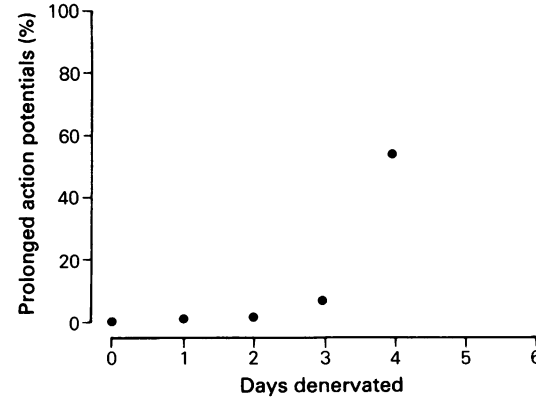

Figure 6 The relationship between time since nerve section and the ability of ATX-II (10^{-8} M) to induce prolongation of action potentials in rat EDL muscle fibres. Action potentials were generated by direct intracellular stimulation and were considered prolonged when the duration at − 50 mV exceeded 4.0 ms (see text). Each point was plotted following observations on at least 20 fibres in at least 2 muscles.

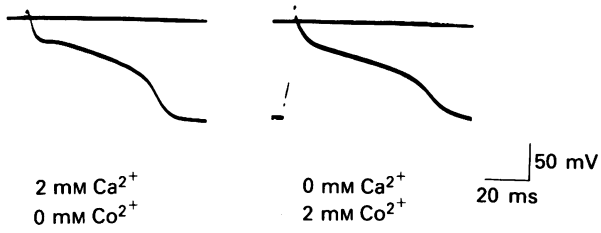

Figure 7 Action potentials generated by direct intracellular stimulation in rat denervated (6-day) EDL muscle fibres in the presence of ATX-II, 10^{-7} M. Note that substitution of Ca^{2+} with Co^{2+} has no effect on the duration of the action potential. The upper trace represents zero potential and the lower trace the voltage record.

action potential was considered to be prolonged if its duration at -50 mV was equal to or greater than 4.0 ms (the mean duration of 6-day denervated EDL muscle fibres in the absence of ATX-II is 3.0 ± 0.1 ms, $n = 20$). The data are summarised in Figure 6.

The prolonged action potentials were not Ca^{2+}-dependent, and substitution of Ca^{2+} by Co^{2+} did not abolish the prolonged portion of the action potentials. For example, in rat EDL muscles denervated for 6 days, action potential duration in the presence of ATX-II (10^{-8} M) was 16 ± 4.6 ms ($n = 8$), in the presence of Ca^{2+} and 20 ± 3.1 ms ($n = 7$) in the absence of Ca^{2+} and the presence of Co^{2+} (see Figure 7).

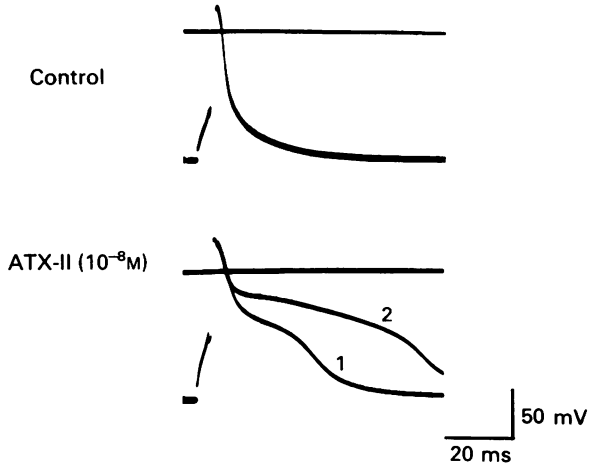

Figure 8 Action potentials generated by direct intracellular stimulation in rat denervated (6-day) SOL muscle fibres before and after exposure to ATX-II, 10^{-8} M. Note the additional prolongation of the action potential caused by repetitive stimulation. In both panels, the upper trace represents zero potential and the lower trace the voltage record. Trace (1) = after first stimulus; trace (2) = after 100 stimuli at 10 Hz.

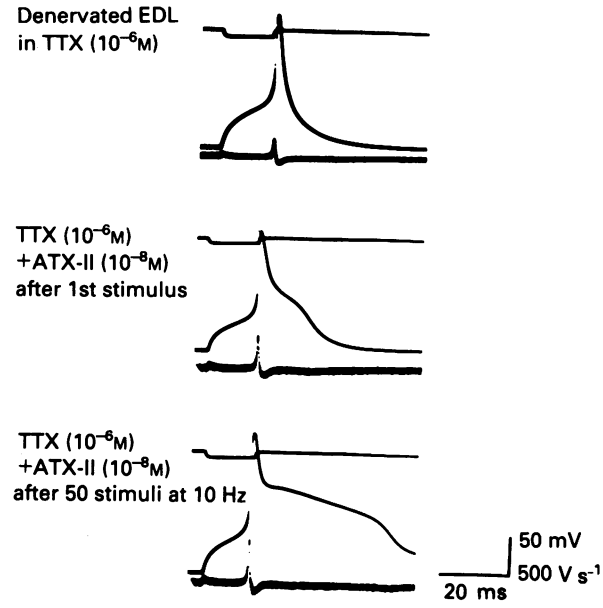

Figure 9 Action potentials generated by direct intracellular stimulation in rat denervated (6-day) EDL muscle fibres illustrating that the tetrodotoxin (TTX)-resistant action potential is prolonged by exposure to ATX-II, 10^{-8} M, and that it is further prolonged by repetitive stimulation. In each panel, the upper trace represents zero potential, superimposed on which is the stimulus (50 nA), the middle trace represents the voltage record and the lower trace the first derivative of the voltage record (i.e. dV/dt). In this experiment, action potentials were generated during the last 1–2 ms of a 10 ms current pulse.

The duration of the action potential in the presence of ATX-II was further prolonged by repetitive stimulation in denervated EDL and SOL muscles of rats (Figure 8). The TTX-resistant action potentials of rat and mouse denervated muscles were prolonged in the presence of ATX-II and these too were prolonged further by repetitive stimulation (Figure 9).

Investigations on regenerating SOL muscles of the rat

Rat muscles regenerating after exposure to the snake venom toxin, notexin, recapitulate the 'normal' pattern of post-natal maturation in terms of both physiological (Harris *et al.*, 1975) and biochemical properties (Whalen *et al.*, 1982). The maturation process in the regenerating muscles is rapid, and 'adult' properties are first seen by 7 days, when the muscle fibres begin to elaborate adult slow myosin. To determine the sensitivity of immature rat muscles to ATX-II the SOL muscles were removed from young adult rats between 4 and 21 days after assault by

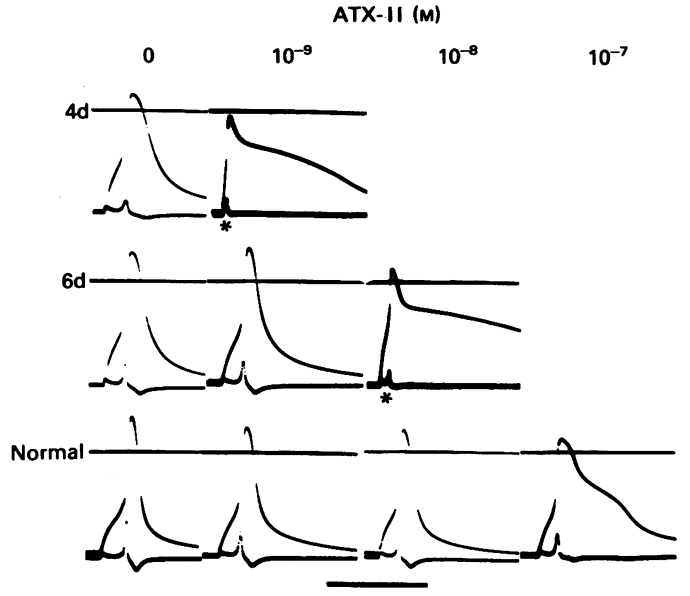

Figure 10 Action potentials generated by direct intracellular stimulation in regenerating and normal muscle fibres in the SOL muscles of rats. Regeneration was induced by producing a toxic necrosis *in vivo* (see Methods) and muscles were removed 4 or 6 days after assault. Note that during the earliest stages of regeneration, muscle fibres are sensitive to very low concentrations of ATX-II, and that the sensitivity declines rapidly as the muscles mature. In each panel, the upper trace represents zero potential, the middle trace represents the voltage record and the lower trace the first derivative of the voltage record (i.e. dV/dt). Horizontal calibration: 80 mV, 1000 V s^{-1}. Vertical calibration: 10 ms (*:20 ms).

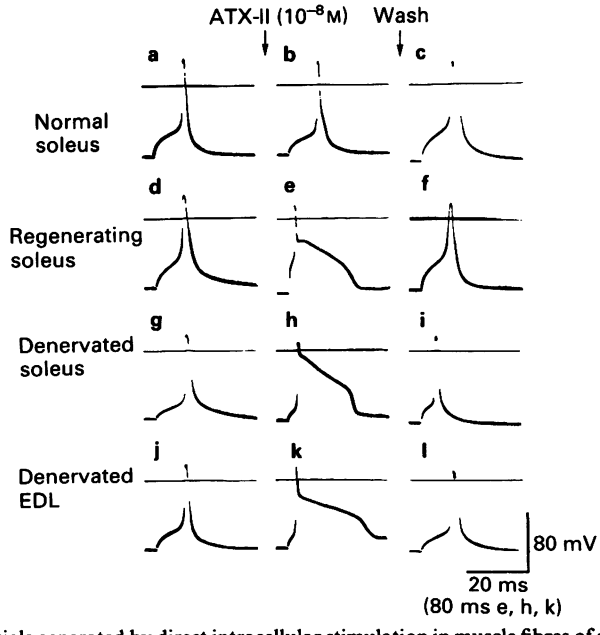

Figure 11 Action potentials generated by direct intracellular stimulation in muscle fibres of normal, regenerating and denervated SOL and denervated EDL of rats. The left hand panels illustrate action potentials generated before exposure to ATX-II, those in the centre panels were generated after ATX-II (10^{-8} M) and those on the right were generated after washing. In each panel the upper trace represents zero potential and the lower trace represents the voltage record. Figure retouched.

notexin (see Methods). At 4 days regenerating SOL muscle fibres were sensitive to ATX-II at concentrations as low as 10^{-9} M. By 6 days the muscle fibres were less sensitive to ATX-II and by 21 days sensitivity was indistinguishable from that in control muscles (Figure 10).

The reversibility of ATX-II

The effects of ATX-II on rat innervated SOL muscles are virtually irreversible (Alsen *et al.*, 1981). By contrast, the effects of ATX-II on regenerating and denervated SOL and denervated EDL muscles of the rat were relatively easily reversed. In a typical experiment, rat innervated SOL, denervated EDL and SOL and regenerating SOL muscles were mounted together. Action potentials were generated by direct intracellular stimulation in 5–10 muscle fibres in each muscle. The muscles were then exposed to ATX-II 10^{-8} M and 15 minutes later another series of action potentials was generated; only the innervated SOL was unaffected by exposure to ATX-II. The preparations were then washed by continuous overflow for 30 min using a total of 30 vol of bathing fluid. Following washing none of the muscle fibres generated prolonged action potentials (Figure 11).

The observation that the effects of ATX-II were often more pronounced after repetitive stimulation suggested that repetitive activity might stabilize the binding of ATX-II. Therefore, rat innervated and denervated EDL and SOL, and rat regenerating SOL muscles were mounted on a bank of platinum electrodes (alternating anode and cathode; inter-electrode distance 1 mm) and stimulated directly (50 pulses at 10 Hz; 1.0 ms duration, supramaximal voltage) in the presence of ATX-II 10^{-7} M. The muscles were then transferred to a toxin-free bathing fluid and washed by continuous overflow for 30 min using a total of 30 vol bathing fluid. After this procedure only innervated SOL muscle fibres generated prolonged action potentials.

Discussion

Our observations, and those of others, on the effects of ATX-II on the resting membrane potential of vertebrate skeletal muscle emphasize the variability of the response of muscle to the toxin. Thus, it now seems clear that although ATX-II depolarizes rat SOL and EDL, mouse SOL, EDL, and diaphragm and chicken PDL, the frog sartorius is insensitive (Alsen *et al.*, 1981; Chang *et al.*, 1983; Erxleben & Rathmayer, 1984). Moreover, the precise degree of depolarization depends not only on the concentration of toxin used, but on the precise muscle. The differences in sensitivity are not the result of using toxin samples obtained from different sources or of different batches, because only one batch was used throughout these experiments. Since the depolarization seems to be due uniquely to an increase in resting Na^+ conductance, ATX-II could cause a depolarization by activating Na^+ channels. Alternatively, if there exists, in a given muscle fibre membrane, a constant proportion of Na^+ channels in the open state, then a depolarization would result from the delayed inactivation of such channels. This second possibility is, in some ways, more attractive, since it is consistent with the view that the primary action of ATX-II is to delay the inactivation of fast Na^+ channels (Neumcke *et al.*, 1980; Ulbricht & Schmidtmayer, 1981; Warashina & Fujita, 1983).

It does not seem probable that the relative insensitivity of some muscles to ATX-II reflects the inaccessibility of the binding site on the gating proteins to the toxin because the prolongation of the action potential can occur in rat muscles at concentrations of ATX-II that do not cause a depolarization (Alsen *et al.*, 1981) and in frog muscles where no depolarization occurs (Erxleben & Rathmayer, 1984; see also Results).

In a variety of neuronal membranes, ATX-II causes a prolongation of the sodium-dependent action potential. There is general agreement that the prolongation of the action potential is due to a delayed inactivation of the fast Na^+ current by ATX-II (Bergman *et al.*, 1976; Neumcke *et al.*, 1980; Ulbricht & Schmidtmayer, 1981; Warashina & Fujita, 1983). The observation that a similar prolongation of the action potential occurred in mammalian muscle fibres exposed to ATX-II was first made by Alsen *et al.* (1981). It is now clear that this prolongation is common to most skeletal muscle fibres. The apparent insensitivity of rat EDL and frog sartorius muscle fibres (Harris & Pollard, 1981; Metezeau *et al.*, 1979) has been shown to be relative. Erxleben & Rathmayer (1984) have also shown that, in contrast to earlier reports, ATX-II prolongs the action potential of frog sartorius muscle fibres.

One controversy on the effects of ATX-II on the action potential of skeletal muscle fibres remains unresolved. In this work and in the independent preliminary observation by Harris & Pollard (1981), mouse EDL muscle fibres were found to be virtually insensitive to ATX-II. Thus, at a concentration of 10^{-7} M, ATX-II caused only a 10% increase in action potential duration, and increasing the concentration of ATX-II to 10^{-6} M was without further effect. By contrast, actin potentials in mouse SOL muscle fibres were prolonged by 80%. These observations conflict directly with those made by Erxleben & Rathmayer (1984), who reported a dose-dependent increase in action potential duration in mouse EDL and further claimed that no prolongation could be seen in SOL muscle fibres even when exposed to ATX-II at concentrations in excess of 10^{-6} M. This may be a relatively

trivial disagreement in the general context of the pharmacology of ATX-II but it is puzzling and difficult to explain. The rather significant differences in experimental technique adopted by Erxleben & Rathmayer (1984) and ourselves may be important. We used pulse durations of 1–2 ms and the action potential was triggered from the final 0.5–1.0 ms of the pulse. We were, therefore, able to study the falling phase of the action potential in the absence of any artefactual distortion, and to measure the duration of the action potential at − 50 mV. By contrast, Erxleben & Rathmayer (1984) measured the time between the maximum rate of rise and the maximum rate of fall of action potentials generated during a stimulating pulse of 5 ms. In these circumstances, the falling phase of action potentials is always distorted by artefact arising when the current pulse is terminated. Moreover, Erxleben & Rathmayer (1984) used concentrations of ATX-II as high as 1.5×10^{-5} M. Few detailed results have been illustrated by Erxleben & Rathmayer (1984) and so it is difficult to discuss the differences further, but on the basis of their illustrations of action potentials in mouse EDL before and after ATX-II, we can only comment that we would not consider the action potentials prolonged.

The muscle most strikingly affected by ATX-II was the chicken PLD. The very large depolarization and the enormous prolongation of the action potential induced by ATX-II at 10^{-7} M suggests that this might be the preparation of choice in the study of the biophysics of Na^+ channel inactivation.

Alsen *et al.* (1981) reported that the maximum rate of rise (dV/dt) and the amplitude of the overshoot of the action potential in rat SOL was suppressed by ATX-II. Erxleben & Rathmayer (1984) reported that in frog satorius, there was an increase of both dV/dt and amplitude. In this paper it has been confirmed that in all mammalian muscles used, and in chicken PLD, there is a suppression of dV/dt and overshoot, but that in frog sartorius, dV/dt if not increased is unchanged. The amplitude of the overshoot also remains unchanged in frog sartorius in the presence of ATX-II, 10^{-7} M to 10^{-6} M. Since the reduction in overshoot and dV/dt can occur when there is relatively little depolarization (see Tables 1 and 2 rat and mouse EDL), it seems unlikely that changes in muscle fibre dV/dt or overshoot arise as a result of partial inactivation caused by depolarization, as suggested by Erxleben & Rathmayer (1984). Rather, the changes presumably result from a reduction in total gNa^+. Such an effect of ATX-II was surmised by Alsen *et al.* (1981) and has been seen in neuronal membranes by Ulbricht & Schmidtmayer (1981).

It has been claimed that the degree of prolongation of the action potential by ATX-II in neuronal membranes is time-dependent (Rathmayer, 1979). This claim was based on the observation that there is a gradual increase in duration of the action potential during trains of low frequency stimulation (Rathmayer & Béress, 1976). In guinea-pig papillary muscle, the effects of ATX-II are strongly influenced by the rate of electrical stimulation (Béress *et al.*, 1982). This suggested to us that rather than being time-dependent, the activity of ATX-II is use-dependent. This was tested in several skeletal muscles, and we have shown unequivocally that in many instances the effect of ATX-II is enhanced by repetitive stimulation rather than by the time of exposure to the muscle. Since the effect of ATX-II is not influenced by prolonged local depolarization or hyperpolarization, it would seem that access or binding to the Na^+ channel gating protein is enhanced when gates undergo dynamic changes in conformation.

In summary, our observations on innervated muscles show that ATX-II causes a sodium-dependent depolarization of vertebrate innervated skeletal muscle fibres and a prolongation of the duration of the action potential. The threshold concentration of ATX-II required for the expression of these effects varies between 10^{-7} M and 10^{-6} M, and the degree of change in any given measurement varies unpredictably between species and between different muscles within a species. In some muscles, the effect of ATX-II is not influenced by local membrane potential, but is influenced by rapid cycles of activity suggesting that ATX-II could be used in the study of conformation changes in the Na^+ channel gating proteins.

It has been known for some time that denervated muscle fibres are depolarized by ATX-II, and that both the 'full' action potential and the tetrodotoxin-resistant action potential of denervated muscle fibres are prolonged by ATX-II (Alsen *et al.*, 1981; Erxleben & Rathmayer, 1984). Some preliminary experiments reported by Tesseraux & Harris (1983) suggested that in rat denervated muscles, there was an increase in sensitivity to ATX-II, and Erxleben & Rathmayer (1984), working on mouse muscles, reported that 'a ten times smaller concentration of ATX-II was sufficient to produce a prolongation of action potentials in denervated SOL muscle . . .'. Our detailed observations on rat denervated EDL and SOL muscles confirm these latter findings. The most dramatic increase in sensitivity was that in rat EDL, in which the increase was two orders of magnitude. The change in sensitivity occurred with a delay of 3–4 days after denervation, and was not fully established until about 6 days after denervation. This is remarkably similar to the time scale of the appearance of the tetrodotoxin (TTX)-resistant action potential in denervated mammalian muscle (Harris & Thesleff, 1971). It is tempting to speculate that the new or modified fast Na^+ channels that appear after denervation (Grampp *et al.*, 1972) are responsible for changes in sensitivity to both ATX-II and TTX. An attempt was made to study the

interaction between the two toxins, but meaningful experiments are difficult to devise given the easy reversibility of ATX-II, the fact that little is known of the kinetics of binding of ATX-II and that the precise structures to which TTX and ATX-II bind have not been formally identified.

Since the properties of mammalian skeletal muscle are, to a large extent, determined by the pattern of stimulation received from the motor innervation (Sreter *et al.* 1973), it has often been suggested that denervation allows the muscle to revert to a more primitive or undifferentiated state. Developing and regenerating muscles may be considered relatively undifferentiated, and both classes of muscles exhibit low muscle fibre resting membrane potentials, enhanced sensitivity to acetylcholine and reduced sensitivity to TTX (Axelsson & Thesleff, 1964; Harris & Marshall, 1974; Harris *et al.*, 1975). It is of interest, therefore, that rat regenerating SOL muscles are also hypersensitive to ATX-II. The sensitivity is highest in the most immature fibres, and declines until by 14–21 days, sensitivity is indistinguishable from that in control muscles. This is identical to the time scale over which the level of the resting membrane potential and the sensitivity to TTX return to normal in regenerating muscles (Harris *et al.*, 1975) and suggests that ATX-II might be of value in the study of developmental changes in fast Na^+ channels in excitable membranes.

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