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MYOTOXIC ACTIVITY OF THE CRUDE VENOM AND THE PRINCIPAL NEUROTOXIN, TAIPOXIN, OF THE AUSTRALIAN TAIPAN, *Oxyuranus scutellatus*

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1 The crude venom of the Australian taipan, *Oxyuranus scutellatus* and its principal neurotoxin, taipoxin, were injected into the anterolateral aspect of one hind limb of the rat.

2 The effects of the venom and toxin on the morphology and physiology on the underlying soleus muscles were examined.

3 Both the crude venom and the toxin caused necrosis and degeneration of the muscle. Damage to the peripheral muscle fibres could be seen at the light microscopic level as early as 3 h after injection of the toxic compounds.

4 The necrotic response was accompanied by an infiltration of phagocytic cells and an extensive oedema. The wet weight of the damaged muscles was almost doubled by 6 h.

5 In individual muscle fibres, necrosis was associated with the disruption of the plasma membrane and the disorganization of the myofibrils. The basal lamina of the muscle fibres was left intact.

6 Denervated mammalian muscles and innervated avian muscles were also destroyed by taipoxin, but immature avian muscle growing in tissue culture was resistant.

7 Of the 3 subunits of taipoxin, only the basic α-taipoxin was itself myotoxic. However, its potency was enhanced by the presence of the acid γ-subunit. The role of the neutral β-subunit is unclear.

8 The period of necrosis and degeneration lasted for approximately 48 h, after which the muscle fibres began to regenerate. Regeneration took place within the surviving basal lamina, with the formation of myotubes by three days, and small, immature muscle fibres by five days. Regeneration was virtually complete by 21 days.

Introduction

The venom of the Australian elapid snake, *Oxyuranus scutellatus* (the taipan), is generally said to cause death by neurotoxic poisoning (Garnet, 1977). Taipoxin, a toxin isolated from the whole venom (Fohlman, Eaker, Karlsson & Thesleff, 1976), has been defined as the principal neurotoxin, acting by inhibiting transmitter release from the motor nerve terminals (Kamenskaya & Thesleff, 1974; Dowdall, Fohlmann & Eaker, 1977). Similar claims were made for the venom of the Australian tiger snake *Notechis scutatus scutatus* (Campbell, 1967; Trinca, 1969) and for the principal neurotoxin, notexin (Harris, Karlsson & Thesleff, 1973). However, it is now clear that a bite by the Australian tiger snake results in a severe, localized necrotizing myopathy (Hood & Johnson, 1975; Sutherland & Coulter, 1977) and that the two major presynaptically active neurotoxins, notexin and notechis II-5, are potent myotoxins (Harris, Johnson & Karlsson, 1975; Harris & Johnson, 1978; Pluskal, Harris, Pennington & Eaker, 1978).

The aim of the present study was to examine the possibility that taipan venom, and the principal neurotoxin, taipoxin, have a myotoxic action. Some of the results have been presented in an abbreviated form to the British Pharmacological Society (Harris, Johnson & MacDonell, 1977) and to the International Society on Toxinology (Harris, Johnson & MacDonell, 1980).

Methods

Animals

The experiments were carried out on female Wistar rats weighing 180–200 g. The rats were anaesthetized with ether, and a single subcutaneous injection

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of either taipan venom, the principal neurotoxin, taipoxin, or the subunits of taipoxin was made into the anterolateral aspect of one hind limb such that the soleus muscle was exposed to the compound(s) used. The venom or the toxic components of the venom were dissolved in 0.9% w/v NaCl solution (saline), and the injection volume was maintained at 0.20 ml. This volume of saline has no effect on the morphology or the physiological integrity of the injected muscle.

At various times after the injection, the soleus muscles of both hind limbs were removed, and subjected to physiological or histological examination. In all cases, the contralateral uninjected muscles served as the control. Muscles processed for histological examination were routinely weighed and any change in the wet weight of the injected muscle was determined using the wet weight of the contralateral muscle as a reference. Muscles used for physiological examination were subsequently frozen, sectioned and processed for histological and histochemical analysis. Some muscles were processed for electron microscopical examination. These muscles were not used for any other form of experiment. The muscles for these various forms of examination were randomly selected from a pool of injected animals.

In some experiments, injections were made into the vicinity of denervated muscles. Denervation was performed under ether anaesthesia, approximately 1 cm of sciatic nerve being removed from the mid-thigh region. The incisions were closed using silk sutures. In the context of these experiments, acute denervation refers to a denervation made at the time of injection of the toxin, and chronic denervation refers to a denervation made four days before the administration of toxin.

Venoms, toxins and anti-venoms used

Crude taipan venom was supplied by Mr E. Worrell (Worrell's Australian Reptile Park, P.O. Box 192, Gosford, N.S.W., Australia). Taipoxin and the α-, β- and γ-subunits of taipoxin were supplied by Drs D. Eaker and J. Fohlman (Department of Biochemistry, University of Uppsala, Sweden). The doses of these compounds administered to the rats were chosen as the result of preliminary experiments. Taipoxin, 2.0 μg in 0.20 ml saline induced clear evidence of muscle damage in every animal injected. At this dose, there was no obvious weakness in the contralateral limb, and no respiratory distress. There was no unusual lacrimation or salivation, and the general behaviour of the animals was indistinguishable from normal. From these observations the inference was drawn that systemic poisoning was minimal. The 'standard' dose of taipoxin was therefore established at 2.0 μg. This toxin comprises about 20% of the whole venom of the taipan (Fohlman *et al.*, 1976) and so the 'standard' dose of crude venom used was 10 μg. The three subunits of taipoxin (Fohlman *et al.*, 1976) appear to be present in the toxin in a molar ratio of 1:1:1. Although the actual molecular weight of each subunit was unclear when these experiments were done, they appear to have marked structural similarity with each other (Fohlman *et al.*, 1976) and so for practical purposes were assumed to have similar molecular weights. The 'standard' dose of each of the subunits was therefore defined as 0.66 μg.

Taipan and tiger-snake anti-venoms were obtained from the Commonwealth Serum Laboratories, Parkville, Victoria, Australia.

The combinations of the various toxins and antivenoms used in these investigations are given in the legend to Figure 7. They are somewhat empirical, but were based on information gathered concerning the 'recommended dose' of antivenom, the 'average yield' of venom and the concentrations of the various toxins and toxin subunits in the venoms. Thus, the contents of one ampoule of antivenom (12,000 units) will neutralize the average venom yield of a taipan (120 mg). Since taipoxin comprises 20% of the venom, it may be calculated that 1 unit of antivenom will neutralize 2.0 μg of taipoxin.

Histology and histochemistry

Blocks of tissue were taken from the belly of both treated and contralateral muscles, sandwiched between thin slices of liver, and then orientated so that transverse sections could be cut. The blocks were frozen in Arcton (dichloro-difluoromethane, I.C.I.) at −150°C in liquid N_2, and sections 10 μm thick were obtained using a cryostat (Dittes, Hamburg) and microtome (Jung).

Muscle architecture was demonstrated by staining with haematoxylin and eosin (H and E) and serial sections were occasionally used to demonstrate the activity of Ca^{2+}-activated myofibrillar adenosine triphosphatase (ATPase; Hayashi & Freiman, 1966), nicotinamide adenine dinucleotide diaphorase (NADH-diaphorase; Pearse, 1960) and cytoplasmic RNA using the pyronin-methyl green technique (Trevan & Sharrock, 1951).

Reference is commonly made to various 'muscle fibre types', identified according to their histochemical properties. The nomenclature in this work is based on that described by Stein & Padykula (1966) and Dubowitz & Brooke (1973).

Ultrastructure of muscle

Both treated and contralateral muscles were removed and pinned out flat on a small piece of dental

wax. The muscles were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 1 h. After this initial fixation period the muscles were removed and the top few layers of muscle fibres were cut away from the muscle. These thin strips were then cut up into pieces of about 1 mm^3 before being returned to the glutaraldehyde fixative for a further hour. The fixed pieces of muscle were rinsed in phosphate buffer before being post-fixed for 1 h in 1% osmium tetroxide in phosphate buffer. The specimens were then dehydrated and infiltrated before being embedded in Spurr resin and cured overnight in the usual manner. Thick sections ($\simeq$ 1 μm) were taken from each muscle block and stained with toluidine blue to allow the preservation and orientation of the specimen to be assessed. Ultrathin sections (60–90 nm thick) were taken from the blocks and stained on copper grids for 15–30 min in 3% uranyl acetate. Counter staining for constrast was achieved using 0.4% modified Reynold's lead citrate. The sections were viewed in a Zeiss EM95 electron microscope.

Physiology

Treated and contralateral muscles were isolated and mounted together on a small Sylgard plate (Dow-Corning 186) in a perspex bath which was continuously perfused with a physiological bathing fluid, maintained at room temperature and equilibrated with 95% O_2/5% CO_2. The bathing fluid had 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 glucose 11.0.

Muscle fibre resting membrane potentials were recorded using intracellular glass microelectrodes filled with 3 M KCl. The electrodes had tip potentials $<$5 mV and d.c. resistances of 5–15 MΩ. Action potentials were generated either indirectly by stimulating the motor nerve or directly by inserting a second current-passing microelectrode into the fibre, 50–100 μm from the recording electrode. In the latter case, the impaled fibre was locally hyperpolarized to a membrane potential of −90 to −95 mV in order to optimize action potential generation (Redfern & Thesleff, 1971). The buffer amplifier and the current passing circuits used in these experiments were home-built and have been described by Allan, Gascoigne, Ludlow & Smith (1977). In most experiments, 3–4 muscles were used at each time point and 10–20 superficial muscle fibres per muscle were sampled. Superficial fibres only were examined because such fibres were always damaged. Any 'spared' fibres existed as a core of undamaged tissue in the middle of the muscle. All physiological data were therefore obtained on an homogeneous population of degenerating or regenerating fibres.

Statistical analysis

Most data are presented in the form of arithmetic mean ± standard error of the mean (s.e.mean). However, in Table 1 the data are presented as arithmetic mean ± standard deviation in order to convey to the reader the variability of the observations made on regenerating muscle fibres.

Results

Muscle wet weight

The subcutaneous injection of taipan venom (10 μg) caused a large and rapid increase in muscle wet weight. The maximum increase (82%) occurred at about 6 h after the injection of the venom. Over the next 48 h, the oedema subsided, and the wet weight of the venom-damaged muscles fell below that of the contralateral muscles. From around seven days after the administration of the venom, muscle wet weight began to return to normal. The injection of 2.0 μg of taipoxin, a constituent toxin representing 20% of taipan venom, caused essentially similar changes in muscle wet weight. These data are summarized in Figure 1.

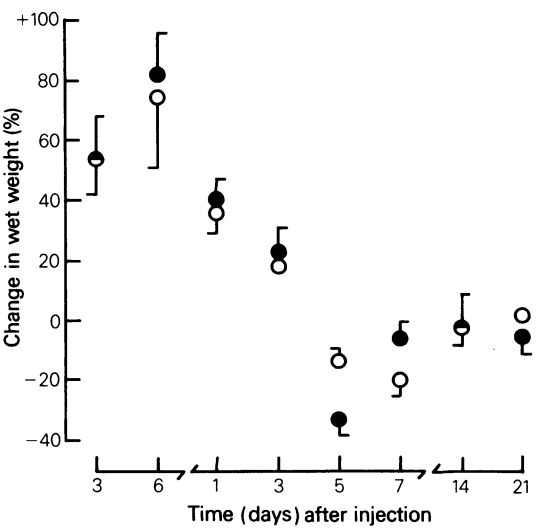

Figure 1 Changes in the wet weight of rat soleus muscles at various times after the injection of taipoxin (2.0 μg: ○) or taipan venom (10.0 μg: ●). The change in wet weight of individual muscles was expressed as a function of the wet weight of the contralateral soleus muscle. Each point represents the mean weight change in 3–6 muscles, and bars indicate s.e.mean. Where no error bar is included, the s.e. was too small to plot.

Taipoxin comprises three subunits, α-, β-, and γ-taipoxin, and the effects of these subunits on muscle wet weight 24 h after administration were also examined. At the dose level of 0.66 μg the β- and γ-subunits of taipoxin were without significant effect, but α-taipoxin caused a marked increase in wet weight. A series of recombination experiments was then carried out. The combinations (0.66 μg $\overline{aa}$) α- plus β-taipoxin and β- plus γ-taipoxin were without significant effect, but the combination α- plus γ-taipoxin induced massive oedema with wet weight changes in the injected muscles similar to those induced by both taipan venom (10 μg) and taipoxin (2.0 μg). The results are summarized in Figure 2.

Muscle histology

The early oedematous response of muscles exposed either to taipan venom or to taipoxin was evident not only from the wet weight changes described above, but also from histological and histochemical studies. The peripheral muscle fibres were always damaged by the toxin, any 'spared' fibres existing as a core of apparently undamaged tissue. The results imply that there is no population of unsusceptible muscle fibres in soleus. The fast-twitch (type IIa) fibres of extensor digitorum longus are spared when exposed to notexin (Harris *et al.*, 1975). It is not known whether they are also resistant to taipoxin.

In general the patterns of degeneration induced by the venom and toxin respectively, were similar, although it seemed on subjective criteria that the venom caused more extensive damage to a muscle than taipoxin. The earliest signs of damage, seen 3 h after the administration of the compounds, consisted of intense interstitial oedema, which was particularly severe at the periphery of the muscle. Some of the peripheral muscle fibres were swollen, and stained darkly with H and E, while others showed various stages of myofibrillar over-contraction and disintegration. Polymorphonuclear leukocytes could be seen in the small blood vessels and in the perivascular spaces (Figure 3b, c). By 6 h both the oedema and muscle fibre necrosis was more extensive and the infiltration of necrotic fibres by phagocytic cells was striking (Figure 3d). By 24 h most of the fibres throughout the muscle showed some evidence of necrosis, although damage was still most striking in the peripheral layers of the muscle fibres. The necrotic fibres were without demonstrable NADH-diaphorase or myofibrillar ATPase activity (Figure 3e). The blood vessels and muscle spindles appeared to be spared in the necrotic muscles.

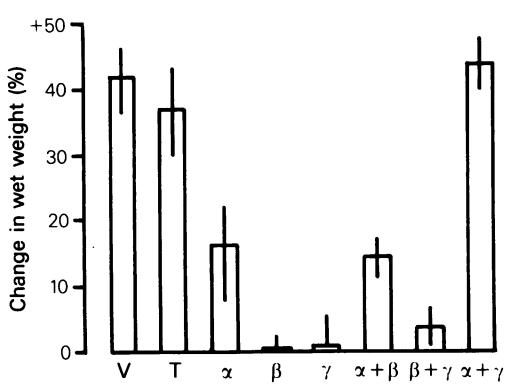

Figure 2 Changes in the wet weight of rat skeletal muscles 24 h after the injection of taipan venom (10.0 μg:V); taipoxin (2.0 μg:T); α-taipoxin (0.66 μg:α); β-taipoxin (0.66 μg:β) γ-taipoxin (0.66 μg:γ); α-taipoxin plus β-taipoxin (0.66 μg $\overline{aa}$: α + β); β-taipoxin plus γ-taipoxin (0.66 μg $\overline{aa}$: β + γ); α-taipoxin plus γ-taipoxin (0.66 μg $\overline{aa}$: α + γ). Each column represents the mean change of 3–6 muscles and vertical lines indicate ± 1 s.e.

Three days after the administration of either crude venom or taipoxin, regeneration was already underway, with large populations of small round basophilic cells with pale vesicular nuclei being clustered together within the membrane boundaries of the original peripheral fibres (Figure 4a). These basophilic cells were strongly pyroninophilic (demonstrated by staining frozen sections with pyronin methyl green) confirming high levels of cytoplasmic RNA, and indicating intense metabolic activity. These results suggested that the peripheral fibres contained actively regenerating cells. Between 5 and 7 days after assault, small immature muscle fibres with centrally located nuclei could be seen (Figure 4b). Although the overall muscle architecture was virtually normal by about 30 days, two pathological features persisted. Firstly, central nucleation persisted, and secondly, a considerable amount of fibre splitting was evident (Figure 4c). The splits were visible as small angular fibres closely associated with and often a part of the general shape of the host fibre. Myofibrillar ATPase staining suggested that differentiation into recognizable fibre types began at about 14 days (Figure 4d).

Muscle ultrastructure

Although histological evidence of muscle fibre damage was rarely seen before 3 h, ultrastructural evidence could be identified as early as 1 h after the injection of taipoxin. The early stages of degeneration were characterized by the disorganization and over-contraction of the myofibrils (Figure 5a). In hypercontracted areas of the fibres, the mitochondria were squeezed into the periphery. Many of these mitochondria contained dark, rod-like structures within their matrix which appeared to be associated

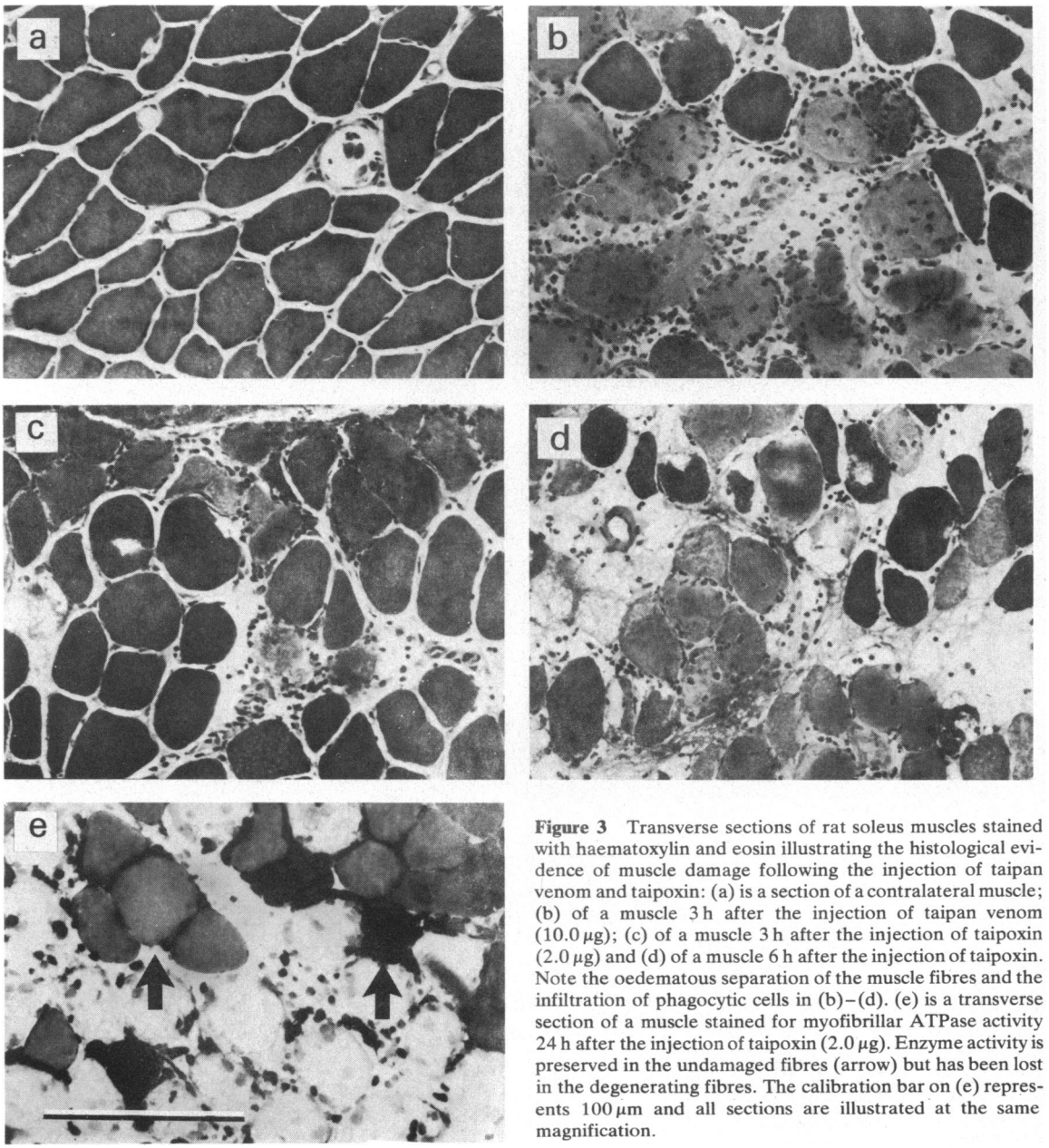

Figure 3 Transverse sections of rat soleus muscles stained with haematoxylin and eosin illustrating the histological evidence of muscle damage following the injection of taipan venom and taipoxin: (a) is a section of a contralateral muscle; (b) of a muscle 3 h after the injection of taipan venom (10.0 μg); (c) of a muscle 3 h after the injection of taipoxin (2.0 μg) and (d) of a muscle 6 h after the injection of taipoxin. Note the oedematous separation of the muscle fibres and the infiltration of phagocytic cells in (b)–(d). (e) is a transverse section of a muscle stained for myofibrillar ATPase activity 24 h after the injection of taipoxin (2.0 μg). Enzyme activity is preserved in the undamaged fibres (arrow) but has been lost in the degenerating fibres. The calibration bar on (e) represents 100 μm and all sections are illustrated at the same magnification.

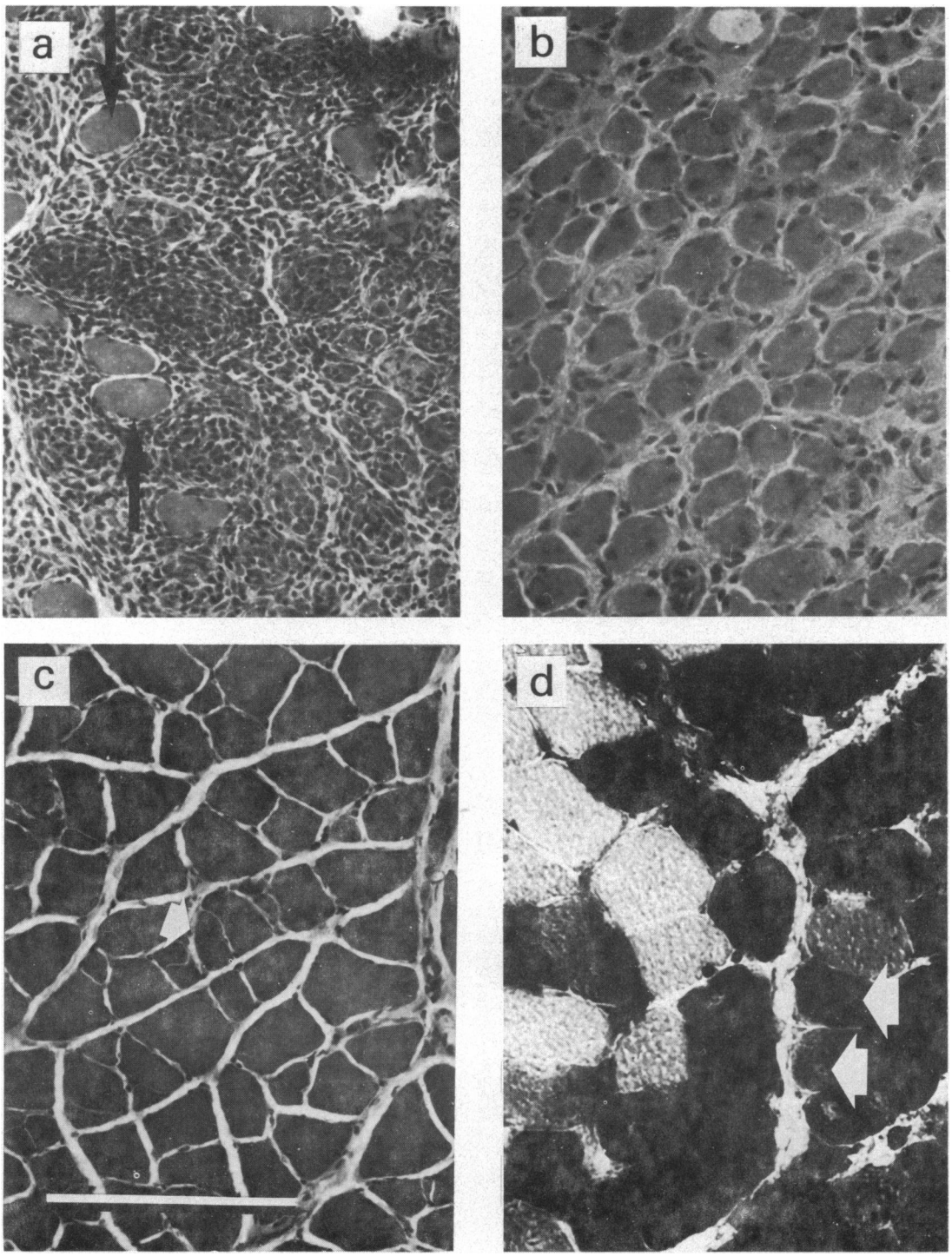

Figure 4 Transverse sections of rat soleus muscles stained with haematoxylin and eosin 3 days (a), 7 days (b) and 28 days (c) after the injection of taipoxin; (d) is a section of a muscle stained for myosin ATPase activity 14 days after the administration of taipoxin. Some fibres were not damaged by the toxin, and these are clearly visible in (a) (arrows). The central nucleation in regenerated muscle fibres and the split fibres (arrow) are clearly visible in (c). Evidence of the metabolic differentiation of regenerating (centrally nucleated) muscle fibres is seen in (d) (arrows). The calibration bar on (c) represents 100 μm, and all sections are illustrated at the same magnification.

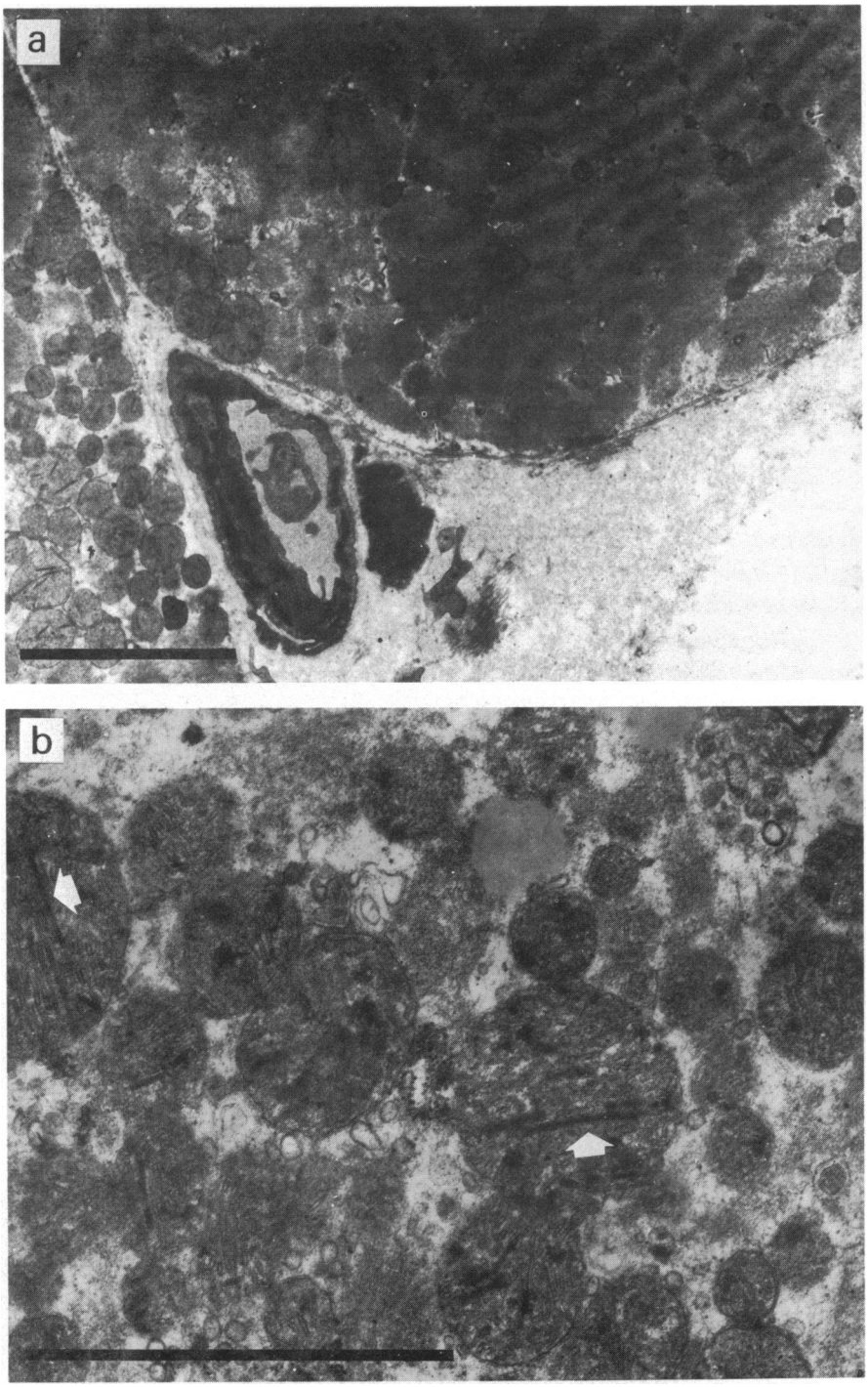

Figure 5a and b Electron micrographs of parts of soleus muscle fibres 24 h after the injection of taipoxin. The micrographs illustrate hypercontraction, and the squeezing of mitochondria into the periphery (a), rod-like abnormalities in mitochondria (b, arrows). The calibration bars each represent 2.5 μm.

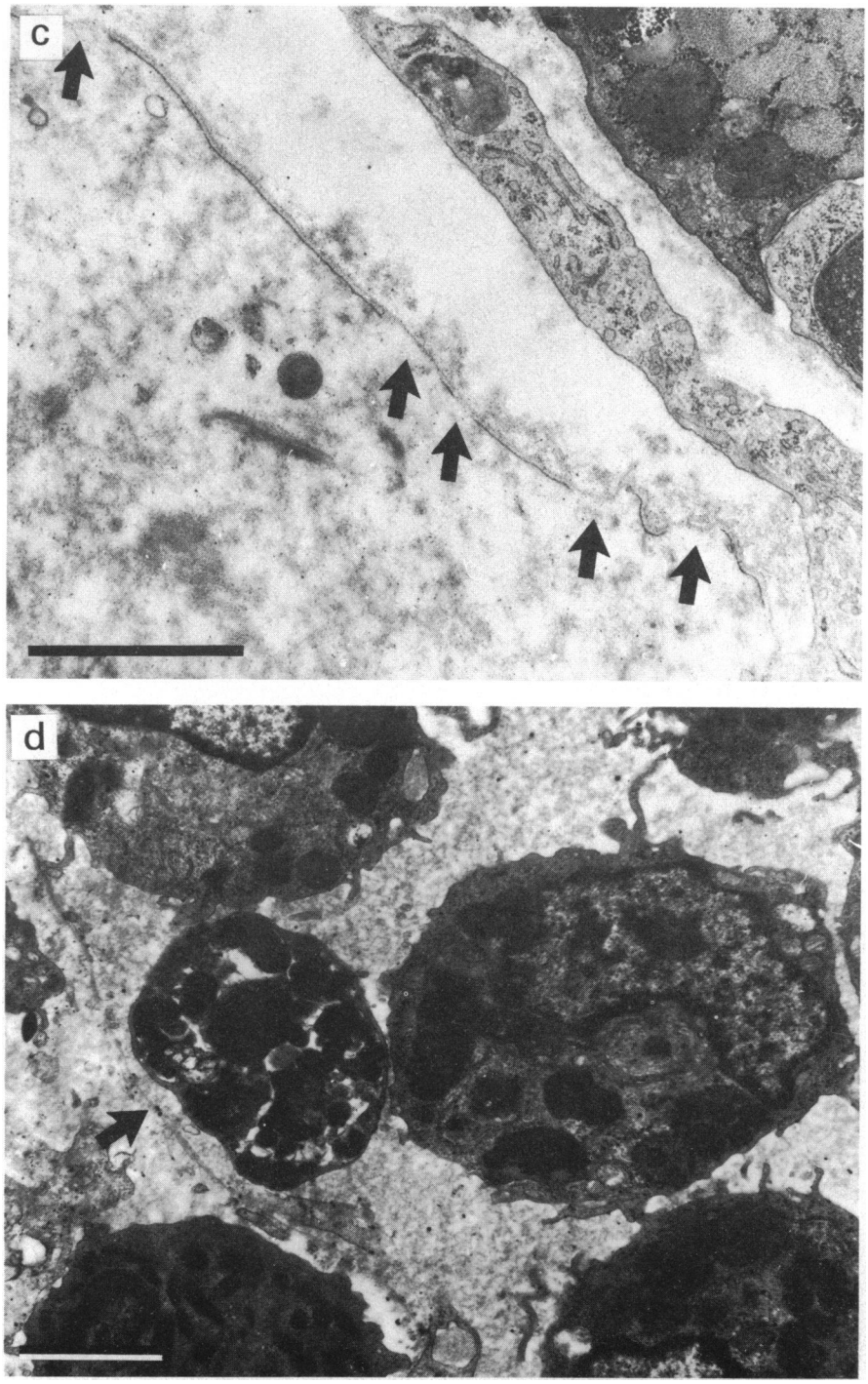

Figure 5c and d Electron micrographs of parts of soleus muscle fibres 24 h after the injection of taipoxin. The micrographs illustrate the disruption of the plasma membrane (arrows) and the preservation of the basal lamina (c), and the infiltration of phagocytic cells into a necrotic muscle fibre (d). Note the large lysosomal vacuole in (d) (arrowed). The calibration bars each represent 2.5 μm.

with disarranged cristae (Figure 5b). One characteristic feature of the necrotic muscle fibres was the loss of the plasma membrane and the preservation of the basal lamina (Figure 5c).

In the majority of the affected fibres the breakdown of the myofibrils was almost complete by 24 h and the remnants of the fibres consisted of basal lamina tubes filled with amorphous sarcoplasm, degenerating cell organelles and large numbers of phagocytic cells. The phagocytic cells appeared to be highly active, with cytoplasmic extensions which ramified extensively through the fibre debris (Figure 5d). The majority of phagocytes contained large lysosomal vacuoles enclosing cellular debris and degenerating muscle mitochondria.

By 3 days after the administration of taipoxin, both fused and unfused myogenic cells were noted at the periphery of the muscle, often in association with phagocytes, and enclosed within the basal lamina tubes left as a result of the initial fibre breakdown and phagocytosis. By 5 days phagocytosis was no longer evident, and the majority of the cells within the original basal lamina tubes had fused and had formed a plasma membrane. From 7 days the maturation of the 'new' fibres was rapid, the fibre diameter increased, the myofibrils became closely packed and the basal lamina was more tightly apposed to the underlying plasma membrane. By 21 days only two features persisted that suggested the muscle had been damaged. Firstly, the persistance of centrally located nuclei, and secondly, fibre splitting – both noted in the histological/histochemical studies. The ultrastructural aspects of the regeneration of the muscle will be described in full elsewhere.

Physiological properties of the muscle fibres

Within 3 h of administration of taipoxin, the resting membrane potential of the superficial fibres fell from a mean of −77 mV to a mean of −16 mV; by 24 h the mean resting membrane potential was only −6 mV (Table 1). During the period 24–28 h, corresponding to the period of muscle fibre necrosis and degeneration, it was impossible to study membrane excitability. However, by 3 days, many of the regenerating muscle fibres could be impaled. They exhibited low and very variable resting membrane potentials (Table 1). A second electrode could be inserted into a few of these fibres and an action potential generated in response to the passage of current. Both the maximum rate of rise and the amplitude of the overshoot of the action potential were lower than normal. From 5 days onwards, resting membrane potentials increased steadily with the maturation of the fibres, and by 14–21 days after assault the measured parameters of the direct action potentials were similar to control values. The results are summarized in Table 1.

Many of the fibres from toxin-treated muscles at 3 days, and a few fibres at 5 days showed considerable spontaneous activity. The spontaneous action potentials generally exceeded zero potential and were preceded by a pacemaker type prepotential, often from a low although apparently stable membrane potential (Figure 6). The potentials occurred rhythmically, and occasionally the membrane potential oscillated before generating a train of repetitive action potentials (see Thesleff & Ward, 1975). The frequency of the fibrillation potentials at 3 days averaged 1.9/s (±0.1

Table 1 Some properties of rat soleus muscle fibres at various times after injection of taipoxin

Muscles	*Functional innervation* (%)*	*Resting potential* (mV)	*Direct action potential* *Overshoot* (mV)	*Max. rate of rise* (V/s)
Contralateral	100	−77± 4.5	+39± 4.7	346±51
24 h	n.m.	−6± 4.5	n.m.	n.m.
3 d	15	−59±11.3	+24±10.6	277±92
5 d	63	−60±10.3	+28±12.2	172±78
7 d	75	−69± 7.3	+33±10.6	294±88
14 d	100	−70± 9.6	+33±13.2	275±50
21 d	n.d.	−77± 3.4	+30± 9.4	305±66
30 d	n.d.	−77± 4.9	+34± 7.8	381±62

*Calculated as the proportion of fibres generating an action potential in response to the electrical stimulation of the motor nerve.

n.m. = not measurable; n.d. = not done.

Each result is calculated from observations made on 10–20 muscle fibres in each of three or more muscles. At 3 d, four muscles were used to assess the degree of functional innervation; all of the innervated fibres were found in one muscle. The calculated values are presented as arithmetic means ± s.d.

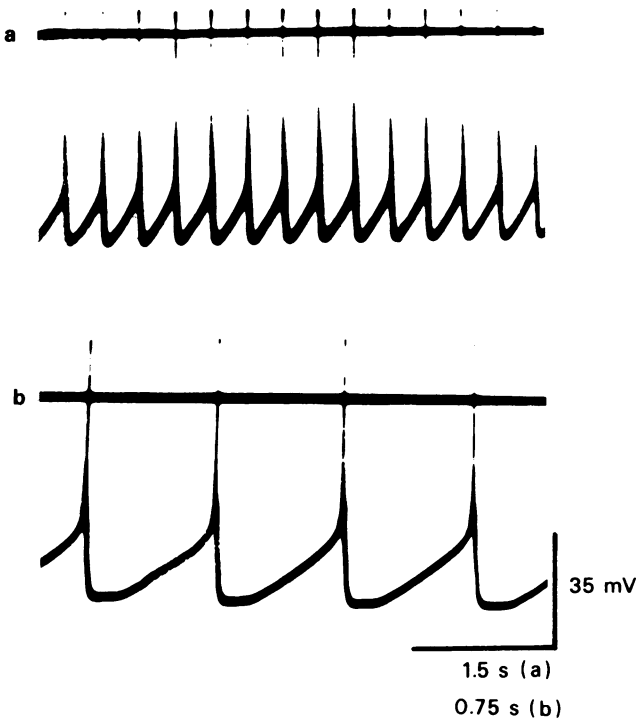

Figure 6 Spontaneous action potentials recorded intracellularly from muscle fibres in two different muscles 3 days following the administration of taipoxin. In each case, the straight horizontal line represents zero potential.

s.e.mean), and their generation could be blocked by tetrodotoxin (10^{-6} M). The potentials in any one fibre had a fairly constant amplitude, and this observation, coupled with that of a stable resting potential, suggested that they were not an artefact caused by mechanical injury to the small regenerating fibres. Fibrillation was not seen if the fibres were hyperpolarized to −90 mV and was seen very rarely after 5 days.

Tetrodotoxin (TTX)-resistant action potentials could be generated in the majority of muscle fibres in the regenerating muscles between 3 and 7 days post-intoxication. From 7 days the percentage of fibres capable of generating TTX-resistant action potentials declined, and by 14 days TTX-resistance was lost (Figure 7).

Innervation of regenerating muscles

The restoration of functional innervation in the regenerating muscles took place extremely rapidly. Thus 15% of sampled muscle fibres responded to indirect excitation by generating an action potential at 3 days, 63% at 5 days, 75% at 7 days and 88% at 10 days after taipoxin administration. By 14–21 days all muscle fibres were functionally innervated.

The spontaneous release of transmitter (measured in terms of frequency of miniature endplate potentials, m.e.p.ps) was low in the early regenerates (0.2 ± 0.05/s at 5 days; cf. normal 1.6 ± 0.09) but the frequency was indistinguishable from normal by between 7 and 10 days after taipoxin administration.

In view of the observation that both immature muscle fibres and mature muscle fibres undergoing reinnervation exhibit a brief phase of polyneuronal innervation (Redfern, 1970; McArdle, 1975) it was of interest that complex e.p.ps indicative of polyneuronal innervation were noted in only two of more than 200 muscle fibres studied.

The speed of reinnervation and the absence of significant polyneuronal innervation both suggest that the extent of damage to the peripheral nervous system in these muscles is limited perhaps to the nerve terminals or to the terminal internodes.

These observations clearly demand extension before a definitive picture emerges of the precise sequence of events underlying the reinnervation of these necrotic muscle fibres. In terms of the maturation of the muscle fibres, however, it is significant that they are virtually fully reinnervated by 7–10 days. Thus the establishment of functional innervation precedes the emergence of histochemically distinctive muscle fibre types by approximately 7 days.

The response of denervated muscle to taipoxin

The subcutaneous injection of taipoxin produced, in both acutely and chronically denervated soleus muscle, an inflammatory response and necrosis that was almost identical to that described for normal innervated muscles. The results suggest that the myotoxic activity of the toxin is not an indirect consequence of its neurotoxic activity and that neurally mediated muscle activity as a whole is not essential for the myotoxic activity of the toxin.

In the chronically denervated muscles damaged by taipoxin, the type IIa fibres appeared to be spared. The significance of this observation is unclear at present.

The response of avian muscle to taipoxin

Both the multiply-innervated anterior latissimus dorsi and the focally-innervated posterior latissimus dorsi muscle of the young adult chicken were damaged following the local subcutaneous injection of taipoxin. However, immature 5-day-old chick myotubes maintained in tissue culture showed no signs of any degenerative changes after 48 h exposure to taipoxin (5 μg/ml; $\triangleq 10^{-7}$ M). These results suggest

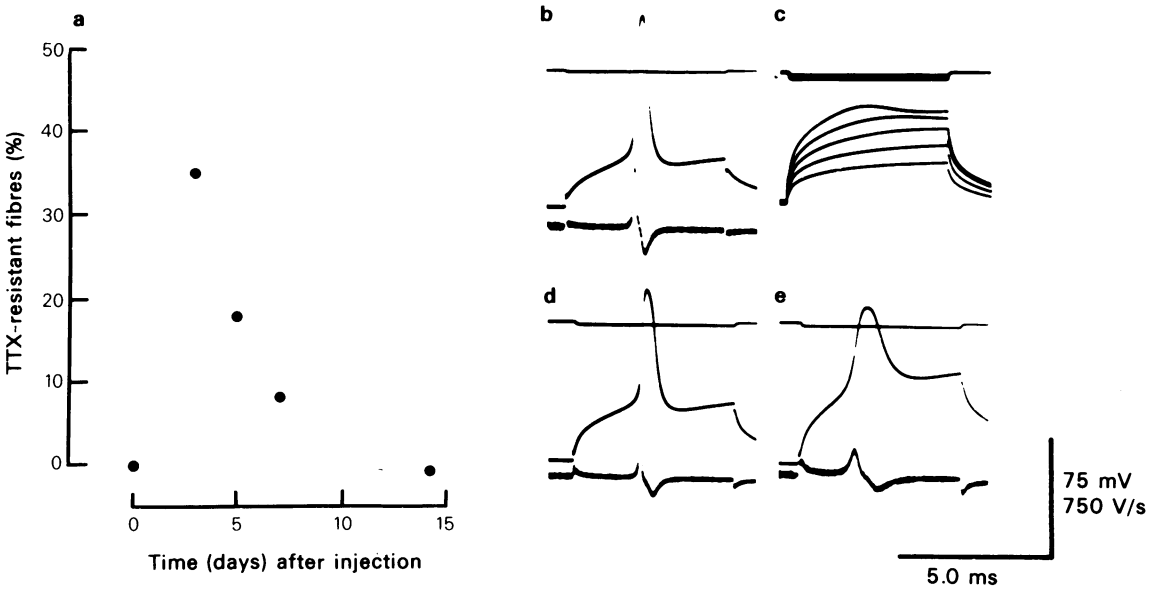

Figure 7 The graph (a) represents the proportion of muscle fibres generating tetrodotoxin (TTX)-resistant action potentials in muscles at various times after the injection of taipoxin. Each point has been calculated from observation on between 10 and 20 muscle fibres in 3–5 muscles; (b) illustrates a typical action potential generated using the double microelectrode technique in a normal muscle fibre, and its inhibition by TTX, 10^{-6} M is illustrated in (c). A similar action potential generated in a 7 day regenerated fibre is illustrated in (d) and a TTX-resistant potential from a regenerated muscle is shown in (e). In (b)–(d), the upper trace is zero potential on which the current pulse is superimposed, the centre trace is the voltage record and the lower trace is the first differential of the voltage record.

that undifferentiated myotubes are not susceptible to toxin assault, whereas fibres which have differentiated are susceptible, an observation similar to that made by Schultz & Lipton (1978) using Marcaine.

The use of antivenom

The incubation of taipan venom, taipoxin and α-taipoxin with the specific taipan antivenom led to a substantial or complete inhibition of myotoxicity. In view of the structural similarity between the subunits of taipoxin and notexin (Fohlman *et al.*, 1976) it was of interest to note that taipan antivenom inactivated notexin and that tiger snake antivenom inactivated taipoxin. The results of these experiments are summarized in Figure 8.

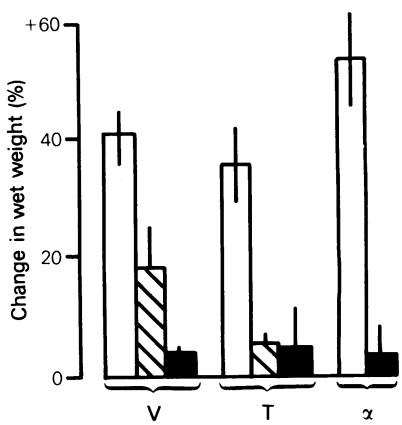

Figure 8 Changes in the wet weight of soleus muscles 24 h after the injection of taipan venom (10.0 μg:V), taipoxin (2.0 μg:T) or α-taipoxin (6.6 μg:α) are illustrated by the open columns. The changes in wet weight were reduced if the venom or toxins were incubated with either taipan antivenom (see hatched column) or with tiger snake antivenom (solid column). The taipan venom was incubated (1 h, 37°C) with either 5 units taipan antivenom or 4 units tiger snake venom; taipoxin was incubated with either 1 unit of taipan antivenom or 4 units tiger snake venom; α-taipoxin was incubated with 13 units of tiger snake antivenom.

Discussion

The data presented in this paper demonstrate that the crude venom of the Australian taipan, and its principal presynaptically active neurotoxin, taipoxin, are myotoxic. Muscle necrosis and degeneration were identified within 3 h of exposure to the myotoxic agents, and were characterized by oedema, the infil-

tration of phagocytic cells and, in individual muscle fibres, by disorganization of the myofibrils, fragmentation of the plasma membrane and a rapid fall in the resting membrane potential. The depolarized muscle fibres were completely inexcitable.

Taipoxin comprises three homologous subunits. Of the three only the basic α-subunit exhibited any significant myotoxicity, neither the neutral β-subunit nor the acidic γ-subunit appearing active in this respect. It is of interest that α-taipoxin is the only neurotoxic subunit and the only subunit with appreciable phospholipase-A_2 activity (Fohlman *et al.*, 1976; Fohlman, Eaker, Dowdall, Lullmann-Rauch, Sjodin & Leander, 1979).

In terms of its structure, strongly basic nature, enzymatic activity, neurotoxicity and myotoxicity, α-taipoxin closely resembles notexin and notechis II/5, the 'myotoxic neurotoxins' from the venom of the Australian tiger snake *Notechis scutatus scutatus* and subunit CA of crotoxin, a neurotoxin isolated from the venom of the South American rattlesnake *Crotalus durissus terrificus* (Lee, 1979).

Fohlman *et al.* (1976) showed that it was not possible to account for the neurotoxicity of taipoxin simply in terms of the activity of the α-subunit. This implied that the presence of the β- and/or γ-subunits in the correct stoichiometry was necessary for the full expression of activity. Similar conclusions might be drawn from the observations presented in Figure 2 (see Results) summarizing the data on myotoxicity. In this case, recombination experiments made it clear that only the acidic γ-subunit is required to raise the toxicity of α-taipoxin to the level of 'native' taipoxin. The potentiation of the activity of the basic α-subunit by the acidic γ-subunit is very reminiscent of the potentiation of the basic CA subunit of crotoxin by the acidic CB subunit (Fraenkel-Conrat, Jeng & Hsiang, 1980). The data presented on the interactions between α- and γ-taipoxin do not allow any speculation on the mechanism of the synergistic behaviour.

Since it was not possible to define precisely the sequence of events that resulted in the degeneration of the muscle fibres after exposure to taipoxin, it is difficult to propose a specific mechanism of action. However, taipoxin is a phospholipase A_2 with a potency similar to that of notexin, and notexin has been shown capable of hydrolysing phospholipids in micelles, fragmented bacterial membranes and intact muscle fibres (Harris & MacDonell, 1981). It is highly probable that the hydrolysis of such lipids is related to the myotoxicity of notexin, and it seems not unreasonable to suppose that the enzymatic activity of taipoxin is somehow related to its myotoxicity. If this is a correct supposition, two possible modes of action exist.

The first possibility is that the toxin produces its pathological effects by altering or destroying the function or structure of the plasma membrane. Since plasma membranes of muscle fibres are rich in phosphatidylcholine and phosphatidyl ethanolamine, both of which may act as substrates for the homologous toxin notexin (Harris & MacDonell, 1981), any alteration in function or structure may be related to the hydrolysis of membrane lipids. This in turn could result in the lesion in the plasma membrane seen routinely in the necrotic muscle fibres (see Figure 5), and in a loss of control of ionic gradients. The free Ca^{2+} concentration of serum is about 10^{-3} M, and that of the cell interior is about 10^{-7} M. A loss of ionic gradients would thus result in a massive increase in internal Ca^{2+} levels. Such an increase has often been implicated in the degeneration of skeletal muscle (see Wrogeman & Penna, 1976; Publicover, Duncan & Smith, 1977 for example) and would be expected to result in hypercontraction, myofibrillary disorganization and mitochondrial abnormalities, all of which were routinely seen in the toxin damaged tissue.

The second possibility is that the toxin is internalized (Kamenskaya & Thesleff, 1974) causing degeneration and necrosis as the result of an indirect action on one or more subcellular organelles. In this context it may be relevant that Ng & Howard (1980) have recently demonstrated that notexin and β-bungarotoxin inhibit calcium uptake by mitochondria and sarcoplasmic reticulum. However, although internalization is theoretically possible (cf. the uptake of cobra neurotoxin by mouse skeletal muscle; Libelius 1975), Howard & Wu (1976) and Dowdall, Fohlman & Watts (1979) have produced evidence to suggest that β-bungarotoxin, notechis II/5 and the α-subunit of taipoxin act on the external surface of sensitive biological membranes.

It is clear, however, that the possession of phospholipase A_2 activity, even if essential for myotoxicity, is not sufficient to confer myotoxicity upon a given substance. Thus β-bungarotoxin is a phospholipase A_2, but it is without significant myotoxic activity against rat skeletal muscle (Harris, unpublished). This must, therefore, lead to the conclusion that non-myotoxic phospholipase A_2 toxins such as β-bungarotoxin, either bind to a different site in the membrane or are unable to establish the necessary relationship with the substrate in order to express myolytic activity.

The regeneration of muscle following assault by taipoxin was rapid and of the discontinuous type (Waldeyer, 1865) occurring within the original basal lamina tubes from myogenic cells which fused and differentiated to form structurally and functionally mature fibres by about 21 days after the initial injury. Several factors probably influence the speed and efficiency of the regenerative response. For example,

neither taipan venom nor taipoxin caused any discernible damage to the small blood vessels and consequently the blood supply and micro-circulation were probably left intact. Moreover, intense and rapid phagocytosis (Aloisi, 1970) and the preservation of an intact basal lamina (Vracko & Benditt, 1972) are reported to stimulate the rapid onset and orderly nature of the regenerative response.

The first sign of regenerating muscle fibres was seen 3 days after the administration of taipoxin. At this stage, the basal lamina tubes contained no more than 11 myoblasts, some of which had fused to form longitudinally orientated myotubes. At least some of these muscle fibres were already functionally innervated, although in terms of resting membrane potential, spontaneous (fibrillatory) activity and resistance to tetrodotoxin, their general physiological state was immature. It has been suggested that some of the variability seen in the properties of these immature fibres may result from the unintentional sampling of spared (i.e. nominally normal) fibres in the periphery. This possibility cannot be excluded but is highly unlikely. Spared fibres have never been observed in the periphery, and this observation dominated the design of the experiments. However, since the techniques used involved the insertion of two microelectrodes into very small fibres (diameter $< 12\ \mu m$), it is possible that the larger or more robust fibres were preferentially sampled. Quite clearly, the physiological data obtained on early regenerates have to be interpreted with caution.

By between 7 and 10 days, the majority of fibres were functionally innervated. The spontaneous (fibrillatory) activity and tetrodotoxin-resistance was correspondingly infrequent. At this stage Ca^{2+}-activated myofibrillar ATPase activity could be demonstrated histochemically. The full histochemical differentiation of the regenerating muscle fibres into recognizable fibre-types occurred between 14 and 21 days, by which time the physiological profile of the fibres was essentially normal. The observations imply that the complete metabolic differentiation of regenerating muscle fibres requires the establishment and maintenance of a functional innervation, but this needs experimental confirmation.

The only obvious morphological abnormalities of the fully regenerated muscle fibres were the persistence of central nucleation, and the relatively high incidence of muscle fibre splitting. The former feature has been observed by several authors (Hall-Craggs, 1974; Schmalbruch, 1976; Harris & Johnson, 1978), and has never been adequately explained. Muscle fibre splitting is a common feature of diseased skeletal muscle and may be observed in a variety of unrelated conditions (Cullen & Mastaglia, 1980). In the case of the taipoxin-damaged muscles, there would appear to be four possible mechanisms to account for the presence of 'split' fibres. Firstly, they might arise from the longitudinal fission of undamaged fibres as a response to a dramatic but transient increase in work-load. Secondly, they might arise as a result of the presence of phagocytic cells or unfused myoblasts within a 'complex' of fusing myoblasts, since this could encourage the formation of a septum in the regenerating fibre. Thirdly, they might arise as the result of the incomplete fusion of two or more regenerating myotubes and fourthly, they might arise by cytoplasmic budding from an undamaged or regenerating muscle fibre. The data obtained in this study do not permit one to favour any of the above hypotheses.

In conclusion, the results of this work demonstrate that both taipan venom and the principal neurotoxin taipoxin are capable of inducing severe myolysis. Myolysis has not been described as a feature of human envenomation by the taipan, and death and distress following a bite are generally considered due to neurotoxic poisoning (Campbell, 1967; Trinca, 1969). However, it is possibly relevant that until 1975 it was believed that envenomation by the Australian tiger snake was not accompanied by myolysis; it is now appreciated that myolysis is a common and perhaps characteristic feature of envenomation by this animal (Hood & Johnson, 1975; Sutherland & Coulter, 1977). The implication of these considerations is that myolysis must be considered a possible clinical feature in patients envenomed by the taipan, *Oxyuranus scutellatus*.

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