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ANODE BREAK EXCITATION IN DENERVATED RAT SKELETAL MUSCLE FIBRES

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SUMMARY

1. Anodal break action potentials were found to be a feature of denervated rat skeletal muscle fibres.

2. Depolarized innervated fibres failed to respond to anodal break stimulation in the same way as denervated fibres.

3. The critical level for action potential generation is related to membrane polarization in denervated fibres but not in innervated fibres. This relationship is inhibited by the I.P. administration of actinomycin D, which also prevents the onset of anodal break action potentials in 3-day denervated fibres.

INTRODUCTION

It has been apparent for more than a century that the termination of an applied anodal current to nerve may lead to 'anode break stimulation'. The phenomenon is less common than is generally believed however.

Frankenhaeuser & Widen (1956) observed that only deteriorating nerve fibres or fibres exposed to high potassium concentrations were able to respond to anode break stimulation. These authors concluded that a small degree of depolarization was a necessary requirement for the observation of this phenomenon. Hodgkin (1951) suggested that only moderately inactivated fibres may respond to anode break stimulation, and that termination of anodal polarization leads to a situation where the inward sodium current is greater than normal, whereas the outward potassium current is less than normal, conditions that favour rapid regenerative depolarization.

Ooyama & Wright (1961) reported that anodal break action potentials could be obtained at the nodes of single frog nerve fibres only under certain special circumstances. There were (*a*) if the experiment was made on preparations bathed in high potassium solutions, (*b*) if the preparations had deteriorated or (*c*) if anodal polarization was applied during a con-

ditioning depolarization which exceeded threshold for cathodal excitation. They found that fibres could respond to true anode break stimulation only if the membrane was broken down and depolarized by the use of very large anodal pulses.

In experiments we were making on denervated rat skeletal muscle, we observed that anodal break excitation, without membrane break-down, was a remarkably common occurrence. In view of this, and as there is a lack of information in the literature on this phenomenon in skeletal muscles, we decided to carry out the following investigation.

METHODS

All experiments were made on isolated extensor digitorum longus (EDL) muscles of 150–200 g female Wistar rats. The rats were anaesthetized with ether and the left EDL was denervated by section of the sciatic nerve in the mid-thigh region. At appropriate times after denervation the animals were killed and both the denervated and contralateral muscles were removed. The muscles were mounted in a Perspex chamber which was continuously perfused with oxygenated fluid (Liley, 1956) maintained at pH 7·2–7·4 and 30° C. In some experiments muscles were bathed with fluid containing tetrodotoxin (10^{-6} M).

Action potentials were generated and recorded with a double micro-electrode technique. With the two micro-electrodes inserted into the same fibre as close together as possible (50–100 μm apart), one micro-electrode was used to pass current and the other to record the resulting potential change. The current micro-electrode was connected to the output of a constant current generator capable of producing a continuous anodal or cathodal current of variable intensity, upon which triggered anodal or cathodal pulses of variable duration and intensity could be superimposed. Conventional glass micro-electrodes, filled with 3M-KCl, were used with DC resistances between 5 and 10 MΩ.

In one group of rats actinomycin D was adminstered I.P. 0·5 mg/kg at the time of denervation and 0·25 mg/kg 2 days later. Actinomycin D was obtained from Sigma Chemical Company, tetrodotoxin from Sankyo Co. Ltd, Tokyo.

RESULTS

Innervated EDL muscles were generally unable to generate anodal break action potentials, even when the membrane was hyperpolarized to about -200 mV above the resting membrane potential (Fig. 1*a*), even though the anode pulse in these experiments was usually large enough to produce membrane break-down, indicated by an irregular decline in input resistance. Occasionally these cells would generate a series of action potentials with large pre-potentials, a decline in amplitude with time and accompanied by a fall in the resting membrane potential, suggesting that they were the result of membrane damage.

Effect of denervation

In some preliminary experiments on 8-day denervated EDL muscle fibres, it was found that anodal break action potentials could always be generated if the muscle fibre membrane was first locally hyperpolarized for 40 msec to a level of approximately -100 mV by the anodal pulse. Consequently, anodal break action potentials were generated routinely in denervated muscle fibres with a 40 msec hyperpolarizing current pulse that raised the membrane potential to about -100 mV (Fig. 1*b*). In

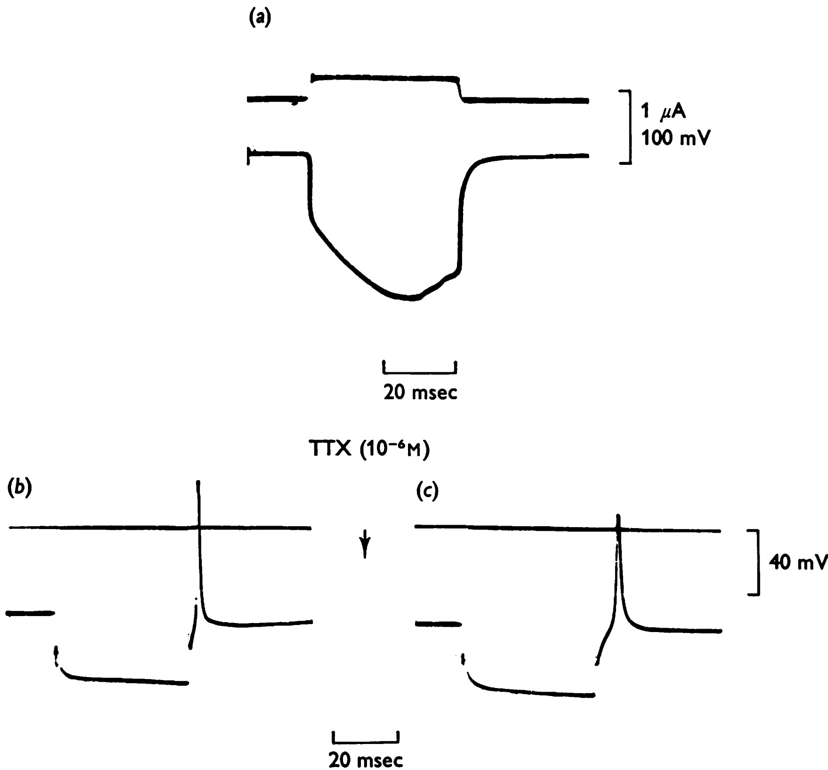

Fig. 1(*a*). The lower trace shows the change in membrane potential of an innervated rat EDL fibre in response to the passage of a rectangular inward current pulse (upper trace) via a second intracellular micro-electrode. Even in the presence of membrane break-down, no anodal break excitation occurs. (*b*) Typical response of a 3-day denervated EDL fibre membrane (lower trace) to an inward current pulse. On termination of the pulse, an action potential is generated which overshoots the zero potential (upper) trace, even though the fibre has been locally polarized to only -100 mM. (*c*) Lower trace shows that the anodal break action potential of a 3-day denervated fibre exhibits partial resistance to tetrodotoxin (TTX, 10^{-6} M). The action potential is seen to overshoot the zero potential (upper) trace.

muscle fibres that had been denervated for only 2 days it was often necessary to hyperpolarize the muscle membrane to a level in excess of -100 mV before action potentials could be generated. In addition action potentials generated in muscle fibres on the second day following nerve section rarely overshot zero potential. By day 3, however, most action potentials overshot zero potential when the membrane potential was pre-set to -100 mV, and by day 4 all action potentials were seen to overshoot zero potential. The onset of anodal break action potentials, as a function of time after denervation, is shown in Table 1. In muscles denervated for 3 days action potentials generated by anodal break excitation were shown to be sodium dependent as they were blocked by the removal of external sodium (in these experiments choline was used to replace sodium).

TABLE 1. The onset of anode break action potentials in rat EDL fibres in normal fluid and in bathing fluid containing tetrodotoxin (TTX) as a function of time after denervation

Days denervated	Normal fluid*	TTX (10^{-6} M)*
Controls	0/25	0/6
1	0/20	—
2	40/90	9/20
3	37/37	27/30
3†	0/10	0/10
4	26/26	10/10
8	24/24	—
9	12/12	—

* No. of fibres responding/no. of fibres examined.
† From two rats receiving actinomycin D.

Tetrodotoxin, at a concentration of 10^{-6} M, completely blocks action potential generation in innervated mammalian muscle fibres, but following nerve section muscle fibres become less sensitive to the effects of tetrodotoxin. Thus action potential generation in denervated muscle fibres becomes partially resistant to the blocking effect of 10^{-6} M tetrodotoxin in that action potentials with a reduced rate of rise and amplitude of overshoot are produced, provided the membrane is first locally polarized to -80 to -90 mV before depolarizing current is applied (Redfern & Thesleff, 1971*b*). Since tetrodotoxin (10^{-6} M) resistant action potentials can also be produced in developing rat muscle before the establishment of the adult pattern of innervation (Harris & Marshall, 1973), it was of interest to note that the action potentials generated by anode break stimulation in denervated muscle fibres were also resistant to the effects of tetrodotoxin (10^{-6} M) (see Fig. 1*c* and Table 1), which would support the hypothesis that the ability of a muscle fibre to respond to anode break stimulation is a direct result of the denervation process.

Effect of polarization on the threshold of action potential generation

The critical level of membrane potential at which an action potential was generated was taken as an indication of threshold (Kita, 1966) and was the measured value (in mV) from zero potential to the positive going point of inflexion of the action potential (Fig. 2).

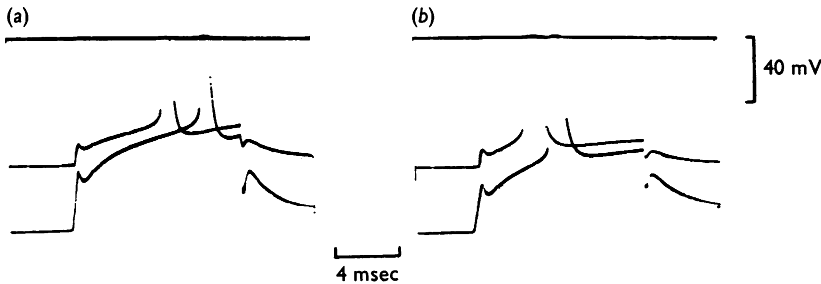

Fig. 2. The effect of membrane hyperpolarization on the critical level for action potential generation by cathodal stimulation. (*a*) In an innervated fibre, there is no change in the critical level when the fibre is polarized to −80 or −120 mV. (*b*) The critical level of a 3-day denervated fibre is increased when the membrane is locally polarized to −120 mV before cathodal stimulation.

TABLE 2. The effect on the critical level for action potential generation of setting the membrane potential to −80, −100 or −120 mV in normal and denervated rat EDL muscle fibres

Days denervated	Set −80 mV	Set −100 mV	Set −120 mV
Controls	−53·45 ± 0·39 (20)	−53·5 ± 0·48 (14)	−53·35 ± 0·92 (20)
Day 3	−53·38 ± 0·61 (16)	−59·67 ± 1·32 (15)	−65·39 ± 1·74 (18)
Day 4	−57·80 ± 0·83 (30)	−67·9 ± 1·14 (10)	−69·47 ± 0·69 (32)
Day 3*	−53·8 ± 1·56 (5)	−51·8 ± 0·58 (5)	−52·2 ± 0·37 (5)

Figures quoted are means ± S.E. Numbers of muscle fibres studied shown in parentheses.

* From rats receiving actinomycin D.

The relation between membrane potential and critical level was investigated in both normal and denervated EDL muscles. The membrane was locally polarized to −80, −100 or −120 mV for approximately 30 sec, before a 10 msec cathodal pulse was superimposed upon the set level. It would have been desirable to record an action potential from each fibre at each of the three set levels; but since cells were occasionally damaged by the micro-electrodes during the first twitch, generally only one action potential was recorded from each muscle fibre penetrated. Table 2 shows

the differences in critical levels at three set levels for innervated and denervated muscles. Hyperpolarizing the membrane of normal muscle fibres brought about no significant change ($P > 5\%$) in the critical level of action potentials generated in fifty-four muscle fibres (see Fig. 2*a* for example). However, muscles that had been denervated for 3 and 4 days showed, using one-way analysis of variance, a statistically significant ($P < 5\%$) increase (towards a more negative value) in critical level as the membrane was hyperpolarized (see Fig. 2*b*). There was no significant difference between the critical levels of innervated and 3-day denervated muscle fibres set to -80 mV ($P > 5\%$), but by day 4 the critical level had increased significantly ($P < 5\%$). Moreover, when the membrane potential was set to -100 and -120 mV the critical levels of action potentials generated in muscle fibres denervated for both 3 and 4 days were significantly different ($P < 5\%$) from these levels obtained in innervated muscle fibres. These observations would suggest that anodal break action potential generation is possible in denervated fibres because the hyperpolarizing pulse brings the threshold for action potential generation to a level similar to (or even more negative than) the resting membrane potential. Thus action potentials are generated as the membrane potential moves to its resting level when the hyperpolarizing current is switched off.

Effect of actinomycin D on action potentials generated by anodal and cathodal stimulation

If actinomycin D (an inhibitor of DNA-dependent RNA synthesis) was administered I.P. to the rats at the time of denervation, the muscle fibres were unable to respond to anode break stimulation (Table 1).

Similarly, action potentials generated in 3-day denervated muscle fibres, taken from rats treated with actinomycin D, showed no significant change ($P > 5\%$) in critical levels when membranes were hyperpolarized prior to the passage of a cathodal pulse (Table 2). This is in contrast to the significant change in critical level that occurs with hyperpolarization in 3-day denervated, untreated muscle fibres.

Grampp, Harris & Thesleff (1972) reported that blockers of protein synthesis inhibit the onset of denervation changes in mouse skeletal muscle, and suggested that the nerve might well exert a regulatory influence over the genome of the muscle cell. It would thus appear that the ability to generate anodal break action potentials also depends either directly or indirectly on the synthesis of new proteins.

Tenotomized muscles

By sectioning the distal tendon of EDL muscles it was possible to render these muscles atrophic. Denervated muscles also become atrophic and it

could have been that the occurrence of anodal break action potentials in denervated muscle fibres is related in some way to the underlying atrophy. However, in a total of sixty-four fibres studied in muscles tenotomized for 7 and 13 days we were unable to demonstrate either anodal break excitation or any changes in the critical level of action potentials.

Effect of depolarizing innervated muscle fibres

After denervation of mammalian muscle fibres, there is a fall in resting membrane potential (Albuquerque & McIsaac, 1970; Redfern & Thesleff, 1971*a*). By day 4, following nerve action, the membrane potential has fallen to about -60 mV. Since it has been proposed that only moderately inactivated nerve fibres respond to anodal break stimulation (see Introduction), the frequency with which we observed anodal break action potentials in denervated muscle fibres might well have been a consequence of their low resting membrane potential. We therefore depolarized innervated muscle fibres to about -60 mV by either increasing the external potassium ion concentration to 10 mM or by passing a steady outward current.

Although it is possible to obtain anodal break excitation in desheathed frog nerves by increasing the external potassium to 10 mM (Frankenhaeuser & Widen, 1956) we were unable to demonstrate anodal break excitation in either normal or 13-day tenotomized muscles where the external potassium had been increased to 10 mM. Similarly, when innervated muscle fibres were locally depolarized for 15 sec by the passage of an outward constant current, superimposing hyperpolarizing pulses of 40 msec duration produced anodal break action potentials in the absence of membrane breakdown in only three out of twenty-six fibres tested in four muscles. Where membrane break-down occurred a train of action potentials were produced. However, we were unable to produce action potentials generated in this manner in the presence of tetrodotoxin (10^{-6} M).

DISCUSSION

Thus depolarized but innervated muscle fibres failed to respond to anode break stimulation in the same way as 4 day denervated fibres. Anode break excitation could only be produced in non-depolarized innervated muscle fibres by using hyperpolarizing pulses sufficiently large to effect membrane break-down (see Introduction; Ooyama & Wright, 1961). On the other hand, denervated fibres were able to respond to anode break stimulation at much lower levels of membrane hyperpolarization at which signs of membrane damage were never observed.

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