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CONTROL OF ACETYLCHOLINE SENSITIVITY AND SYNAPSE FORMATION BY MUSCLE ACTIVITY

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SUMMARY

1. The formation of ectopic junctions between the 'foreign' superficial fibular nerve and the soleus muscle of adult rats, and its relation to changes in extrajunctional sensitivity to acetylcholine (ACh), has been studied by denervating the muscle 3–6 weeks after implanting the foreign nerve.

2. The earliest signs of nerve–muscle transmission were seen 2·5–3 days after denervation, in those fibres where the extrajunctional ACh sensitivity first reached its full post-denervation level. The number of innervated fibres continued to increase throughout the first week after denervation until 70–100 % of fibres underlying the foreign nerve growth were innervated.

3. Direct stimulation of muscles with chronically implanted electrodes from the time of denervation prevents the formation of functional neuromuscular junctions (n.m.j.s). If stimulation begins 2 or 4 days after denervation, some functional n.m.j.s are formed which can be detected 7–9 days after denervation, though not as many as in the absence of stimulation.

4. Direct stimulation of muscles from the time of denervation prevents the development of detectable extrajunctional ACh sensitivity. If stimulation begins 2 days after denervation nearly maximal sensitivity develops during the third day and then rapidly declines to undetectable levels by the beginning of the eighth day after denervation.

INTRODUCTION

Following denervation, many changes in the surface of vertebrate skeletal muscle occur. One of these is the development of sensitivity to acetylcholine (ACh) outside the vicinity of the nerve-muscle junction (n.m.j.) (Axelsson & Thesleff, 1959; Miledi, 1960*a*). This sensitivity is known to depend upon the presence of a complex glycoprotein receptor for ACh which appears in the extrajunctional membrane after denervation (see Brockes, Berg & Hall, 1976, for review). Another change in the extrajunctional region is the appearance of the ability to make new n.m.j.s. with experimentally introduced nerves (Elsberg, 1917; Aitken, 1950; Fex & Thesleff, 1967). In contrast to ACh sensitivity, nothing is known about the molecular basis of this property.

The possibility that the ACh binding site on the receptor might itself play an essential role in initiating the events in n.m.j. formation, though attractive (cf. Katz

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& Miledi, 1964), appears to be ruled out by experiments in which pharmacological block of that site in a non-innervated muscle cells fails to prevent differentiation of the terminal of an ingrowing motor axon (Crain & Peterson, 1971; Cohen, 1972; Jansen & Van Essen, 1975). On the other hand, a number of experiments show that sensitivity to ACh and the ability to make new n.m.j.s often appear together. For example, block of action potentials in the motor nerve (Jansen, Lømo, Nicolaysen & Westgaard, 1973) or local treatment with botulinum toxin (Fex *et al.* 1966) both lead to increased extrajunctional ACh sensitivity and to the ability to form new n.m.j.s. Since direct stimulation of denervated muscles prevents both these events (Lømo & Rosenthal, 1972; Jansen *et al.* 1973) a key feature of these treatments may well be the resulting paralysis of the muscle. While apparently having different molecular bases, these properties of the muscle fibre surface may thus be subject to similar controlling influences.

In this study, we have examined in greater detail how the formation of new ectopic n.m.j.s. in denervated adult muscle is related to the development of extrajunctional ACh sensitivity, and how both processes are influenced by imposed electrical stimulation.

METHODS

All experiments were made on male white rats which weighed approximately 200 g at the time of the initial operation.

Surgical procedures. Under Nembutal anaesthesia, the superficial fibular nerve was cut away from the fibular muscles and the central end placed in the space between the soleus and gastrocnemius muscles, near the proximal end of the soleus. Subsequent denervation of the soleus was accomplished by removing several millimetres of the tibial bundle of the sciatic nerve in the thigh, under ether anaesthesia.

Chronic stimulation. The procedure for chronic implantation of electrodes and subsequent stimulation has been described in detail elsewhere (Lømo & Westgaard, 1975). In all the experiments reported here, the temporal pattern of stimulation was as follows; once every 100 sec a train of 100 stimuli, each 1 msec long, at a frequency of 100 Hz was given (over-all mean frequency 1 Hz). To ensure adequate stimulation of the entire soleus muscle, careful positioning of the electrode in the leg was important, and currents of 20–25 mA were used. Inadequate stimulation of denervated muscles was indicated by the presence of fibres with high ACh sensitivity, and such muscles were not included in the results.

Acute experiments. Both soleus muscles, together with the soleus and/or fibular nerve, were removed from each rat, and mounted in a chamber (*ca.* 10 ml. volume) perfused with gassed (95% O_2–5% CO_2) mammalian saline solution at a rate of 1–2 ml/min. The composition of the bathing solution was (mM): Na^+, 149; K^+, 5; Ca^{2+}, 2; Mg^{2+}, 1; $H_2PO_4^-$, 1; HCO_3^-, 12; Cl^-, 147; D-glucose, 11.

The preparations were transilluminated so that the extent of the fibular nerve could be seen (see Results). In determining the extent of new innervation by the fibular nerve, only those surface muscle fibres which were clearly within the visible zone of nerve growth were considered. Intracellular membrane potentials were recorded with conventional micropipettes filled with 4 M-K acetate, which had resistances of 20–60 MΩ.

Sensitivity to ACh, applied iontophoretically, was determined as previously described (Lømo & Westgaard, 1975) and expressed as mV depolarization per nC of charge passed through the drug pipette (Miledi, 1960*a*).

The pulse which ejected ACh was generally kept at 10^{-8} A and the duration adjusted to give a response of less than 5 mV.

RESULTS

During the first 2 weeks after implanting the proximal end of the cut fibular nerve onto the soleus muscle, the nerve grows over the surface of the muscle and forms a highly vascularized growth zone which contains numerous axonal sprouts (Frank, Jansen, Lømo & Westgaard, 1975). The extent and character of the growth is rather variable, depending, it seems, on exactly how the nerve comes to lie over the muscle. In about 25% of the operations we performed (31/132) the axonal sprouts either grew off into the intramuscular connective tissue or into the connective tissue

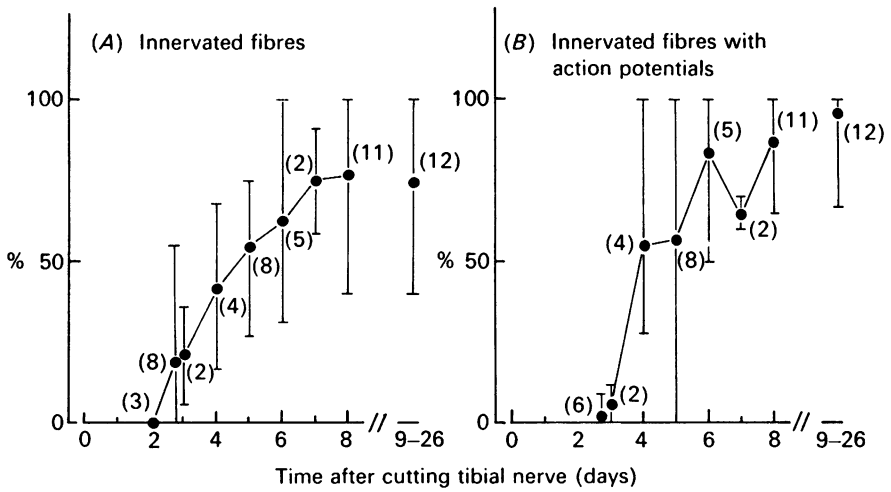


Fig. 1. Development of new innervation of denervated soleus muscles by previously transplanted fibular nerves. *A*, fraction of soleus muscle fibres underlying visible sprouts of the fibular nerve which showed some signs of innervation by the fibular nerve (either evoked or spontaneous). *B*, fraction of innervated fibres which responded to fibular nerve stimulation with an action potential. Values were calculated for each muscle, and the average and range of these are shown (number of muscles in brackets). Usually fifteen to thirty fibres were studied in each muscle.

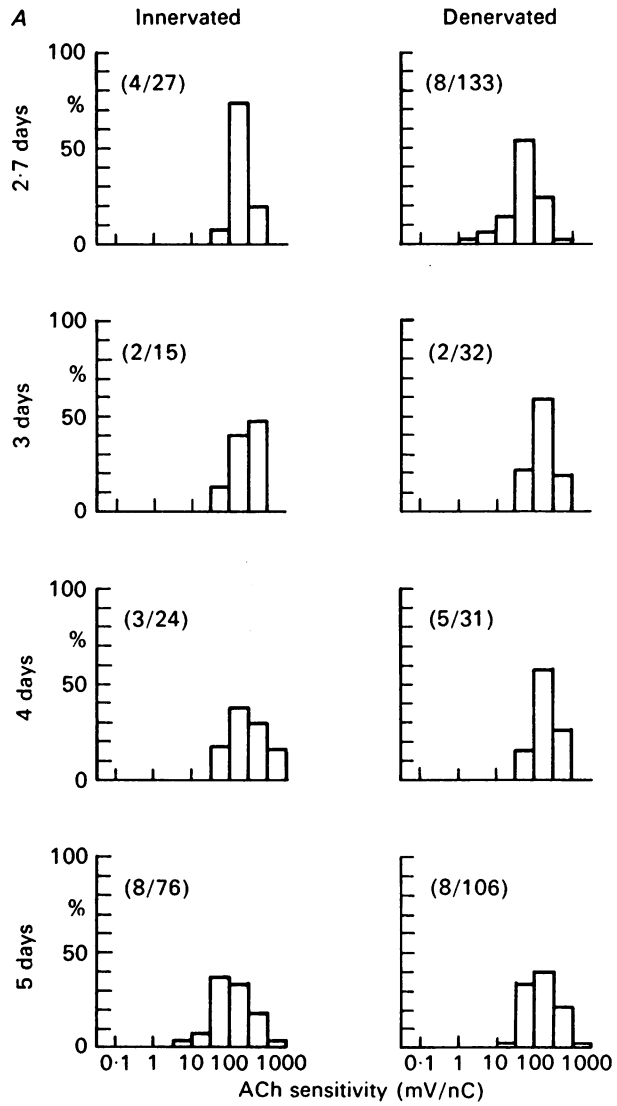
immediately overlying the muscle, in which case the entire growth could be dissected away. In most cases, however, the neural growth adheres tightly to the muscle and the axons seem to establish intimate contact with the muscle fibres. Within several weeks, many of these sprouts become myelinated, sometimes to within 100 μm or less of their terminations (as visualized after staining with methylene blue, Waerhaug & Korneliussen, 1974). The spatial limits of the adherent growth zone can usually be determined fairly unambiguously by inspection of the freshly isolated preparation with the dissecting microscope (see Methods). It rarely comes closer to the original end-plate band than 1–2 mm and usually extends about half-way across the muscle.

So long as the soleus nerve was intact, very few of the fibular nerve axons innervated soleus muscle fibres. In ten out of thirty-one muscles with an intact soleus nerve, studied 15–60 days after nerve transplantation, a few isolated muscle fibres (usually fewer than five in any one muscle) could be seen to contract on stimulation of the fibular nerve. It was only after some searching that these fibres could be

identified and impaled with a micro-electrode, and they might well have been missed during a routine study of the surface fibres.

Development of foreign innervation following denervation

To initiate extensive formation of new n.m.j.s, the tibial nerve was cut, after allowing a period of 3–6 weeks for the fibular nerve to grow into the muscle (Fex & Thesleff, 1967). Neuromuscular transmission had failed at most of the isolated soleus



Figs. 2*A* and *B*. Distribution of extrajunctional ACh sensitivity of soleus muscle fibres at different stages during the development of ectopic innervation by the fibular nerve. Fibres innervated by the fibular nerve are shown in the left column, while those remaining fully denervated are shown on the right. The time (days) after cutting the tibial nerve is shown for each pair of histograms. Numbers in brackets are the number of muscles and fibres in each sample.

nerve endings 18 hr later. The first signs of a response to stimulation of the fibular nerve were seen 2·5–3 days after denervation. During the next few days, the number of innervated fibres steadily increased and by the end of the first week after denervation, about 75% of the fibres underlying the visible growth zone showed some signs of innervation (Fig. 1*A*).

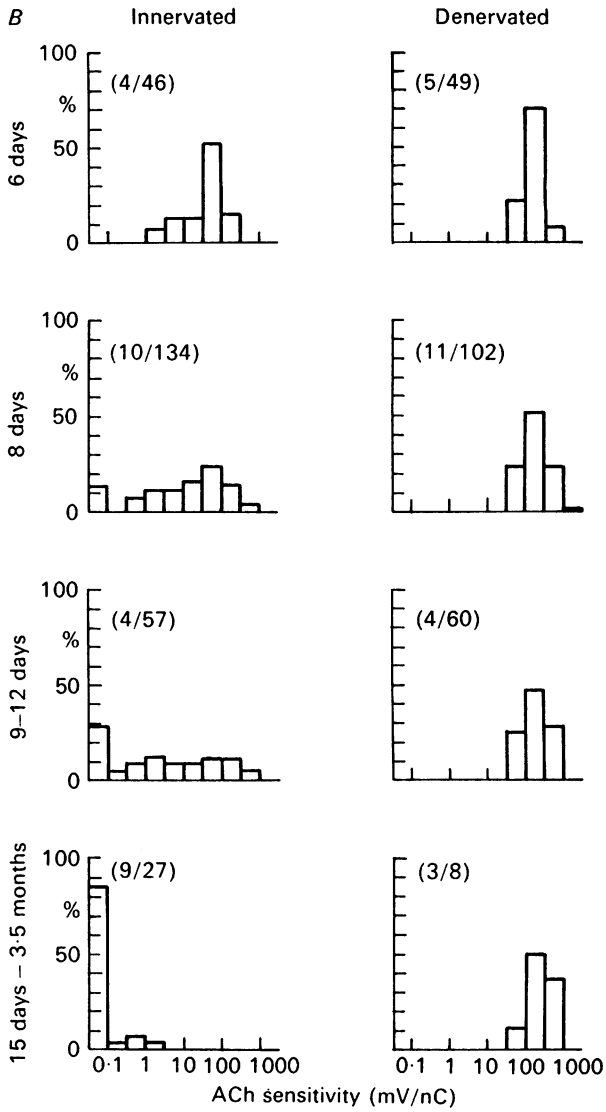


Fig. 2*B*. For legend see facing page.

When tested in isolated nerve-muscle preparations at room temperature, the efficiency of transmission at newly formed synapses was low. Only about 10% of the innervated fibres examined 2·5–3 days after cutting the tibial nerve responded to fibular nerve stimulation with an action potential. In the rest, only subthreshold end-plate potentials, similar in amplitude to the spontaneously occurring miniature

end-plate potentials, were seen. During the following few days, the efficiency of transmission increased markedly, so that 8 days after cutting the tibial nerve, an average of more than 85 % of innervated fibres (or about 65 % of all fibres underlying the growth zone) gave action potentials (Fig. 1*B*).

While not studied in detail here, it is evident that the morphology of newly formed ectopic n.m.j.s is still primitive even 8–10 days after denervation of the soleus (Korneliussen & Sommerschild, 1976). Our preliminary observations show that histochemically detectable cholinesterase activity (demonstrated with the method of Buckley & Heaton, 1968) develops gradually at the new n.m.j.s. Signs of enzyme activity were generally first seen 6–7 days after denervating the soleus, but a pattern of staining qualitatively similar to that at mature n.m.j.s was not seen for a further week or so.

Changes in extrajunctional ACh sensitivity associated with new innervation

To study how the development of new innervation is related to changes in extrajunctional ACh sensitivity, the response of muscle fibres to locally applied ACh was determined at different times after cutting the tibial nerve. To reduce the influence of any local effects which the new n.m.j.s might have had on ACh sensitivity, fibres were tested at the edge of the zone of fibular nerve growth. Those fibres which responded to fibular nerve stimulation with action potentials or a detectable end-plate potential were considered to be innervated. Fibres which gave no detectable response were considered still denervated, though this group would also have included any fibres with a subthreshold end-plate potential too small to be detected at the recording site.

The results of these studies are shown in Fig. 2, in which the distribution of sensitivities in samples of fibres deemed innervated or denervated by the above criteria is shown as a function of time after denervation. For ease of comparison, the median sensitivity of each distribution is plotted against time after denervation in Fig. 3.

Development of sensitivity. In confirmation of earlier studies (e.g. Axelsson & Thesleff, 1959; Lømo & Rosenthal, 1972) the ACh sensitivity of denervated soleus fibres increased approximately 1000-fold during the second and third days after denervation (Figs. 3, 4).

When the first responses to stimulation of the fibular nerve were seen, 65–66 hr after denervation, many fibres were not yet fully sensitive (Fig. 2). However, those fibres which were innervated at this time were as sensitive as expected for fibres innervated 3 days or more, and as a group were therefore slightly more sensitive than the non-innervated fibres in the same muscles (Fig. 3). Further, at this time, the innervated fibres represented nearly half (25/58) of all the fibres tested whose sensitivity was greater than 100 mV/nC. In contrast, virtually no fibres (2/102) with sensitivities less than 100 mV/nC were innervated. Thus, signs of transmission at the new nerve-muscle junctions were not detectable until after the extrajunctional sensitivity had reached its final high level.

Decline of sensitivity. Fibres which became innervated by the fibular nerve lost their extrajunctional ACh sensitivity during the first 2 weeks after cutting the soleus nerve. The first newly innervated fibres with sensitivities lower than 30 mV/nC

(a value encountered in only 2/406 denervated fibres studied 3 days or more after denervation) were seen 5–6 days after cutting the soleus nerve (Fig. 2). By the 8th day after denervation, the sensitivity of some fibres was already too low to be detected, and by the 15th day, nearly all innervated fibres were insensitive.

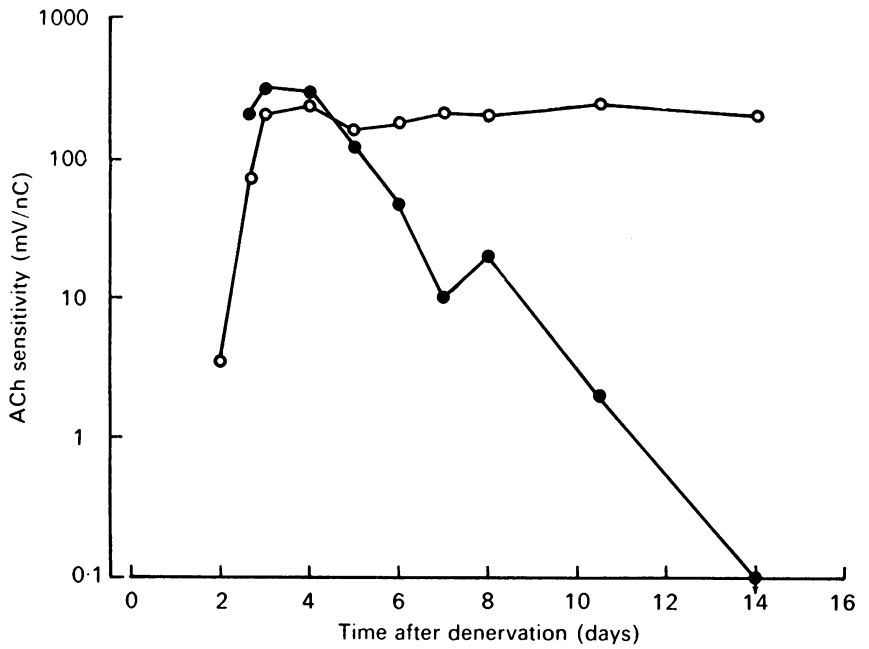


Fig. 3. Extrajunctional ACh sensitivity of soleus fibres at different stages during the development of ectopic innervation by the fibular nerve. Values are the medians of the samples shown in Fig. 2, as well as those from several additional times. ○, denervated fibres; ●, fibres innervated by the fibular nerve. (Point with downward arrow indicates no detectable sensitivity.)

Effects of stimulation on n.m.j. formation

In their study of the effect of direct stimulation on the ectopic reinnervation of rat soleus muscle, Jansen, Lømo, Nicolaysen & Westgaard (1973) assessed the extent of innervation by measuring the strength of contraction elicited by fibular nerve stimulation. We have examined the state of innervation of individual fibres with intracellular recordings and have fully confirmed their basic findings. When direct stimulation was started at the time of denervation and was deemed fully effective (see Methods) no innervated fibres were found 4–6 days later (Table 1). In contrast, unstimulated control muscles were well innervated at this time (Fig. 1, Table 1).

It was of interest to learn how long a period of muscle inactivity was required to allow the formation of stable innervation. Experiments were therefore made in which direct stimulation was started either 2 or 4 days after denervating the soleus muscle. Once again, the extent of innervation was tested 4–6 days after stimulation began, when an average of more than 70% of muscle fibres underlying the foreign nerve growth were innervated in control muscles.

Whether stimulation was started 2 or 4 days after denervation, an appreciable

number of innervated fibres was found (an average of 20 or 35% respectively, Table 1). Thus, a period of inactivity lasting only 2 days is adequate to allow formation of some functional n.m.j.s. At the same time, though the sample sizes are small, the data in Table 1 suggest that fewer muscle fibres were innervated in the stimulated muscles than in the unstimulated controls.

TABLE 1. Effect of chronic stimulation of denervated soleus muscles on the development of innervation by a previously transplanted fibular nerve

Stimulation (for parameters see Methods) began 0, 2, or 4 days after cutting the original soleus innervation. In the acute experiments the response of a number (n) of muscle fibres in the stimulated muscle and in the unstimulated contralateral muscle to stimulation of the fibular nerve was tested. Any evoked response was taken as a sign of innervation. In some cases, the foreign nerve had not grown over the control muscle and these are indicated by blanks in the Table

Onset of stimulation (days after denervation)	Acute experiment (days after denervation)	Fibres with foreign innervation			
		Stimulated muscle		Unstimulated muscle	
		%	n	%	n
0	4	0	(No contraction)	32	25
0	6	0	10	—	—
0	4	0	20	17	18
0	4	0	28	50	28
2	8	28	32	82	33
2	8	23	47	94	18
2	8	22	40	96	29
2	8	17	12	—	—
2	7	20	50	—	—
4	8	24	17	—	—
4	9	33	48	—	—
4	8	50	32	100	37

Effects of stimulation on extrajunctional ACh sensitivity

It is known that direct stimulation of denervated soleus muscles from the time of denervation prevents the appearance of extrajunctional ACh sensitivity, if the pattern of stimulation we have used is employed (Lømo & Westgaard, 1976). If stimulation does not begin until 5 days after denervation, when the muscle is fully sensitive, then that sensitivity is abolished within about 10 days (Lømo & Rosenthal, 1972; Lømo & Westgaard, 1975). The experiments in the previous section show that even when direct stimulation begins 2 days after denervation – that is, before full ACh sensitivity has developed – synapses can form. To see how ACh sensitivity changes in these circumstances we stimulated denervated soleus muscles, which had not received a fibular nerve transplant, starting 2 days after denervation (Fig. 4).

In spite of the vigorous activity of the muscles during the third day after denervation, the median ACh sensitivity (tested in the region of the muscle far from end-plates) rose to very nearly the full post-denervation level, only to begin to decline

almost immediately. Within 5 days of the onset of stimulation, the median sensitivity was once again in the range expected for a normally innervated muscle.

In contrast, when stimulation was started only one day after denervation, only a very small increase in sensitivity was seen at the end of the third day after denervation. If the onset of stimulation was delayed until 3 days after denervation, when full sensitivity had already developed, than the decline of sensitivity occurred at a rate similar to that seen after 2 days or 5 days (Lømo & Westgaard, 1975). In each of these three cases, the maximum rate of decline of sensitivity begins about 2 days after the onset of stimulation and corresponds roughly to an exponential decline with a half-time of 6–9 hr.

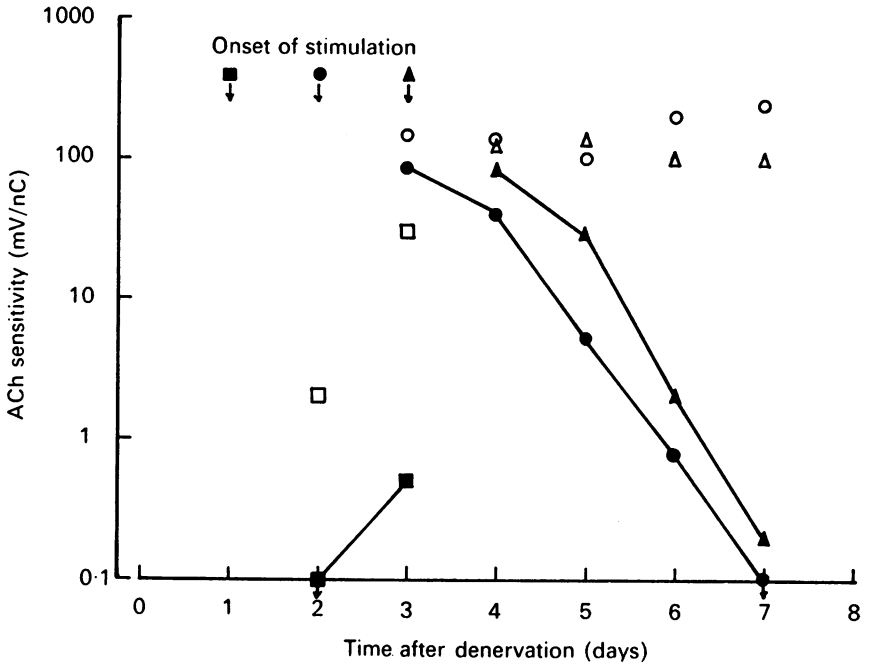


Fig. 4. Effect on extrajunctional ACh sensitivity of denervated soleus fibres of direct electrical stimulation with chronically implanted electrodes (see Methods) starting at different times after denervation. Stimulation started 1 (■), 2 (●), or 3 (▲) days after denervation. Open symbols show results from unstimulated contralateral muscles denervated at the same time. Values are medians of samples of 20–120 fibres in one to six muscles (average, sixty fibres in three muscles). Points with downward arrows indicate no detectable sensitivity.

DISCUSSION

These experiments confirm the close parallels between extrajunctional sensitivity to ACh and the ability to form new functional n.m.j.s. Thus, in the development of ectopic innervation, the first fibres to become innervated were also those which first became fully sensitive to ACh. In the stimulation experiments, 2 days of inactivity was long enough to allow some new n.m.j.s to form, though not as many as in unstimulated muscles, and was also just long enough to allow the development of full ACh sensitivity in most fibres. This suggests that the kinetics of the response of both these properties of the muscle to imposed activity is similar.

Nonetheless, for reasons cited in the Introduction, it seems unlikely that the ACh receptor site itself plays a key role in the earliest stages of synapse formation. Yet it is clear that for effective transmission to occur, an adequate number of ACh receptors must be present at the junction. It seems very plausible that these should be drawn from the extrajunctional 'pool' of receptors found in uninnervated muscles, and somehow fixed in place at the site of nerve–muscle interaction, possibly after some chemical modification. This would provide a clear role for the phenomenon of denervation sensitivity without assuming that the receptors are involved in any sort of cell recognition event. Indeed, it has recently been shown that extrajunctional receptors accumulate at sites of nerve-muscle contact in cultured amphibian tissues (Anderson, Cohen & Zorychta, 1977; Anderson & Cohen, 1977).

Sensitivity to ACh is clearly related to the presence of membrane receptors. In reducing sensitivity, stimulation presumably reduces the number of those receptors. The extrajunctional ACh receptors of denervated muscle are thought to have a half-life in the membrane of 12–24 hr (Berg & Hall, 1974, 1975; Chang & Huang, 1975; Devreotes & Fambrough, 1975). Since, at its fastest, the decline of sensitivity during direct stimulation has a half-decay time of less than 12 hr, stimulation with the pattern we have used must slow or even stop the appearance of new receptors in the membrane. This is the more true since recent reports suggest that stimulation may actually reduce the rate of loss of receptors from the membrane (Hogan, Marshall & Hall, 1976; Fambrough, Devreotes & Card, 1977).

The events in the very early stages of n.m.j. formation are unclear, and it is therefore impossible to interpret the inhibitory effects of stimulation on n.m.j. formation in molecular terms. What our results do show is that the period of inactivity required to allow n.m.j. formation need be no more than 48 hr. Thus, even if muscle activity resumes before the developing n.m.j.s are functional their further development is possible. In interpreting this result, it is worth noting that in the same circumstances, ACh sensitivity continues to develop for at least 24 hr ultimately reaching nearly its maximum post-denervation level, and any change in the surface of the muscle required for interaction with the nerve might do the same. The key events underlying both increased ACh sensitivity and the ability to make new n.m.j.s thus appear to be initiated during the first 2 days of inactivity and then to require a period of several days activity to be fully reversed. Whatever the basis of these observations may be, it is clear that muscle inactivity is only required during the earliest, pre-functional stages of n.m.j. formation. This makes good sense since the new n.m.j. must be able to survive the effects of the activity it generates.

The striking effects of direct stimulation on the denervated muscle suggest that normal activity plays an important role in maintaining muscle properties. In this study, for example, the loss of extrajunctional ACh sensitivity following reinnervation may well be a result of the muscle activity induced by the new ectopic innervation. Certainly, there was little indication of any decline of sensitivity prior to the time when signs of reinnervation could be detected, confirming the results of Tonge (1974) and Dennis & Ort (1977) (but see Miledi, 1960*b*; Bray & Harris, 1975). Furthermore, the rate of decline of sensitivity following reinnervation could be accounted for by the rate seen during stimulation (Fig. 4) even when the asynchrony of reinnervation is taken into account. However, in the absence of detailed infor-

mation about the activity of the muscles *in vivo*, it is not possible critically to test the idea that the activity of the muscles is the sole factor contributing to the decline in sensitivity.

In conclusion, we suggest that activity is able to maintain the extrajunctional region of the muscle fibre surface in a normal state with respect both to ACh sensitivity and to the ability to accept new innervation. However, even a short period (2 days) of inactivity allows the expression of the full post-denervation effect, if only transiently. The nature of the process of n.m.j. formation ensures that during such a brief period of altered conditions, stable nerve–muscle interactions can form which, though primitive in both structure and function, can develop into transmitting junctions even if intense muscle activity resumes.

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