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Twitch responses with acetylcholine in the isolated innervated and chronically denervated rat diaphragms and their modification by neuromuscular blocking agents

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The increased sensitivity to acetylcholine after chronic denervation was investigated by retrograde injection of the acetylcholine into the venous drainage of the isolated diaphragm of the rat set up in Krebs solution at 38°. Supersensitivity of the twitch response was observed 3 days after nerve section and developed further to reach a peak at the eighth to tenth day after denervation and then slowly declined with atrophy of the muscle. At the time of the onset of supersensitivity the degenerating peripheral nerve stump failed to respond to electrical stimulation and fibrillation of the muscle was observed. The muscle also responded to acetylcholine and other depolarising drugs by giving a contracture. After denervation, the response to injected acetylcholine was more sensitive to block by depolarising neuromuscular blocking agents but not to block by competitive blocking drugs. Tubocurarine did not cause a stimulation on injection into denervated muscle, while decamethonium gave an acetylcholine-like response. The significance of the observations is discussed.

ISOLATED mammalian muscle does not under normal conditions give a twitch response to the application of acetylcholine to the fluid bathing the external surface of the muscle. With low concentrations (5×10^{-6} to 2×10^{-5} w/v) there is some potentiation of responses to nerve stimulation and with higher concentrations (10^{-4} w/v) this gives way to neuromuscular block. With none of these concentrations is a twitch response obtainable as a result of the action of the acetylcholine. The reason may lie in the rate of change of concentration of acetylcholine at the motor end-plate.

It is well known that if acetylcholine is injected into the vascular supply of a skeletal muscle, *in vivo*, a twitch can be produced (Brown, Dale & Feldberg, 1936). Similarly electrophoretic application of acetylcholine by micropipette is also capable of inducing a twitch response. With "close-arterial" injection, the twitch has been shown to be a short asynchronous tetanus (Brown, 1937). "Close-arterial" injection is impracticable in the isolated rat diaphragm muscle because of its diffuse arterial supply. However, the venous drainage of the right hemidiaphragm affords a suitable channel for retrograde injection into the muscle. This was developed as a technique by Burgen, Dickens & Zatman (1949) in their studies on the mode of action of botulinum toxin. The method lends itself well to the study of the twitch responses to acetycholine before and after denervation and for the investigation of substances modifying these twitch responses.

Experimental

Albino rats, 150–250 g, were stunned and bled. The entire diaphragm with the right phrenic nerve and the thoracic inferior vena cava and

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hepatic vein intact was dissected into cold Krebs solution gassed with 95% oxygen and 5% carbon dioxide. The right phrenic nerve was separated from the thoracic inferior vena cava and the abdominal aspect of the diaphragm cleared of fascial attachments to give access to the abdominal inferior vena cava. This vein was then tied just below the diaphragm, care being taken to leave the right phrenic vein patent. The liver was then dissected to leave the tied stump of the abdominal inferior vena cava. The veins draining the left hemidiaphragm and the crura were then tied and these muscles dissected. A polythene cannula was tied into the thoracic inferior vena cava and the preparation set up in Krebs solution at 38° and gassed with 95% oxygen and 5% carbon dioxide (Fig. 1).

Drugs were injected through the cannula into the muscle as follows: the cannula and its attached length of tubing was filled by injecting sufficient of the drug solution to fill the dead space (about 0·15 to 0·2 ml); 0·15 ml quantities of the solution could then be displaced rapidly into the right phrenic vein. Further 0·15 ml quantities of the solution were then injected at 3 min intervals in normal muscle and 2 min intervals in denervated muscle.

The injection of acetylcholine into the muscle gives rise to a tetanic twitch of short duration and tension dependent upon the dose. With constant speed of injection a uniform response to a given dose can be obtained, providing adequate time for recovery between doses is given.

The uniformity of the response to injected acetylcholine is maintained for up to 8 hr provided the bath fluid is changed after a series of injections is made. The diffusion of the drug into the bath after injection gives a a negligible final concentration with the smaller doses used and has no discernible effect on the responses to subsequent injections. Between injections the responses to nerve stimulation in normal muscle and direct stimulation in denervated muscle were recorded. Frequency of stimulation was 5 per min.

DENERVATION

Adult rats 150–300 g were anaesthetised with diethyl ether and the right phrenic nerve exposed where it crosses the brachial plexus. After section, the distal end of the nerve was avulsed slowly. In most animals the nerve broke at the level of the right atrium. The rats were then allowed to recover and were killed at intervals after nerve section. The denervated diaphragm was then set up in the same way as for the innervated preparation. The success of the denervation can be judged at the time of operation by the absence of diaphragmatic respiration on the right side and by the presence of fibrillation when the muscle was subsequently prepared for recording.

STIMULATION

In the innervated preparation, the nerve was stimulated by square wave pulses of 10 or 30 μsec duration and 4 to 5 V at a frequency of 5/min delivered from a Multitone stimulator.

The denervated muscles were stimulated between a platinum wire

on the Perspex rod to which the central tendon margin of the diaphragm was attached, and a stainless-steel wire tied into the costal margin which served both as an electrode and as a connecting link to the lever system (Fig. 1). Pulses were 0·3 to 1 msec in duration and 120 V.

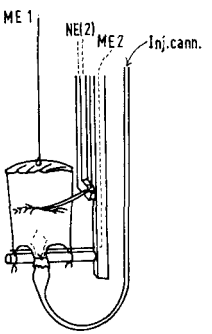

FIG. 1. Electrode and injection assembly for intravenous injection into the isolated rat diaphragm. ME1: thin gauge stainless steel wire acting as one electrode for muscle stimulation and for attachment to spring lever. ME2: second muscle electrode; platinum wire making contact with central tendinous margin of the diaphragm. NE (2): two platinum wires over which the phrenic nerve, if present, is laid for stimulation. Inj. cann: small bore polythene tubing distended where it inserts into the right phrenic vein. Injections are made through this into the venous drainage of the muscle.

Twitches were recorded on a smoked drum with a semi-isometric spring lever.

Results

SENSITIVITY TO ACETYLCHOLINE

Normal diaphragm. The responses of the innervated diaphragm to injected acetylcholine are shown in Fig. 2. A small injection artifact was obtained when 0·15 ml of Krebs solution was injected through the phrenic vein. With 0·32 μg of acetylcholine the response on injection was no different from the artifact; with 1·25 μg, there was a twitch about 50% of that of nerve stimulation and with 10 μg there was a twitch about equal to that of the nerve-induced twitch.

Diaphragm denervated 6 days previously (Fig. 2). Again, control injections of 0·15 ml of Krebs solution gave small artifacts. After denervation much less acetylcholine was required for a given height of contraction than in the normal diaphragm. After the larger doses (100 ng to 1 μg; Fig. 2) the denervated muscle also showed a contractural response accompanied by a decrease in the response of the muscle to direct stimulation, from which it recovered rapidly.

RELATION OF SUPERSENSITIVITY TO ACETYLCHOLINE TO THE LENGTH OF TIME AFTER NERVE SECTION

Log dose-response curves were obtained at various lengths of time after nerve section. Results are shown graphically in Fig. 3. Three days

after denervation the muscle began to show increased sensitivity to injected acetylcholine. Before this time no supersensitivity could be detected. Three days was also the earliest time at which fibrillation of the muscle was observed during dissection. At this time the muscle failed to respond to electrical stimulation of the peripheral end of the degenerating nerve. Thereafter the sensitivity increased to reach a peak at the eighth to tenth day after denervation and then declined slowly as atrophy of the muscle progressed. Eighty-four days after denervation the sensitivity of one muscle was still about one hundredfold greater than that of an innervated muscle. The maximum twitch tension in this muscle was very much reduced because of atrophy.

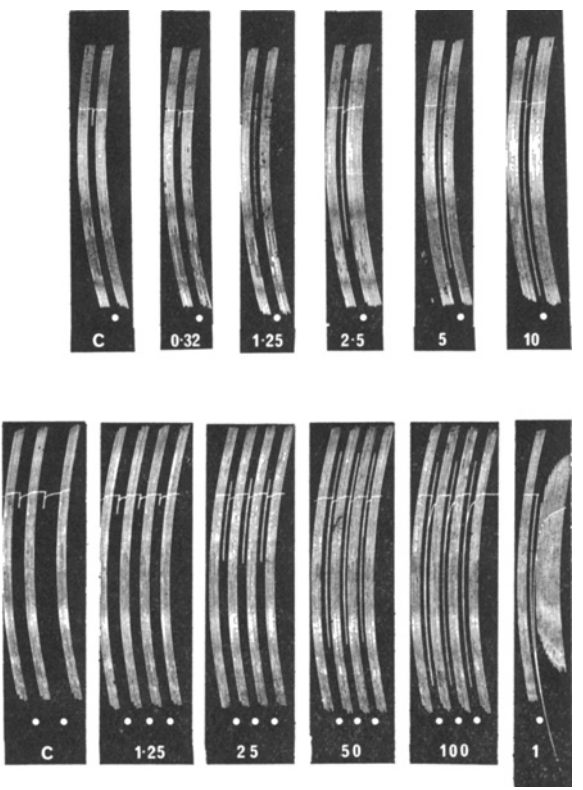

FIG. 2. Responses of innervated and denervated diaphragm to retrograde intravenous injection of acetylcholine. Responses are shown between blocks of nerve stimulation or direct stimulation (rate 5/min) of the muscles. Upper row: normal diaphragm; Responses to 0·15 ml Krebs (C) and 0·32, 1·25, 2·5, 5 and 10 μg Ach in 0·15 ml Krebs. Lower row: diaphragm denervated for 6 days; Responses to 0·15 ml Krebs (C) and 12·5, 25, 50, 100 ng, and 1 μg Ach in 0·15 ml Krebs.

EFFECTS OF NEUROMUSCULAR BLOCKING AGENTS ON THE TWITCH RESPONSE TO ACETYLCHOLINE

Neuromuscular blocking agents added to the bathing fluid reduced the responses to injected acetylcholine in both normal and denervated muscles.

In the innervated muscle, tubocurarine in a concentration of 10^{-6} reduced to a greater extent the response to acetylcholine than the response to a single nerve volley (Fig. 4A). This concentration of tubocurarine caused a similar reduction of the response to acetylcholine in a diaphragm denervated 7 days previously (Fig. 4B). After washing, the responses to acetylcholine returned slowly to normal in both preparations. The difference in blockade between the endogenous and exogenous acetylcholine probably lies in the difference in nature of the two responses, one being a single impulse phenomenon and the other a tetanic response. It is known that tetani are more easily reduced than single nerve volley responses. Blockade of the acetylcholine response by gallamine is also

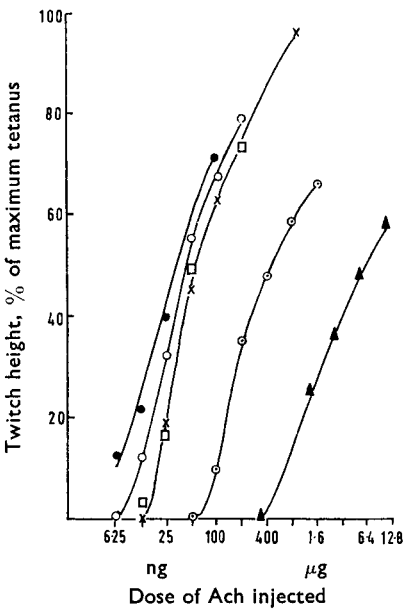

FIG. 3. Log. dose-response curves of rat diaphragms, innervated and at varying stages of denervation. Abscissa: dose of Ach injected in 0·15 ml Krebs, log range from 6·25 ng to 12·8 μg. ▲ Normal diaphragm. ○ Denervated 3 days. X Denervated 6 days. ○ Denervated 7 days. ● Denervated 18 days. □ Denervated 84 days.

unchanged after denervation (Fig. 5). If anything, both tubocurarine and gallamine are slightly less effective after denervation.

Depolarising blocking agents on the other hand, have a greater effect on the response to acetylcholine after denervation. In the innervated diaphragm, about 80% block of the response to acetylcholine was obtained with suxamethonium (10^{-6}), whereas in the denervated preparation complete block of its action was recorded with 2×10^{-7} suxamethonium (Fig. 6).

Decamethonium (Fig. 7) in a concentration of 10^{-5} caused a block of responses to acetylcholine in the normal diaphragm comparable with that of a concentration of 10^{-6} in the denervated muscle. Similar results

were obtained with carbachol where the block of responses to acetylcholine of the normal diaphragm with $2{\cdot}5 \times 10^{-6}$ was equivalent to a block with $2{\cdot}5 \times 10^{-7}$ in a muscle denervated 14 days previously. In Figs 7C, 8, 9, a further characteristic of the actions of depolarising agents is shown. In all of these instances the response to direct stimulation of the denervated diaphragm is depressed. This is seen to be maintained in Figs 8, 9, and can be reversed on washing. It is easily reversible (Fig. 7C) by hexamethonium 10^{-5} and by tubocurarine 5×10^{-7} whereas the response to injected acetylcholine still remains depressed.

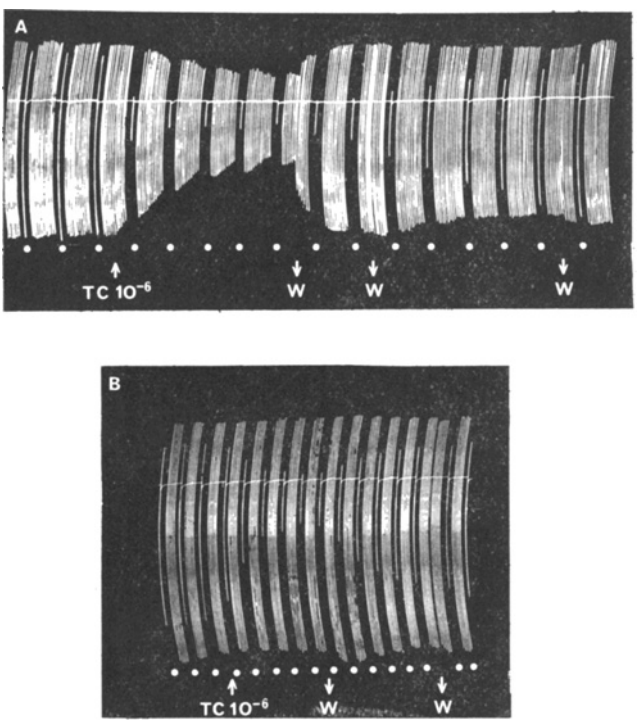

FIG. 4. Blockade of injected Ach by (+)-tubocurarine. (A) Normal diaphragm. At dots, 5 μg Ach was injected every 3 min between blocks of responses to nerve stimulation. At TC, (+)-tubocurarine was added to bath to give a final concentration of 10^{-6}. The drug was washed out at W. (B) Diaphragm denervated 7 days previously. At dots, 50 ng Ach was injected every 2 min between blocks of direct stimulation of the muscle. At TC, (+)-tubocurarine was added to the bath to give a final concentration of 10^{-6}.

SENSITIVITY OF DENERVATED MUSCLE TO RETROGRADE INTRAVENOUS INJECTION OF SUBSTANCES OTHER THAN ACETYLCHOLINE

Decamethonium and potassium chloride both gave twitch and contractural responses on injection into the phrenic vein of a diaphragm denervated 21 days previously (Fig. 10). However, even in large doses (1 mg) tubocurarine did not cause contraction, contracture or depression of direct stimulation.

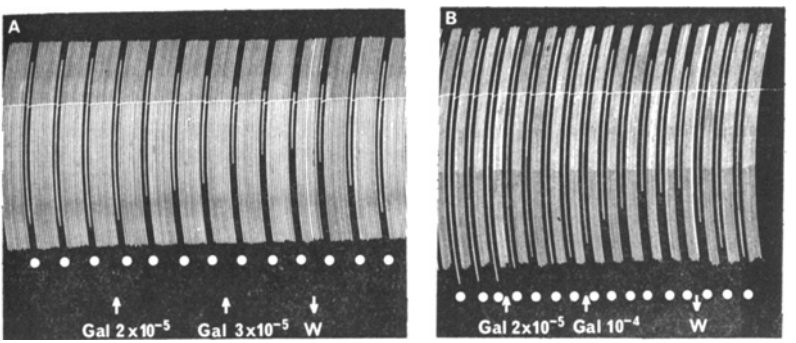

FIG. 5. Blockade of injected acetylcholine by gallamine. (A) Normal diaphragm. At dots, 2 μg Ach was injected every 3 min between blocks of responses to nerve stimulation. At Gal, gallamine was added to the bath to give final concentrations shown, 5×10^{-5} *in toto*. (B) Diaphragm denervated 8 days previously. At dots, 25 ng Ach was injected every 2 min between blocks of responses to direct stimulation. At Gal, gallamine was added to the bath to give final concentrations shown, $1{\cdot}2 \times 10^{-4}$ *in toto*.

EFFECT OF NEOSTIGMINE ON TWITCH AND CONTRACTURAL RESPONSES TO ACETYLCHOLINE

Fig. 11 shows responses to acetylcholine before and after treatment with a concentration of neostigmine (10^{-7}) which potentiates considerably the response to nerve stimulation in the innervated diaphragm. The twitch response was little affected but the contractural action was enhanced and prolonged.

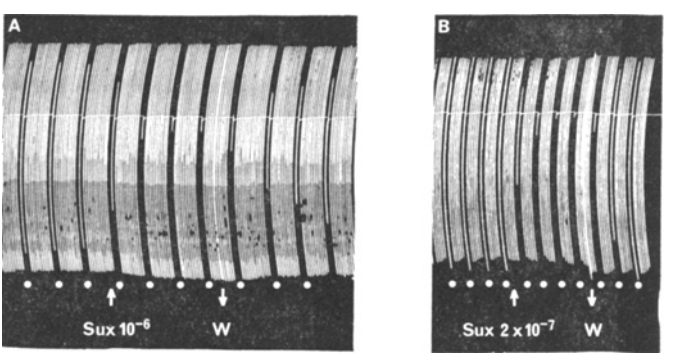

FIG. 6. Blockade of injected acetylcholine by suxamethonium. (A) Normal diaphragm. At dots, 2 μg Ach was injected every 3 min between blocks of responses to nerve stimulation. At Sux, suxamethonium was added to the bath to give a final concentration of 10^{-6}. (B) Diaphragm denervated 21 days previously. At dots, 25 ng Ach was injected every 2 min between blocks of responses to direct stimulation. At Sux, suxamethonium was added to the bath to give a final concentration of 2×10^{-7}.

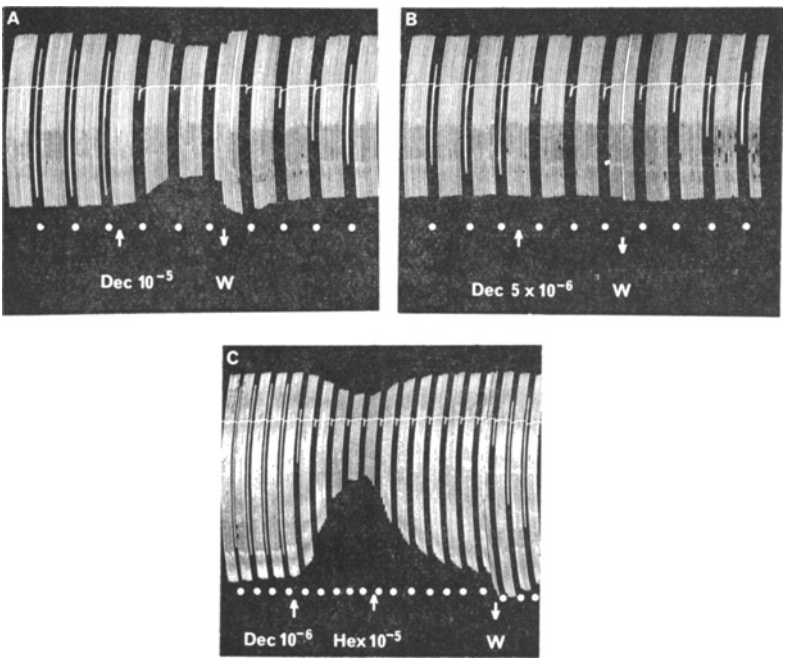

FIG. 7. Blockade of injected acetylcholine by decamethonium. (A) and (B) Normal diaphragm. At dots, 2·5 μg Ach was injected every 3 min between blocks of responses to nerve stimulation. (A) At Dec 10^{-5}, decamethonium was added to the bath to give a final concentration of 10^{-5}. (B) At Dec 5×10^{-6}, decamethonium was added to the bath to give a final concentration of 5×10^{-6}. (C) Diaphragm denervated 18 days previously. At dots, 25 ng Ach was injected every 2 min between blocks of responses to direct stimulation. At Dec, decamethonium was added to the bath to give a final concentration of 10^{-6} and at Hex, hexamethonium to give a final concentration of 10^{-5}.

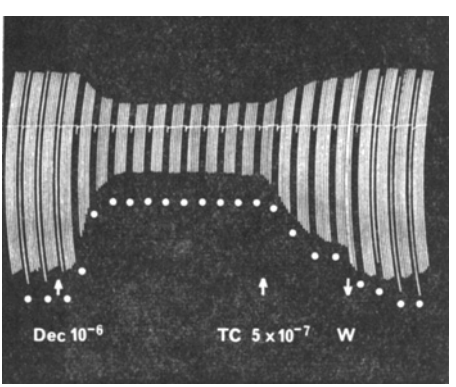

FIG. 8. Reversal by tubocurarine of depression of direct stimulation with decamethonium. Diaphragm denervated 23 days previously. At dots, 25 ng Ach injected every 2 min between blocks of direct stimulation. At Dec, decamethonium was added to the bath to give a final concentration of 10^{-6}. At TC, (+)-tubocurarine was added to give a final concentration of 5×10^{-7}.

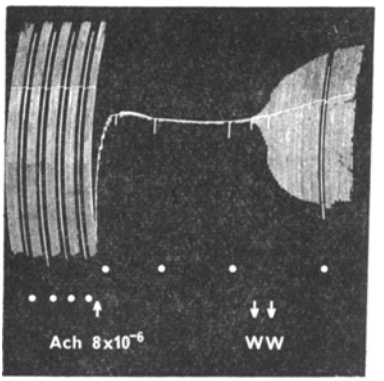

FIG. 9. Blockade of responses to injected acetylcholine by acetylcholine added to the bath. Diaphragm denervated 21 days previously. At dots, 25 ng Ach was injected intravenously into the muscle. At Ach, acetylcholine was added to the bath to give a final concentration of 8×10^{-6}.

Discussion

The chronically denervated diaphragm increases in sensitivity to acetylcholine injected intravascularly; the increase is gradual and becomes evident on the third day after denervation, that is, at a time when the degenerating stump of the phrenic nerve fails to elicit a twitch response to electrical stimulation. At the same time contractural properties develop and the contraction of the muscle on direct stimulation is depressed. No abrupt change occurs from the low sensitivity of the innervated muscle to the much greater sensitivity after denervation. Instead, a gradual transition takes place, which although rapid in the first few days after nerve degeneration, is nevertheless progressive up to the eighth to tenth day after nerve section. Reid & Vaughan Williams (1949) in their experiments on the denervated tibialis anterior muscle of the cat, found that sensitivity of the twitch response to acetylcholine increased rapidly on the sixth and seventh days after denervation while the contractural response appeared earlier and continued to develop after increased sensitivity had been established. In the rat gastrocnemius muscles, sensitisation and contractural responses to acetylcholine appeared between the second and third days after section of the nerve; both these increased to a maximum at 7 days after denervation. The onset of supersensitivity to acetylcholine after nerve section seemed to depend, to some extent, on the length of the peripheral nerve stump left after cutting the nerve. Luco & Eyzaguire (1955) noted that it appeared earlier when the nerve was cut nearer the motor end-plate. In the present experiments the length of nerve that remained after avulsion was about 2 to 2·5 cm since at phrenectomy the nerve broke at the level of the right atrium.

The reason for the supersensitivity is not certain, but one main factor has emerged and has gained considerable support in recent years (Ginetzinsky & Shamarina, 1942, Axelsson & Thesleff, 1959, Miledi, 1960a).

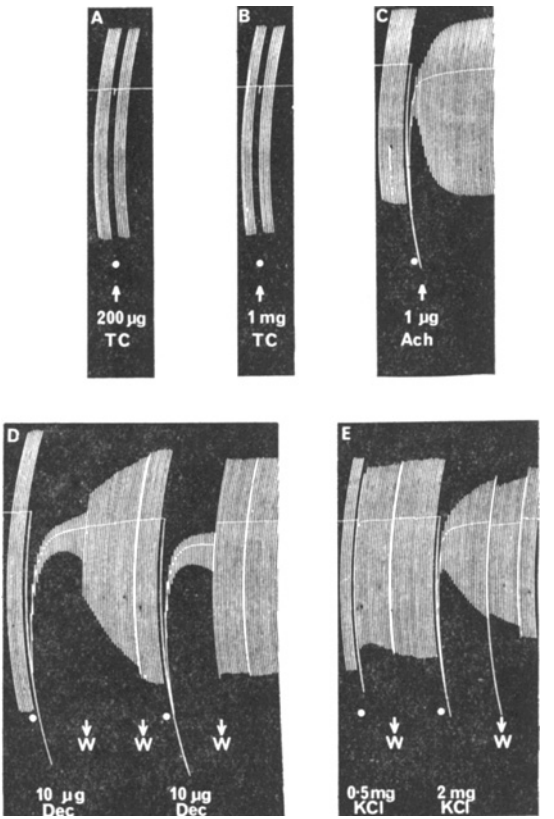

FIG. 10. Effects of injections of substances other than acetylcholine into the phrenic vein of a diaphragm denervated 21 days previously. TC, (+)-Tubocurarine. Ach, Acetylcholine. Dec, Decamethonium. KCl, Potassium chloride.

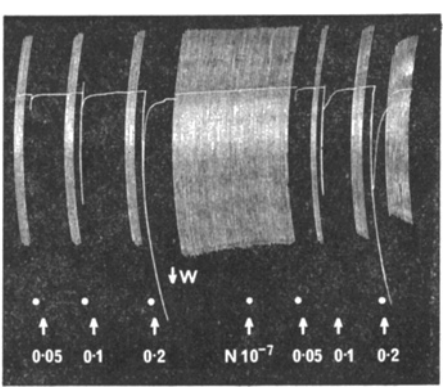

FIG. 11. Effect of neostigmine on the responses to injected acetylcholine. Diaphragm denervated 6 days previously. At 0·05, 0·1 and 0·2, injections of Ach were made through the cannula of 0·05 μg in 0·05 ml Krebs, 0·1 μg in 0·1 ml Krebs and 0·2 μg in 0·2 ml Krebs respectively. At N, neostigmine was added to the bath to give a final concentration of 10^{-7}.

This is the concept that after nerve section and degeneration, extra-junctional receptors appear, spreading with time after denervation over the entire surface of the muscle. The end-plate area still seemed to show a greater sensitivity than did the rest of the fibre (Miledi, 1962) but the existence of the extra-junctional receptors appeared to explain the overall contractural effects and the depression of direct stimulation seen with depolarising drugs. It is probable that in the same way this accounted for the increased sensitivity for the twitch response to injected acetylcholine. The mechanism by which these changes are brought about is also open to dispute. Thesleff (1960, 1961) believed that the absence of the transmitter is the governing factor since blockade of release of acetylcholine from nerve terminals with botulinum toxin induced changes indistinguishable from denervation. His concept received some support from experiments by Emmelin & Stromblad (1956) on salivary glands in which they induced supersensitivity with a muscarinic blocking agent which prevented the effects of acetylcholine released by nerve action. On the other hand, Miledi's experiments (Miledi 1960a, c; Katz & Miledi, 1961, 1964) have led him to conclude that a substance other than the transmitter is responsible for the effect, this substance exerting a controlling influence on the spread of receptors from the end-plate. Whatever the reason for the induction of the extra-junctional receptors, their presence altered the response of the muscle to a state which in many ways resembles that of foetal muscle (Paterson, 1957a; Diamond & Miledi, 1959, 1962).

The changes shown in response to acetylcholine are also seen with other depolarizing drugs and this is reflected in three ways. These drugs can induce contracture in denervated muscle (Muscholl & Lüllman, 1955; Paterson, 1957a, b). Depression of the effects of direct electrical stimulation and blockade of the action of injected acetylcholine are also seen. There is no evidence that these effects are not facets of the same action.

When the blockade of injected acetylcholine by depolarising blocking agents is compared in normal and denervated muscles, much less of the depolarising drug is required to block in the denervated than in the innervated muscle. There is no such difference in the action of competitive blocking agents. Maclagan & Vrbova (1964) described an increase in sensitivity of reinnervated motor-end plates to depolarisation blockade. Sensitivity was greatest immediately after reinnervation and gradually returned to normal during 9 weeks. This was seen although no difference in receptor area could be detected between normal and reinnervated muscles even when the period of denervation had been 3 weeks. They ascribed the increased sensitivity to the effects of denervation. The change may be the result of altered efficacy of the depolarising blocking drugs since in some instances, although not in the present experiments, tubocurarine and gallamine induced contractural effects in denervated muscle (McIntyre, King & Dunn, 1945; Jarcho, Berman, Eyzaguirre & Lilienthal, 1951; Bülbring & Depierre, 1949). Bowman (1964) could find no contractural or twitch-depressant action of 0·5 mg/kg

tubocurarine on a cat tibialis muscle denervated 14 days previously. It is surprising that Waser (1962), using radioactive curarine, was unable to detect by autoradiographic means the spread of receptors from the end-plate region in denervated mouse diaphragms, since competitive drugs can block all of the effects of acetylcholine seen after denervation.

Tubocurarine and hexamethonium reversed the depression by decamethonium of direct stimulation in the denervated muscle. This would suggest that the receptors which appeared after denervation had qualitatively similar properties to those of the normal motor end-plate, an observation which agrees with the findings of other groups of workers (Axelsson & Thesleff, 1959; Miledi, 1960a; Klaus, Kuschinsky, Lüllman & Muscholl, 1959; Jenkinson, 1960; Letley, 1960). It also means that the sustained depression of contraction caused by the decamethonium is the result of a sustained specific reaction of the decamethonium with these receptors and not the result of the ionic changes which accompany the contracture caused by depolarisation of the muscle membrane.

The sustained depression with decamethonium seen in Fig. 8 emphasises one difference between the response of innervated and denervated muscle *in vitro*. On isolated innervated muscle, depolarising blocking agents have a diphasic action, where the neuromuscular block develops in about 10 min (phase I block), but is not maintained. A reversal of block appears which then continues into a further block (phase II block) (Jenden, Kamijo & Taylor, 1954; Jenden 1955; Dillon & Sabawala, 1959; Maclagan, 1962; Huskisson & Paterson, 1965, in preparation). During Phase I block there is a concomitant reduction in the response of the muscle to direct stimulation. This transitional action of the depolarising blocking drugs *in vitro* has led Zaimis (1962) and Maclagan (1962) to urge caution in the comparison of the actions of depolarising drugs *in vitro* and *in vivo*. No transitional effect is seen in Fig. 8 and this suggested that this action of decamethonium was different from that seen during phase I in the innervated muscle.

The suggestion that acetylcholine receptors have a role in conduction along muscles (Hinterbuchner & Nachmansohn, 1960) must also be examined in the light of these results. If the depression of conduction by depolarising drugs were to arise from the ability of the drug to cause a reduction in the activity of endogenous acetylcholine, then the same should be true for any competitive blocking agent reaching the same site. That this is not so, when taken together with the inability of tubocurarine to excite or depress the muscle on intravenous injection of large doses, suggests that acetylcholine is not concerned in impulse conduction in this muscle. Also, Hebb, Krnjević & Silver (1964) have shown that in the denervated diaphragm of the rat acetylcholine was practically undetectable in that part of the muscle, which in the innervated diaphragm was designated to be "nerve-free". They also found that choline acetyltransferase activity fell in the denervated diaphragm to 4% of normal values; even this residuum of activity might be accounted for by Schwann cell activity in denervated muscle (Birks, Katz & Miledi, 1960). In agreement with the conclusion that acetylcholine has no role in conduction

are the results of Cooke & Grinnell (1964) who, investigating the effects of tubocurarine on frog sartorii and rat intercostal muscle, were unable to demonstrate any significant difference between action potentials of normal and denervated muscle fibres in Ringer solution with or without tubocurarine up to 10^{-3} M. The depression of direct stimulation by acetylcholine, suxamethonium, carbachol and decamethonium might be thought to contradict the hypothesis (Grundfest, 1957, 1963) that acetylcholine sensitive areas of muscle were electrically inexcitable, but Grundfest (1961, 1964) proposes an explanation which supposed chemosensitive areas to develop in patches after denervation, but not to cover the entire membrane. The results of the present experiments agree with this supposition when we take into consideration the sustained transmitter-like action of the depolarising drugs and the ease by which it is reversed by competitive blocking drugs. It would be difficult to envisage a "short-circuit" transmitter-like action which involved all of the active membrane and yet was sustained for prolonged periods of time.

Neostigmine did not enhance the twitch caused by acetylcholine in the denervated diaphragm, but did prolong the contracture. The temporal difference between the two forms of response may be one explanation for this observation since there is still much cholinesterase activity in the rat diaphragm for up to 42 days after denervation (Lüllmann & Muscholl, 1955). Since the contractural action relies more on a sustained ambient concentration than does the twitch response, protection from hydrolysis would be expected to prolong the contracture. The twitch response being tetanic would remain little changed. Miledi (1962) found a reduction by edrophonium of the effect of ionophoretically-applied acetylcholine at the end-plate region in a frog sartorius fibre 58 days after denervation, so it would seem that at that stage little if any cholinesterase remained.

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References

Axelsson, J. & Thesleff, S. (1959). *J. Physiol.*, **147**, 178–193.
Birks, R., Katz, B. & Miledi, R. (1960), *Ibid.*, **150**, 145–168.
Bowman, W. C. (1964). In "*Evaluation of drug activities: Pharmacometrics.*" London: Academic Press.
Brown, G. L. (1937), *J. Physiol.*, **89**, 438–461.
Brown, G. L., Dale, H. H. & Feldberg, W. (1936). *Ibid.*, **87**, 394–424.
Bülbring, E. & Depierre, F. (1949). *Brit. J. Pharmacol.*, **4**, 22–28.
Burgen, A. S. V., Dickens, F. & Zatman, L. J. (1949). *J. Physiol.*, **109**, 10–24.
Cooke, I. M. & Grinnell, A. D. (1964). *Ibid.*, **175**, 203–210.
Diamond, J. & Miledi, R. (1959). *Ibid.*, **149**, 50 P.
Diamond, J. & Miledi, R. (1962). *Ibid.*, **162**, 393–408.
Dillon, J. B. & Sabawala, P. B. (1959). *Acta anaesth. scand.*, **3**, 83–100.
Emmelin, N. & Stromblad, B. C. R. (1956). *Acta. physiol. scand.* **36**, 171–174.
Ginetzinsky, A. G. & Shamarina N. M. (1942) *Uspekhi Sovremennoi Biologii*, **15**, 283–294, (DSIR translation RTS 1710).
Grundfest, H. (1957). *Physiol. Rev.*, **37**, 337–361.
Grundfest, H. (1963). In *The General Physiology of Cell Specialization.* New York: McGraw-Hill.
Grundfest, H. (1964). *Ann. Rev. Pharmacol.*, **4**, 341–364.
Grundfest, H. (1961). In *Regional Neurochemistry*, 378–403. London: Pergamon.
Hebb, C. O., Krnjević, K. & Silver, A. (1964). *J. Physiol.*, **171**, 504–513.

Hinterbuchner, L. P. & Nachmansohn, D. (1960). *Biochem. Biophys. Acta*, **44**, 554–560.
Jarcho, L. W., Berman, B., Eyzaguirre, C. & Lilienthal, J. L., Jr. (1951). *Ann. N.Y. Acad. Sci.*, **54**, 337–346.
Jenden, D. J. (1955). *J. Pharmacol.*, **114**, 398–408.
Jenden, D. J., Kamijo, K. & Taylor, D. B. (1954). *Ibid.*, **112**, 229–240.
Jenkinson, D. H. (1960). *J. Physiol.*, **152**, 309–324.
Katz, B. & Miledi, R. (1961). *Ibid.*, **156**, 24–25P.
Katz, B. & Miledi, R. (1964). *Ibid.*, **170**, 389–396.
Klaus, W., Kuschinsky, G., Lüllmann, H. & Muscholl, E. (1959). *Med. exper.*, **1**, 8–11.
Letley, E. (1960). *Brit. J. Pharmacol.*, **15**, 345–350.
Luco, J. V. & Eyzaguirre, C. (1955). *J. Neurophysiol.*, **18**, 65–73.
Lüllmann, H. & Muscholl, E., (1955). *Arch. exp. Path. Pharmak.*, **225**, 486–490.
Maclagan, J. (1962). *Brit. J. Pharmacol.*, **18**, 204–216.
Maclagan, J. & Vrbová, G. (1964). *J. Physiol.*, **170**, 72P–73P.
McIntyre, A. R., King, R. E. & Dunn, A. L. (1945). *J. Neurophysiol.*, **8**, 297–307.
Miledi, R. (1960a). *J. Physiol.*, **151**, 1–23.
Miledi, R. (1960b). *Ibid.*, **151**, 24–30.
Miledi, R. (1960c). *Ibid.*, **154**, 190–205.
Miledi, R. (1962). In *Enzymes and Drug Action* 220–236, Ciba Foundation Symposium, London: Churchill.
Muscholl, E. & Lüllmann, H. (1955). *Arch. exp. Path. Pharmak.*, **226**, 88–97.
Paterson, G. (1957a). Ph.D. thesis. University of London.
Paterson, G. (1957b). *J. Physiol.*, **136**, 4–5P.
Reid, G. & Vaughan Williams, E. M. (1949). *J. Physiol.*, **109**, 25–31.
Thesleff, S. (1960). *Ibid.*, **151**, 598–607.
Thesleff, S. (1961). *Ann. N.Y. Acad. Sci.*, **94**, 535–546.
Waser, P. G. (1962). In *Enzymes and Drug Action*, 207–217, Ciba Foundation Symposium. London: Churchill.
Zaimis, E. (1962). In *Curare and Curare-like Agents*, 75–82, Ciba Foundation Study Group No. 12, London: Churchill.