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Some properties of the denervated anterior gracilis muscle of the rat

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1. Microdrops of acetylcholine (ACh) were topically applied to denervated, mammalian skeletal muscle *in vivo*, and ACh-evoked depolarization and contracture were recorded simultaneously.

2. Contracture tension was directly proportional to the degree of ACh-elicited depolarization.

3. Atropine and (+)-tubocurarine increased the amount of ACh required to produce a given amount of depolarization, but these anticholinergic agents did not alter the relationship between the degree of ACh-evoked depolarization and contracture tension.

4. Topically applied catecholamines did not produce either depolarization or contracture, despite the fact that parenterally administered catecholamines elicited both responses.

After denervation, skeletal muscle becomes supersensitive to acetylcholine (ACh) and other agents (see Cannon & Rosenblueth, 1949). In the vast number of studies of denervation phenomena, drugs have been administered in a wide variety of ways. They have been injected parenterally (Dale & Gasser, 1926), applied locally in microdrops to denervated muscles both *in vivo* and *in vitro* (Ginetzinsky & Shamarina, 1942; Kuffler, 1943), applied iontophoretically to muscles *in vitro* (Axelsson & Thesleff, 1959; Miledi, 1960), as well as added to muscle bathing solutions (Miledi, 1960). In the present study microdrops of ACh solution were applied to denervated, mammalian skeletal muscle by means of a new microapplicator system, and the resulting depolarization and contracture were recorded simultaneously. This technique permitted the analysis of the relationship of ACh-evoked depolarization and contracture in a denervated muscle *in vivo*. In addition, the effects of atropine and (+)-tubocurarine on the responses to ACh were investigated.

In normal skeletal muscle catecholamines are known to affect indirectly evoked twitch tension but do not cause contracture (Bowman & Zaimis, 1958; Bowman & Raper, 1966), while in chronically denervated skeletal muscle catecholamines do produce contracture (von Euler & Gaddum, 1931; Luco & Sánchez, 1959; Bowman & Zaimis, 1961; Bowman & Raper, 1965). It has been clearly demonstrated that denervation supersensitivity to ACh results from the development of cholinergic receptors over the entire muscle-fibre surface (Axelsson & Thesleff, 1959; Miledi,

1960). The possibility of a similar mechanism for catecholamine-evoked contracture of denervated skeletal muscle was examined in the present study.

Methods

Preparation

The test object was the anterior gracilis muscle of adult, female Sprague-Dawley rats ranging in weight from 200 to 260 g. This muscle is well suited for the investigation of end-plate and end-plate-free sites *in situ* because the end-plates of the muscle in the rat are located in two discrete, 1 mm bands easily located by anatomical and electrophysiological criteria (Jarcho, Eyzaguirre, Berman & Lilienthal, 1952; Belmar & Eyzaguirre, 1966). In order to reconfirm the location of the end-plates, six normal muscles were stained for acetylcholinesterase by the method of Koelle & Friedenwald (1949) and examined microscopically.

Rats were anaesthetized by intraperitoneal administration of pentobarbitone 50 mg/kg. The muscle was prepared by a modification of the procedures described by Jarcho *et al.* (1952). Muscles were denervated by section of the left obturator nerve at the site where it disappeared under the muscle, and at least 5 mm of the proximal stump of the nerve was removed.

Electrical recording

Muscle action potentials were recorded extracellularly by means of a pair of platinum-iridium electrodes, amplified by a capacitance-coupled preamplifier, displayed on an oscilloscope, and photographed on moving film. Muscle action potentials and drug-evoked changes in membrane potential were recorded intracellularly and extracellularly by means of conventional 3M KCl-filled glass microelectrodes (3 to 20 MΩ), calomel cells, and a unity-gain, solid-state electrometer probe (Fein, 1964). An indifferent electrode was positioned on the contralateral muscle. The output of the electrometer probe was amplified by a d.c. amplifier, displayed on an oscilloscope, and photographed on moving film or recorded on a polygraph.

Tension recording

The distal end of the dissected muscle was fastened to a tension transducer that was sufficiently sensitive and stable to measure reproducibly 5 mg of tension change. The transducer was a pair of Micro-systems Type DC6A7-16-350 strain gauges mounted on a flexible brass plate (Electro-Optical Company, Pasadena, California). The dissected muscle was adjusted to the resting length in each experiment. The output of the gauges was amplified by a carrier amplifier and recorded on moving film or on a polygraph.

Administration of drugs

For topical application of drugs, a microapplicator was designed to apply uniform microdrops of solution to the preparation. It consisted of a Hamilton PB600-1 repeating dispenser (The Hamilton Company, Whittier, California) and a 25 or 50 µl. Hamilton syringe connected by fine-bore, polyethylene tubing to a blunted 27 gauge hypodermic needle. The Hamilton dispenser can eject repeatedly one-fiftieth

of the total volume of a microsyringe. The needle was placed adjacent to a low-resistance, extracellular microelectrode located on the surface of the muscle, and a microdrop of solution (0·5 or 1·0 μl.) was ejected in order to surround the tip of the recording electrode. In some experiments involving catecholamines, the microdrop was positioned around the shaft of an intracellular microelectrode. The microdrop of solution was removed from the muscle by a miniature cotton ball moistened with 0·9% NaCl solution. When the drug solution was applied repeatedly to the same site, the area of application was washed three times with 0·9% NaCl solution, and at least 20 min was allowed between drug applications. This topical method of drug application is subsequently referred to as the microdrop method. Drug solutions were also injected in the direction of the heart into the contralateral external iliac artery and the contralateral femoral vein.

The drugs used were: sodium pentobarbitone (Abbott Laboratories), acetylcholine chloride (ACh) (Merck and Company, Inc., and Sigma Chemical Company), atropine sulphate (Sigma Chemical Company and A. H. Robins Company, Inc.), (+)-tubocurarine chloride (DTC) (Eli Lilly and Company), and (−)-noradrenaline bitartrate monohydrate, (−)-adrenaline bitartrate, and (−)-isoprenaline (+)-bitartrate dihydrate (Sterling-Winthrop Research Institute). Doses and concentrations of drugs refer to the free form; solutions were freshly prepared in 0·9% NaCl solution.

Neural stimulation of normal muscle

The obturator nerve was electrically stimulated through a pair of platinum-iridium electrodes. The stimulus intensity was twice that required to evoke maximum twitch tension; the frequency of stimulation was once every 2 sec. During electrical stimulation DTC or atropine was administered intravenously, and the effect of the agent on maximal twitch tension was recorded on a polygraph.

Experimental procedures

Certain criteria were used in an attempt to standardize the procedures of the present investigation. The physiological condition of the preparation was a prime consideration. Fibrillatory movements or potentials were monitored in all experiments on denervated muscle because fibrillation is a good index of the physiological condition of the preparation (Hník & Skorpil, 1962; Belmar & Eyzaguirre, 1966). If the untreated denervated muscle was not fibrillating or stopped fibrillating during an experiment, the preparation was discarded. Animals treated with DTC were artificially respired to maintain a constant fibrillatory-potential rate; this rate is extremely sensitive to changes in respiration. Furthermore, it was observed that the preparation usually failed to respond to ACh when the mean blood pressure fell below 70 mm Hg.

Glass microelectrodes used for extracellular recording were required to maintain a steady baseline while in contact with the muscle. A few denervated muscles responded with small depolarizations or hyperpolarizations to the repeated application of 0·9% NaCl solution and such preparations were not used. Furthermore, responses that resulted from rolling microdrops, as indicated by microscopic observation, were not included in the results. Heat from a 100 W bulb in a reflector was used to maintain the rectal temperature within the normal range 37°–39° C. The temperature of the mineral-oil pool surrounding the preparation was usually 1·5°–2° C lower than the rectal temperature.

Results

Histology

Microscopic examination of small bundles of fibres and individual fibres from normal muscles stained for acetylcholinesterase revealed that the obturator nerve innervates two discrete end-plate zones approximately 1 mm wide. One end-plate zone is located near the entrance of the nerve into the muscle ; the other is more distal and is adjacent to the saphenous vessels. Most of the muscle fibres extend the entire length of the muscle bundle, are usually 40 to 70 μ in diameter, and contain only one end-plate. Jarcho *et al.* (1952), using different histological methods, found similar results. During experiments, the end-plate zones were readily located anatomically by following the degenerating nerve and functionally by recording intracellularly a fibrillatory potential preceded by a prepotential (*see* Belmar & Eyzaguirre, 1966). The locations of the end-plate zones by means of functional and anatomical criteria agree favourably.

Development of ACh-induced contracture

Two days after denervation, ACh applied topically anywhere on the muscle evoked a contracture ; thereafter, the ACh threshold of the muscle at both end-plate and end-plate-free regions decreased until a minimum was attained at 20 days after nerve section. Hence, the subsequent experiments involving ACh were conducted on animals denervated for a period of 20–25 days.

Relationship between depolarization and concentration of topically applied ACh

The data, as illustrated in Fig. 1, indicate that in denervated muscle the magnitude of the extracellularly-recorded depolarization is quantitatively related to the concentration of ACh topically applied by the microdrop method. The concentration of ACh needed to produce a response on the linear portion of the concentration-depolarization curve varied within an order of magnitude from one experiment to another if the volume of the drop and the time after denervation were kept constant in each experiment. In addition, the concentration of ACh required to produce a minimal response at end-plate zones was similar to that required at end-plate-free areas. Figure 1A shows the results from one of eleven similar experiments.

The maximal responses obtained from end-plate zones and end-plate-free areas of any given preparation usually differed by a few mV (Fig. 1A). In six denervated muscles the end-plate zones showed greater maximal sensitivity than did the end-plate-free areas ; in five denervated preparations the reverse was true. In this series of experiments, however, the repeated application of ACh solution to a given test site on a denervated muscle produced desensitization, manifested as a decrease in depolarization at high concentrations of ACh (Fig. 1A). Differences in the time of onset of desensitization may explain the variation in the maximal depolarization obtained at the two test sites within a given muscle (Fig. 1A). Desensitization of cholinergic receptors in denervated muscle has been reported previously (Axelsson & Thesleff, 1959 ; Miledi, 1960).

In spite of the desensitization, the data from eleven experiments suggest that the ACh sensitivities of end-plate-free regions and end-plate zones of the denervated preparations were very similar. With the microdrop technique, however, small

differences between end-plate and end-plate-free membrane may have been missed. The measured size of the 0·5-μl. microdrop was approximately 0·5 mm in diameter; consequently, the size of the microdrop was far greater than that of an individual end-plate. Also the end-plates *per se* comprise a relatively small area compared with the total area of the end-plate zone. Therefore data obtained from end-plate zones reflect the sensitivity of end-plate as well as end-plate-free membrane and, in fact, may reflect mainly the sensitivity of end-plate-free membrane. Axelsson & Thesleff (1959) reported that the sensitivity of end-plate-free regions of denervated, mammalian skeletal muscle to iontophoretically applied ACh increases until it reaches the sensitivity of the end-plate. In contrast, Miledi (1962; personal communication, 1969), using a mammalian preparation and iontophoretically applied ACh, found that the sensitivity of the end-plate remains higher than that of end-plate-free regions even some weeks after denervation. For the reasons given, the findings of the present study do not resolve the differences between the results of the two above-mentioned investigations.

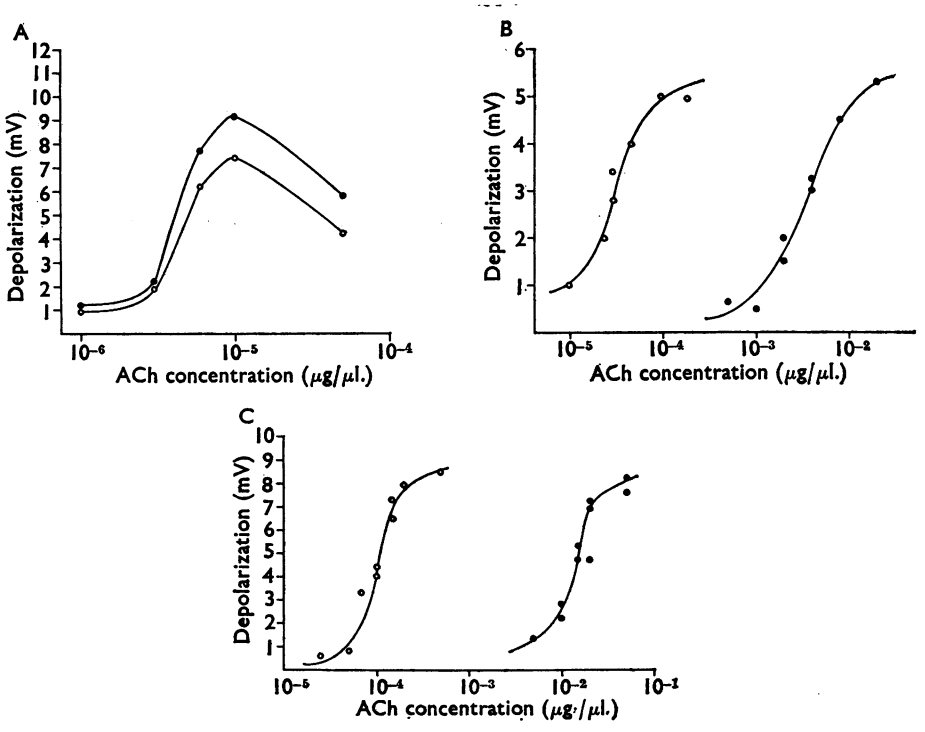

FIG. 1. Relationship between degree of depolarization (recorded extracellularly) and concentration of ACh. ACh solutions were applied by the microdrop technique; concentrations of ACh are plotted logarithmically. In A, data were obtained from one site within one proximal end-plate zone (●) and one end-plate-free area (○) of a 20 day denervated muscle. The volume of each microdrop of drug solution was 1 μl. In B, each response was obtained from a different end-plate-free site of a 21 day denervated muscle. ○, Control; DTC 0·5 mg/kg (●) was administered intravenously; the volume of each microdrop of ACh solution was 0·5 μl. In C, each response was obtained from a different end-plate-free site of a 20 day denervated muscle. ○, Control; atropine 0·5 mg/kg (●) was administered intravenously, and the volume of each microdrop of ACh solution was 0·5 μl.

In subsequent experiments application of ACh was restricted to end-plate-free areas and, by avoidance of repeated application of ACh to any given site on the muscle, desensitization was circumvented (Fig. 1B and C). In such experiments it was observed that the maximal response of end-plate-free sites of fifteen different 20 to 25 day denervated preparations had a mean potential of 8·3 mV and varied from less than 1 mV in one muscle to 18 mV in others. The variability observed from one muscle to another may be caused by differences in the rate of development of sensitivity to ACh, or in the amount of connective tissue that proliferated in the denervated preparations, or in the blood flow.

ACh-evoked depolarization and contracture

The relationship between ACh-evoked depolarization (recorded extracellularly) and the contracture tension was determined by the microdrop method at the proximal end-plate zone and at one end-plate-free site in each of six denervated preparations (Fig. 2A). From the results of these experiments several points emerged. First, contracture never occurred without prior depolarization, as illustrated in Fig. 2A. Secondly, the contractures were transient, although the muscles remained depolarized (Fig. 2D). The short-lived nature of the contracture elicited by ACh is clearly not the result of the repolarization of the membrane. Thirdly, the contracture tension was directly proportional to the degree of depolarization evoked by ACh. Finally, depolarization and tension relationships were similar at both end-plate-free regions and end-plate zones.

Effects of (+)-tubocurarine and atropine

The intravenous administration of DTC or atropine 0·5 mg/kg caused a marked shift to the right in the ACh concentration-depolarization curve obtained at end-plate-free regions of denervated muscles. As can be seen in Fig. 1B and C, approximately two hundred times as much ACh was required to produce a given intensity of depolarization after DTC or atropine as compared with the controls. The magnitude of the shift in the ACh concentration-depolarization curves produced by DTC and atropine in other experiments was approximately fifty to two hundred times. The findings observed after DTC in six denervated muscles were indistinguishable from those obtained after atropine in another six.

Although DTC and atropine increased the amount of ACh required to produce a given amount of depolarization, these cholinoceptor blocking drugs did not alter the relationship between the degree of ACh-evoked depolarization and contracture tension (Fig. 2B and C). These results were observed after DTC in three denervated preparations and after atropine in another three.

It was quite unexpected that low doses of atropine would antagonize the effects of ACh on the denervated skeletal muscle, and so a few experiments were conducted in order to determine the effects of atropine on neuromuscular transmission in the normal anterior gracilis muscle. In five of seven rats given atropine intravenously in doses ranging from 0·5 to 1·5 mg/kg, neuromuscular transmission was completely abolished (Fig. 3B). In one of the remaining two rats, atropine 1·0 mg/kg only partially decreased the amplitude of maximal twitch tension and subsequent doses (up to 6 mg/kg) had no additional effect; the residual twitch tension was readily abolished by DTC 0·4 mg/kg intravenously administered. In a single experiment

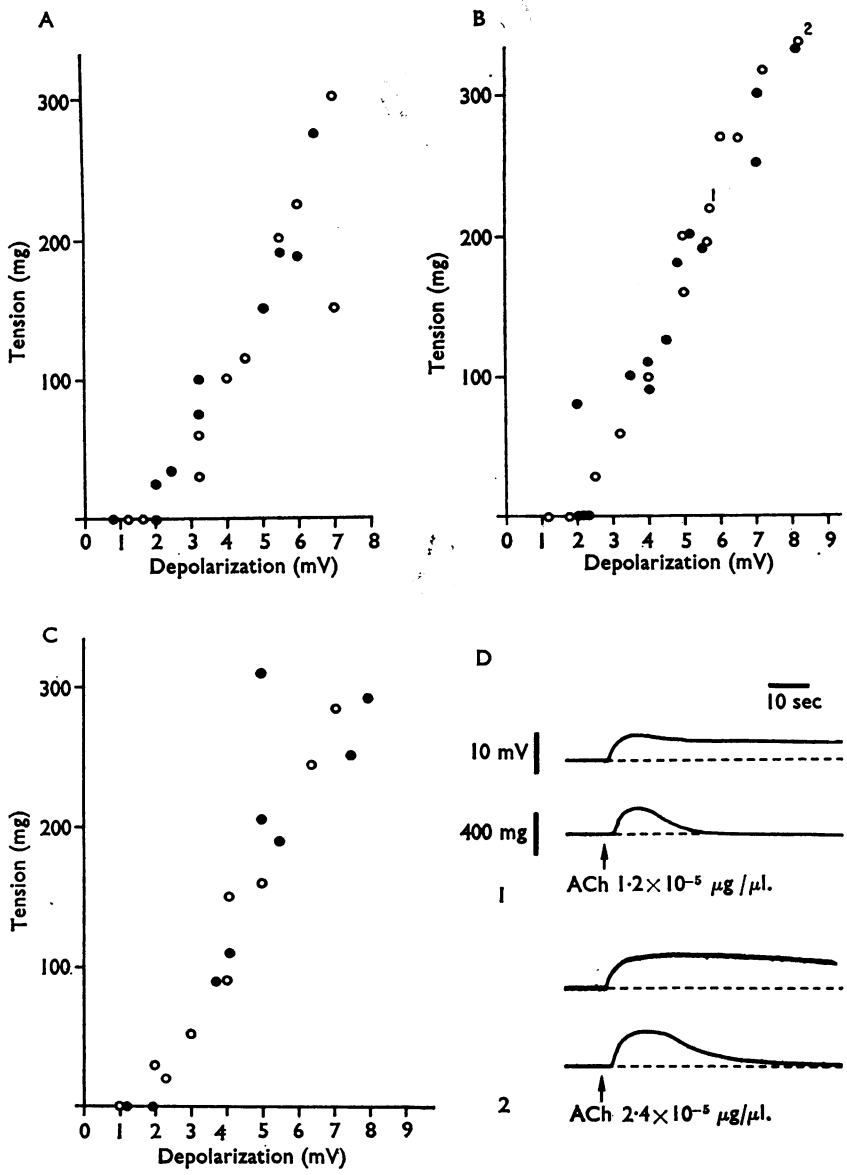

FIG. 2. Relationship between ACh-induced depolarization and contracture tension. Depolarization (mV) was measured extracellularly; ACh solution (0·5 μl.) was applied by the microdrop method. In A, data were obtained from one site within a proximal end-plate zone (●) and one end-plate-free area (○) of a 20 day denervated muscle. In B, each set of depolarization-tension responses was obtained from a different end-plate-free site of a 21 day denervated muscle. ○, Control; DTC 0·5 mg/kg (●) administered intravenously increased the concentration of ACh required to produce a given amount of depolarization by approximately a hundredfold. In C, each set of depolarization-tension responses was obtained from a different end-plate-free site of 21 day denervated muscle. ○, Control; atropine 0·5 mg/kg (●) administered intravenously increased the concentration of ACh required to evoke a given amount of depolarization by approximately a hundredfold. In D, the responses illustrated correspond to the experimental observations as indicated by the numbers depicted in B.

atropine 10 mg/kg had no effect on neuromuscular transmission. Intravenously administered DTC, in a total dose of 0·1–0·5 mg/kg, completely blocked neuromuscular transmission in three rats (Fig. 3A).

Effects of noradrenaline, adrenaline, and isoprenaline

Since cholinoceptors develop in the end-plate-free regions of chronically denervated skeletal muscle (Axelsson & Thesleff, 1959 ; Miledi, 1960), the possibility that adrenoceptors might also develop was investigated. In this series of experiments,

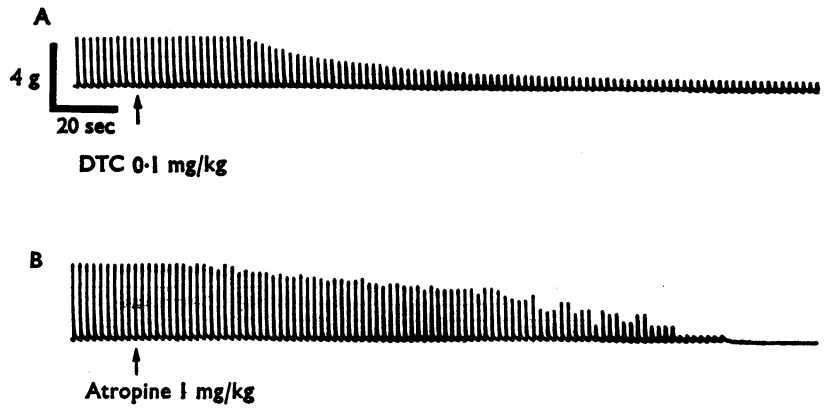

FIG. 3. Effects of cholinergic blocking agents on normal neuromuscular transmission. During electrical stimulation of the obturator nerve the drugs were administered intravenously and their effects noted on maximal twitch tension. The stimulus intensity was twice that required to produce maximal twitch tension ; the frequency of stimulation was once every 2 sec. The effect of DTC on maximal twitch tension is shown in A ; that of atropine, in B.

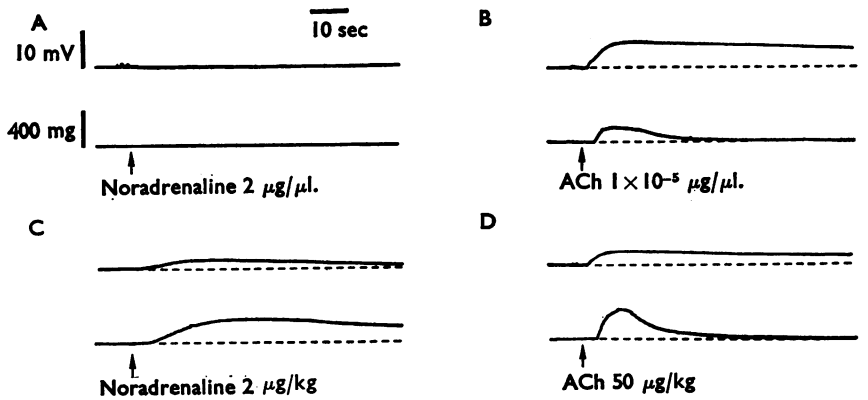

FIG. 4. Effects of noradrenaline on denervated muscle. Depolarizations were recorded extracellularly with a glass microelectrode ; drugs were applied by the microdrop method and the volume of the drop was 1 μl. Each drop was applied to a different end-plate-free site. Drugs were also administered intra-arterially. Responses shown were all obtained from the same 24 day denervated muscle. Upper trace in each set is depolarization ; lower trace is tension. In A, topically applied noradrenaline produced no effect. In B, topically applied ACh evoked both depolarization and contracture. In C, intra-arterially administered noradrenaline elicited both depolarization and contracture. In D, intra-arterially administered ACh caused both depolarization and contracture.

the effects of noradrenaline, adrenaline, and isoprenaline administered parenterally and applied topically by the microdrop technique were examined in eleven muscles 5–30 days after section of the motor nerve. Microdrops of these catecholamines (1–20 μg/μl.) were applied to both end-plate zones and end-plate-free regions of denervated muscles. The drugs did not produce a contracture, and there was usually no potential change observed with either intracellular or extracellular recording (Fig. 4A). Occasionally, however, the drugs caused small hyperpolarizations and less frequently minute depolarizations. Even though topically applied catecholamines did not produce a contracture, they markedly increased the frequency of the fibrillatory potentials. In all the experiments, ACh applied topically evoked both depolarization and contracture (Fig. 4B).

Bhoola & Schacter (1961) found that adrenaline produced contracture of the isolated, denervated diaphragm muscle of the rat. The reason why the diffuse application of adrenaline to a denervated muscle *in vitro* produced a contracture whereas the discrete application of microdrops of adrenaline in the present study did not evoke contracture is unknown. The difference between the results of the present and previous investigations may be due to the use of different muscles.

In contrast to topical administration, intra-arterial administration of 1 to 15 μg/kg of catecholamine usually caused depolarization and contracture (Fig. 4C). Of the eleven preparations included in the study, only in three muscles did intra-arterially administered catecholamines fail to evoke depolarization or contracture. Tachyphylaxis quickly developed to the effects of the catecholamines and cross-tachyphylaxis with intra-arterially administered ACh appeared to occur. Bowman & Raper (1965) demonstrated that parenterally administered catecholamines caused hyperpolarization and contracture in denervated skeletal muscle of the cat. The reason for the differing results are not clear, though they might be due to the use of different muscles or species.

Discussion

In the present study the microdrop technique for the local application of drugs permitted an analysis of the relationship between ACh-induced depolarization and contracture of the denervated, anterior gracilis muscle *in vivo*. The small size of the microdrop as well as the discrete location of the end-plate zones enabled a comparison to be made of the effects of ACh at end-plate areas and the end-plate-free regions. The data support the long-assumed and generally accepted view that ACh-elicited depolarization is related to contracture in denervated skeletal muscle. The findings also suggest that the relationship between depolarization and tension is similar at both end-plate zones and end-plate-free regions (Fig. 2A). Furthermore, atropine and DTC do not affect the depolarization-tension relationship but merely antagonize the depolarizing effects of ACh.

Dale & Gaddum (1930) and Beránek & Vyskočil (1967) clearly demonstrated that relatively high concentrations of atropine are able to antagonize the effects of ACh on denervated, mammalian skeletal muscle *in vitro*. In addition, Beránek & Vyskočil (1967) found that more than 300 times more atropine than DTC was required to produce an equal effect on the ACh-induced potentials in the denervated diaphragm muscle of the rat. Since normal skeletal muscle is endowed with ACh receptors of the nicotinic type, it is not surprising that denervated muscle develops receptors of a similar nature.

In contrast to the results of previous studies, atropine and DTC in the same dose range produced similar antagonizing effects on the ACh-evoked potentials of the denervated anterior gracilis muscle (Fig. 1B and C). These data suggest that the ACh receptor that develops on the end-plate-free regions after denervation has both muscarinic and nicotinic properties. An alternative suggestion is that separate muscarinic and nicotinic receptors develop on the denervated muscle. The anterior gracilis muscle of the rat may in fact be unique because of the demonstrated ability of low doses of atropine to block neuromuscular transmission in some normal preparations (Fig. 3B). Atropine is able to antagonize some of the nicotinic effects of ACh but usually high doses are required. (For a more complete discussion, see Dale & Gaddum, 1930; Abdon, 1940; Koelle, 1965; and Beránek & Vyskočil, 1967.)

The reason why topically applied catecholamines did not produce either depolarization or contracture despite the fact that parenterally administered catecholamines elicited both responses is yet unknown. However, these findings suggest that catecholamine-evoked contracture in the denervated, anterior gracilis muscle is not the result of the development of adrenoceptors on the muscle membrane. Since ACh-evoked contracture is the result of the development of receptors over the entire muscle-fibre membrane, catecholamines and ACh appear to cause contracture in denervated muscle by different mechanisms.

Catecholamine-evoked contracture is not the result of some vascular phenomenon because the contractile effects are independent of changes in blood flow in denervated skeletal muscle of both rat and cat (Bowman & Zaimis, 1961; Bowman & Raper, 1965). The mechanism by which catecholamines produce contracture is not completely clear. They are known to cause an increase in the fibrillatory activity of denervated skeletal muscle (Luco & Sánchez, 1959; Miledi, 1960, unpublished results; Bowman & Zaimis, 1961; Rushworth, 1964; Bowman & Raper, 1965). Bowman & Raper (1965) concluded that there is a causal relationship between the catecholamine-evoked increase in the frequency of the fibrillatory potentials and contracture. In the present study, topically applied catecholamines markedly increased the fibrillatory potential frequency without evoking contracture. Further research is clearly necessary to explain the disparate results. It is possible that catecholamines may induce contracture by some other mechanism. For example, catecholamines may elicit contracture by releasing some contracture-producing substance.

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