THE EFFECT OF TEMPERATURE ON THE DIRECT MUSCLE TWITCH RESPONSE AND THE ACTION OF DRUGS ON THE ISOLATED DENERVATED RAT DIAPHRAGM

BY

E. LETLEY

From the Department of Pharmacology, King's College, London

(RECEIVED MARCH 16, 1960)

The effect of temperature on the direct twitch response of the normal and denervated rat diaphragm, and the sensitivity of the contractural response of the denervated muscle to acetylcholine, suxamethonium, decamethonium, and potassium salts, was studied. Cooling the normal diaphragm from 39 to 20° increased the size of the direct twitch, but below this temperature the twitch response of the muscle was progressively depressed. The denervated muscle twitch was, by contrast, maximal at 39° and decreased as the temperature was lowered. This property of the denervated muscle first appeared about the third day after denervation. The contractural response of the denervated diaphragm to applied acetylcholine was enhanced by cooling, while the response to potassium salts was depressed. The depression of the contracture induced by potassium followed closely the depression of the direct muscle twitch of the denervated muscle over the same temperature range.

Previous work on the relation between temperature and the size of the direct muscle twitch response has indicated that generally the tension developed in a twitch is greater at low temperature. This has been found for the frog sartorius over temperature ranges from 0 to 20° (Hill, 1951), whereas studies on the rat triceps (Walker, 1949) have indicated that an 80 to 90% increase in the size of the indirect muscle twitch occurs on cooling from 37 to 24°. These findings have been confirmed by Doudoumopoulos and Chatfield (1959), who found that the twitch tension of the rat gastrocnemius was greatest between 26 and 18°. Work on the cat tibialis anterior and rat diaphragm muscles (Maclagan and Zaimis, 1957; Bigland, Goetzee, Maclagan, and Zaimis, 1958), however, indicates that in these muscles lowering of temperature decreases the size of the twitch response. The present studies were made to investigate whether both the normal and denervated rat diaphragm showed similar or dissimilar changes in the size of the direct twitch response when cooled.

Recently it has been shown that cooling markedly increases both the magnitude and duration of action of depolarizing blocking agents in cat muscle (Zaimis, 1958; Bigland *et al.*, 1958), whereas the blocking action of competitive agents

is reduced by cooling (Holmes, Jenden, and Taylor, 1951). In view of these findings it seemed important to establish whether the contractural response produced by acetylcholine (Frank, Nothmann, and Hirsch-Kaufmann, 1922; Gasser and Dale, 1926; Dale and Gaddum, 1930; Knowlton and Hines, 1937; Brown, 1937) and potassium salts (Frank, Nothmann, and Guttman, 1923; Gasser and Dale, 1926; Brown, 1937) in denervated mammalian muscle was materially affected by cooling.

METHODS

The experiments were made on the isolated rat diaphragm preparation (Bülbring, 1946), denervated some days previously by removal of the right phrenic nerve under ether anaesthesia. The muscle was suspended in a bath of 50 ml. capacity and bathed with Krebs solution aerated with 95% oxygen and 5% carbon dioxide. Stimulation of 3 milliseconds duration at 120 volts was delivered at 5 per min. Twitch responses to electrical stimulation, together with contractural responses to various drugs, were recorded by means of a semi-isometric lever on a smoked drum. The doses of drugs are expressed as their final bath concentrations.

In the experiments on the effect of temperature on the direct twitch response of the denervated muscle, the normal diaphragm of the opposite side was used as a control.

FIG. 1.—The effect of cooling on the direct twitch response of (a) a normal rat diaphragm, and (b) a 21-day denervated diaphragm. Both muscles were suspended in Krebs solution containing 2×10^{-6} tubocurarine. The normal muscle was cooled in 4° steps (left to right) from 39° to 3° . The denervated muscle was cooled in 3° steps from 39° to 12° .

FIG. 2.—The relation between the direct twitch response and temperature for normal and denervated diaphragms in the presence of tubocurarine. The curves represent: (Δ—Δ) the mean for normal muscles, (Δ---Δ) the mean for muscles denervated from 3 to 28 days, (○—○) 1-day denervated muscle, (○—○) 2-day denervated muscle, and (●—●) 3-day denervated muscle.

RESULTS

The Effect of Temperature on the Size of the Direct Twitch Response of the Normal and Denervated Diaphragm

In all experiments on both normal and denervated muscles a concentration of 2×10^{-6} tubocurarine was maintained to abolish any neurally mediated effects, thereby ensuring that the twitch responses recorded were direct muscle responses.

Fig. 1 shows records from (a) a normal muscle, and (b) a muscle denervated for 21 days. The size of the direct twitch response of the curarized denervated muscle at 39° was much greater than that of the curarized normal muscle at the same temperature. As the bath temperature was lowered the size of the direct twitch response of the normal muscle increased down to a temperature of approximately 20° , while below this temperature the twitch response became sluggish (Li, 1958). In marked contrast to the normal diaphragm, the direct twitch response of the denervated muscle underwent a progressive decline as the temperature was lowered; also the twitch itself became sluggish in form at a higher temperature in the denervated muscle than in the normal muscle.

The results of a large number of experiments on normal and denervated diaphragms are graphically presented in Fig. 2. The mean curves which relate the size of the direct muscle twitch to temperature for the normal and denervated muscles (from 3 to 28 days) are significantly different. The normal diaphragms showed their greatest twitch responses at nearly 20° , whereas the denervated muscles gave their greatest twitch responses around 39° . The curve for the muscle denervated for 1 day shows but little departure from the normal, unlike that for the muscle denervated 3 days which corresponds closely to the mean curve for muscle denervated from 3 to 28 days. These findings therefore show that denervation brings about a characteristic change in the relation between the size of the direct muscle twitch response and temperature; this change first appears about the second day after denervation and is complete soon after the third day.

The Effect of Temperature on the Contractural Response of the Denervated Diaphragm to Acetylcholine, Suxamethonium, and Decamethonium

Cooling the denervated muscle to 30° , 20° , and 10° , respectively, progressively increased the magnitude and duration of the contractural response which the standard (2×10^{-6}) dose of acetylcholine elicited (Fig. 3). Further, the threshold dose of acetylcholine which excited the denervated muscle at 39° was reduced by at least one log-dose on cooling to 20° . When the muscle was rewarmed the original sensitivity to acetylcholine was regained, indicating that the sensitization of the muscle to acetylcholine by cooling was a reversible process. Similar experiments carried out in the presence of 5×10^{-7} neostigmine have shown that the presence of an anti-cholinesterase in no way modifies the sensitization of the muscle to acetylcholine at low temperature, an observation which suggests that the hypersensitivity was not caused by the inactivation of the muscle cholinesterase by cooling. The contractural responses of the denervated diaphragm to acetylcholine were incompletely blocked by tubocurarine, 2×10^{-6} .

Suxamethonium and decamethonium also showed increased activity on the denervated diaphragm at low temperature. Generally, it was found that the degree of sensitization seen for

FIG. 3.—Contractural responses of the denervated diaphragm to acetylcholine are interposed between groups of direct twitch responses. On the left are contractures to doses of 1, 2, 4, and 8×10^{-6} acetylcholine. The test dose of 2×10^{-6} acetylcholine was then given to the muscle at 39° . Cooling the preparation to 30° , 20° , and 10° progressively enhanced the contractural response of the denervated muscle to the test dose of acetylcholine.

FIG. 4.—At (●) potassium chloride was added to the bath to give a concentration of 5×10^{-3} . Contractural responses of the same denervated diaphragm to 5×10^{-3} potassium chloride at (a) 38° , (b) 34° , (c) 30° , and (d) 22° . Rewarming the muscle to 39° (e) restored the original sensitivity of the preparation to the test dose of potassium chloride.

FIG. 5.—The dose-response relations for the contractures produced in the denervated rat diaphragm at 37° by acetylcholine (Δ — Δ), potassium chloride (●—●), potassium bromide (○—○), potassium iodide (○—○), potassium nitrate (□—□) and potassium sulphate (■—■). The contractural response elicited by a sucrose solution isosmotic with a solution of 5×10^{-3} potassium chloride is represented (—·—).

suxamethonium was about the same as that found for acetylcholine. The sensitization seen with decamethonium was usually much smaller than that with acetylcholine.

The Effect of Temperature on the Contractural Response of the Denervated Diaphragm to Potassium Salts

Large doses of potassium salts (1×10^{-3}) were found to depress the electrical excitability of the denervated muscle, confirming the findings of Hajdu, Knox, and McDowall (1950). However, still larger doses, 5×10^{-3} , in addition to depressing the electrical excitability of the muscle also produced a typical contracture (Frank, Nothmann, and Guttman, 1923; Gasser, 1930). These doses of potassium salts produced contractures which were attributed specifically to the presence of the potassium ion since the nature of the potassium salt used only slightly influenced the responses obtained. Control experiments showed that the effect was not caused by any effect of the potassium salts on the pH of the Krebs solution, or an osmotic action, since an osmotic solution of sucrose produced only a very small contracture (Fig. 5).

Cooling, instead of producing enhancement of the contractural response as was found to be the case with acetylcholine, suxamethonium, and, to a lesser extent, with decamethonium, depressed the contractural response to potassium chloride (Fig. 4). The depression of the contractural response to potassium salts on cooling paralleled the simultaneous depression of the twitch response.

The contractural responses of the denervated diaphragm to doses of 5×10^{-3} potassium chloride were unaffected by tubocurarine, 2×10^{-6} .

DISCUSSION

Frog skeletal muscle is known to give a greater twitch at low temperature attributed to the temperature coefficient of the velocity of shortening being less than the temperature coefficient of relaxation (Hill, 1951). It is possible to account for some of the present findings in the normal rat diaphragm in this way. This might well be the reason for the increase in size of the twitch response of the normal diaphragm on cooling to 20°, but additional factors probably come into play when the muscle is cooled below 20° to cause the twitch response to gradually decline. Thus below 20° the oxygen consumption of the rat is decreased and at the same time there is a rise in the blood sugar and in the level of the plasma inorganic phosphate (Andjus and Smith, 1955). In addition to these findings, Li (1958) reported that the efficiency of the rat neuromuscular junction was greatest at temperatures above 15° while below this temperature changes in the size and duration of the muscle action potentials could be observed.

It would seem from Hill's (1951) work that, in the rat, denervation may, in effect, cause the temperature coefficient of the velocity of shortening to become greater than the temperature coefficient of relaxation, possibly by bringing about fundamental changes in the biochemical processes of the muscle related to contraction. Whatever the true explanation of these phenomena may be, it is of considerable interest that the appearance of these new properties of the denervated muscle, with respect to the change in its twitch response on cooling, should be on or about the third day after denervation, as it is at this time also that the denervated rat diaphragm is known to develop contractural properties to depolarizing drugs (Paterson, 1957). This would seem strong evidence for regarding the progressive depression of the twitch response of denervated muscle on cooling as a property *per se* of denervated muscle. Axelsson and Thesleff (1959) have recently drawn attention to the fact that denervated mammalian muscle will display increased sensitivity to humoral agents and other drugs only when there is a complete lack of connexion between the muscle and living nerve tissue. In support of this view, Jefferson, Phillips, and Necheles (1950), Jefferson, Ogawa, and Necheles (1958), and Jefferson, Ogawa, Toman, Scruggs, and Necheles (1959) have shown that the degenerative changes which normally appear in the diaphragm of the dog after phrenic nerve section can be prevented by either anastomosing the peripheral cut end of

the phrenic to the other cut end of the same phrenic, or to the vagus nerve. These findings suggest that the degenerative changes appear in denervated mammalian muscle only when the trophic influence of a functional, or potentially functional, nerve supply has been lost. If this is so, the appearance of the characteristic twitch responses of the denervated rat diaphragm might well be associated with the loss of the trophic influence of the phrenic nerve innervation.

The probable explanation why cooling enhances the contractural responses elicited with acetylcholine and suxamethonium and yet depresses those produced by potassium salts is that these two groups of compounds produce contracture in denervated muscle by different mechanisms. Some evidence in favour of the dissimilar modes of action of acetylcholine and potassium ions has already been put forward in the literature. Brown and Feldberg (1936) found that 7×10^{-4} tubocurarine blocked the response of the perfused superior cervical ganglion to pre-ganglionic impulses and to applied acetylcholine but not to potassium chloride. Buchthal and Lindhard (1939) were also impressed by the fact that lizard muscle could still be stimulated with potassium chloride when it was refractory to nerve stimulation and applied acetylcholine. They suggested, on this basis, that acetylcholine and potassium chloride acted upon different regions of the muscle endplate. In agreement with these workers it was found in the present study that the contractural responses of the denervated diaphragm produced by acetylcholine were blocked by a dose of 2×10^{-6} tubocurarine whereas those produced by doses of 5×10^{-3} potassium chloride were completely unaffected by the tubocurarine. Examination of the log-dose curves for acetylcholine, potassium chloride, potassium bromide, potassium nitrate, and potassium sulphate for this preparation (Fig. 5) shows that the curve for acetylcholine is not parallel to those for the potassium salts which are grouped closely together, and are themselves parallel. Further, the graph indicates that acetylcholine can evoke a contracture from the denervated muscle in doses approximately 1,000 times smaller than those required for potassium salts (Brown, 1937).

The evidence presented above indicates that acetylcholine and related substances trigger a contracture in denervated mammalian muscle by combining with receptors which, according to Axelsson and Thesleff (1959), are situated diffusely over the whole of the outer surface of the muscle membrane. Potassium salts appear to produce contracture in the denervated muscle either by

acting directly on some other region of the former endplates or muscle membrane generally, or by an intracellular mechanism. An intracellular mechanism cannot be ruled out since massive doses of potassium salts have to be given before any contracture is seen, and potassium sulphate, the least permeable of the potassium salts used, was the weakest of the potassium salts in producing contracture in the denervated rat diaphragm.

The results suggest that the biochemical processes concerned with the production of a mechanical twitch after an electrical stimulus, together with those which produce a contracture in the denervated muscle on the application of potassium salts, are similarly depressed by cooling. That enhancement of the contractures produced by acetylcholine is seen on cooling may therefore be explained by specific sensitization of the muscle receptors by cooling.

I am greatly indebted to Professor G. Brownlee for his advice and criticism. The experiments were carried out during the tenure of a Medical Research Council Scholarship.

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