Br. J. Pharmac. (1976), **58**, 109–116

THE EFFECT OF CATECHOLAMINES ON THE INFLUX OF CALCIUM AND THE DEVELOPMENT OF TENSION IN DENERVATED MOUSE DIAPHRAGM MUSCLE

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1 The nature of the catecholamine-induced contracture of chronically denervated mouse diaphragm muscle has been investigated and compared with the contractural response evoked by acetylcholine.

2 The time course of onset of catecholamine-sensitivity in denervated diaphragm muscles was similar to the development of acetylcholine sensitivity. However, catecholamine contractures were absent in tissues denervated for periods longer than 90 days whereas acetylcholine-sensitivity was still evident several months after denervation.

3 The catecholamine-induced contracture of the denervated muscle was inhibited specifically by β-receptor blocking drugs and was unaffected by α-receptor blocking drugs and cholinoceptor antagonists.

4 Catecholamine-induced contractures of denervated muscles, unlike contractures to acetylcholine, were dependent upon the presence of spontaneous fibrillation and the amplitude of spontaneous fibrillation was increased by catecholamines. Fibrillation was absent in the presence of tetrodotoxin (1 μM), 2,4-dinitrophenol (10 μM), potassium cyanide (10 μM), ouabain (100 μM), in lithium chloride Ringer solution and at low temperature. Under these conditions catecholamine-induced contractures, but not those to acetylcholine, were abolished.

5 Labelled calcium was found progressively to enter denervated muscle fibres and this entry of calcium was increased by catecholamines. It is suggested that this calcium entry may represent either an increased calcium permeability of denervated muscle fibres which is increased further by catecholamines or the presence of a calcium current that occurs during the fibrillatory potentials of denervated muscle.

Introduction

Chronically denervated mammalian skeletal muscle has been shown to produce contractures to applied catecholamines both *in vivo* (Bowman & Zaimis, 1961; Bowman & Raper, 1965; Turkanis, 1969) and *in vitro* (Montagu, 1955; Bhoola & Schachter, 1961; Paterson, 1963; Bhoola, Evans & Smith, 1972). The mechanism underlying this contractural response is poorly understood. Following denervation, mammalian skeletal muscle also produces a contracture in the presence of acetylcholine (Brown, 1937; Rosenbleuth & Luco, 1937). It has been demonstrated that this response is the result of development of cholinoceptors over the entire muscle fibre surface (Axelsson & Thesleff, 1959). The present experiments were designed to compare the pharmacology of the catecholamine-induced contracture of denervated muscle with the acetylcholine contracture, in order to see if there were any similarities in the mode of action of these drugs.

It was observed in the course of these experiments that the denervated muscle contracted in response to the catecholamines provided that the bathing fluid contained calcium. Similar findings were reported by Bhoola & Schachter (1961). Much evidence suggests that calcium ions play an important part in the linkage between excitation and contraction in muscles with normal resting potentials (see Sandow, 1965). Consequently, it was of special interest to study the calcium-dependence of these contractures and the effect of catecholamines on calcium fluxes.

The results obtained indicate that there may be a similarity between the response of denervated muscle to the catecholamines and the known effect of these drugs on the twitch tension of innervated muscle (Bowman & Zaimis, 1955, 1958; Bowman, Goldberg

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& Raper, 1962) in that activation of fibres is a prerequisite for both processes. In denervated muscle the activation of fibres occurs spontaneously and is known as fibrillation (Tower, 1939). Some of these results have been presented in a preliminary form (Bhoola *et al.*, 1972).

Methods

Diaphragm muscles from adult mice of either sex, body weight 25 to 35 g, were used. The diaphragms were denervated by intrathoracic section of the left phrenic nerve under chloroform anaesthesia at various intervals before the mice were killed. Isolated hemidiaphragms were suspended in a 10 ml organ bath which was filled with Ringer solution of the following composition (mM): NaCl 130, KCl 5, NaH_2PO_4 1, $CaCl_2$ 2.5, $MgCl_2$ 1, $NaHCO_3$ 12 and glucose 12. This solution was gassed with 95% O_2 and 5% CO_2 at all times and the temperature was maintained at 37 ± 2°C unless stated otherwise. In some experiments the effects of lithium ions on the contractile response of denervated skeletal muscle to acetylcholine and isoprenaline and on the influx of calcium into denervated muscle was investigated. In these experiments lithium Ringer solution of the following composition was used (mM): LiCl 130, KCl 5, NaH_2PO_4 1, $CaCl_2$ 2.5, $MgCl_2$ 1, $KHCO_3$ 12 and glucose 12.

Isometric tension developed by the muscles in response to drugs or electrical stimulation was measured with Sangamo displacement transducers (type D1/100) under an applied resting tension of 2 grams. The output from these transducers was led through a preamplifiier with a pre-set gain of 1 to 10 into a Devices heated stylus pen recorder (type DC5H).

In order to stimulate muscles electrically, silver wire electrodes were placed at each end of the muscle and rectangular pulses of 1 ms duration and supramaximal intensity at a frequency of 0.05 Hz were applied.

To investigate the effect of isoprenaline and acetylcholine on calcium influx into denervated diaphragm muscles, paired segments of hemidiaphragms or groups of whole diaphragms were incubated for 3 min with ^{45}Ca Ringer solution and drug was then added to one of the segments or groups of muscles. After a given period of incubation, the muscles were rinsed briefly in tracer-free Ringer solution and the ^{45}Ca content was measured as described by Evans (1974).

The extent of the extracellular space in mouse diaphragm muscles was determined by the use of $^{35}SO_4$ as an extracellular marker (Walser, 1954; Robert, 1956). In these experiments, diaphragm muscles together with the rib cage were excised and incubated in Ringer solution to which $Na_2\ ^{35}SO_4$ (0.1 μCi/ml Ringer) was added. After a given period of incubation, the diaphragm muscles were rinsed in $^{35}SO_4$-free Ringer solution, blotted, and dissected free of the rib cage and tendon. Each diaphragm was then separated into right and left hemidiaphragms and the wet weight of the hemidiaphragms was determined on a torsion balance. The weighed muscle samples were digested with 0.3 ml 0.3 M NaOH, then 2 ml of methanol and 10 ml of phosphor (2, methoxyethanol, 3 litres, toluene 7 litres, 2,5-diphenyloxazole 40 g, 1 : 4-di-2 (5-phenyloxazolyl)-benzene 1 g) was added to the samples before measurement of ^{35}S by liquid scintillation counting. The $^{35}SO_4$ space was calculated as

$$\frac{\text{ct/min per g wet weight of muscle}}{\text{ct/min per ml of Ringer}}$$

Results

Characteristics of the contractural response

Responses of denervated mouse diaphragm muscle to acetylcholine and (±)-isoprenaline are shown in Figure 1a, and the time course of contracture in the continued presence of both drugs is illustrated in Figure 1b. It can be seen that the response to isoprenaline is slower in onset and has longer duration than that to acetylcholine. The thickness of the base-line in Figure 1a and in subsequent records was produced by the spontaneous fibrillatory activity of the muscles. The catecholamine contractural response was accompanied by an increase in fibrillatory activity which can be seen as the increased amplitude of the base-line that occurs at the peaks of the isoprenaline responses in Figure 1a. It was found that the increase in tension and fibrillation produced by the application of isoprenaline to denervated diaphragm muscle was maintained for up to 45 min following washout of the drug. Tachyphylaxis to successive doses of isoprenaline given at intervals of less than 30 min was observed, and there appeared to be a deterioration of the catecholamine response compared to the acetylcholine response in tissues that had been mounted in Ringer solution for longer than six hours.

The response of denervated mouse diaphragm to the catecholamines was not always a simple increase in tension. Occasionally the response was biphasic and the contraction was preceded by a small and short lasting reduction in tone. Similar findings were reported by Bowman & Raper (1965) for denervated soleus and tibialis anterior muscles of the cat *in vivo*.

The maximum tension developed by denervated mouse hemidiaphragm muscles to acetylcholine was $1.71\ g \pm 0.16$ s.e. mean ($n = 5$). Isometric tensions produced by isoprenaline were smaller and were usually about 20% of those produced by acetylcholine.

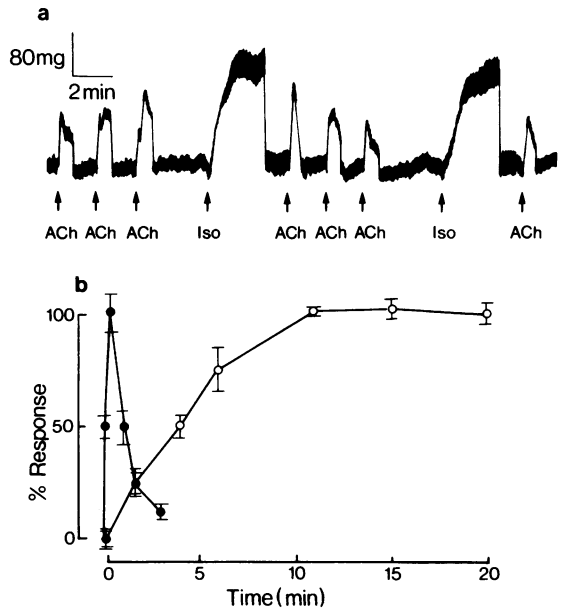

Figure 1 (a) Contractural responses of denervated diaphragm muscle of mouse evoked by acetylcholine, 5 μM (ACh) and isoprenaline, 3 μM (Iso). The recording was stopped 1 min after addition of acetylcholine and 2 min after addition of isoprenaline to the bathing fluid and fresh Ringer solution perfused until the tension had returned to the base-line. (b) Time course of the change in isometric tension of denervated mouse diaphragm muscle in the presence of acetylcholine and isoprenaline. The percentage of the maximum tension developed is plotted against time after application of drug. (●) Acetylcholine, 4 μM; (○) isoprenaline, 2.5 μM. Each point is the mean response of five muscles. Vertical lines show s.e. mean.

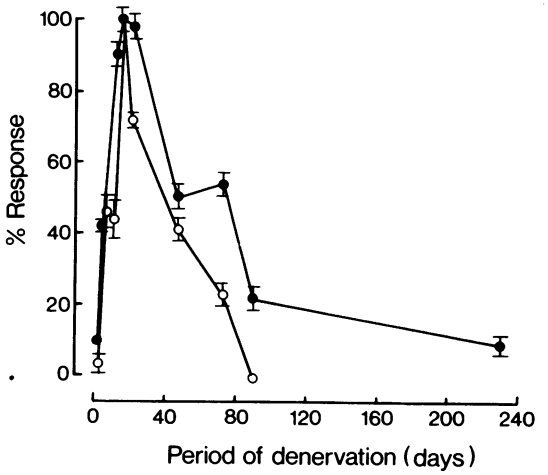

Figure 2 Time course of the development of sensitivity to acetylcholine and isoprenaline of denervated diaphragm muscle of mouse. Maximum contractural responses to 2 mM acetylcholine (●) and 2.5 μM isoprenaline (○) expressed as a percentage of the mean response at 15 days are plotted against time after denervation. Each point is the mean response of five muscles. Vertical lines show s.e. mean.

Development of the contractural response

In order to see if catecholamine-sensitivity of denervated diaphragm muscle developed in a similar way to acetylcholine-sensitivity, maximum contractural responses of denervated mouse diaphragm muscle to isoprenaline and acetylcholine in Ringer solution at 37°C were measured at various times after denervation. Isoprenaline was chosen as the most suitable catecholamine stimulus because the tissue showed highest specificity towards this catecholamine (see Figure 3). Figure 2 shows the time course of the development of the response to both drugs. Two days after denervation both acetylcholine and isoprenaline produced detectable contractural responses in the denervated muscle. The maximum effect of both drugs appeared after 14 days denervation. However, isoprenaline-induced contractures were absent in muscles that had been denervated for longer than 90 days, whereas acetylcholine contractures were still present in muscles 230 days after denervation.

The fact that isoprenaline contractures were absent in muscles which still responded to acetylcholine, suggested that the development of sensitivity to acetylcholine and to isoprenaline in denervated diaphragm muscles involved separate processes. Further indication of the difference between the development of acetylcholine and isoprenaline-sensitivity in skeletal muscle was obtained from studies on foetal and neonatal tissues. In these experiments it was found that whereas acetylcholine produced contractures in diaphragms taken from eight 22-day old foetal and six 2 to 3 day old neonatal rats, no contractures to isoprenaline were observed in these tissues.

Specificity of the contractural responses to acetylcholine and the catecholamines

To show how specific the contractural responses of denervated muscles to acetylcholine and isoprenaline were, the effects of several other potential agonists were tested. It was found that denervated mouse diaphragm muscle failed to contract in the presence of histamine (10 μM), 5-hydroxytryptamine (10 μM), bradykinin (0.01 μM), vasopressin (0.2 u/ml), γ-aminobutyric acid (2 mM) and L-glutamate (2 mM). However, the cholinomimetic drugs carbachol,

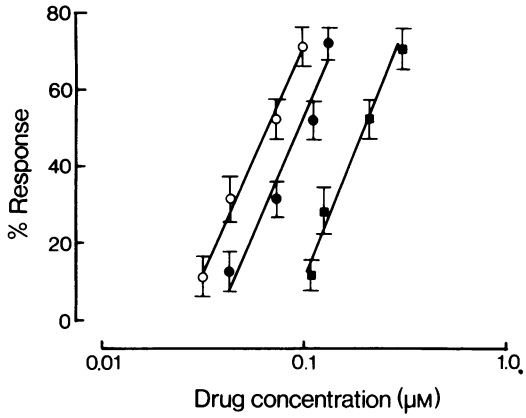

Figure 3 Dose-response curves of denervated diaphragm muscle of mouse to (±)-isoprenaline (○), (–)-adrenaline (●) and (–)-noradrenaline (■). Each point is the mean result from at least four muscles. Vertical lines show s.e. mean.

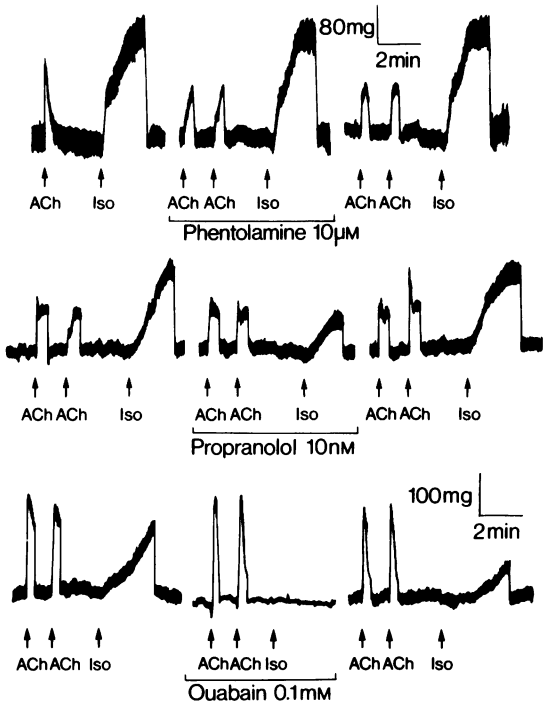

Figure 4 The effect of propranolol, phentolamine and ouabain on contractural responses of denervated diaphragm muscle of mouse evoked by acetylcholine, 5 μM (ACh), and isoprenaline, 2.5 μM (Iso). The muscles were preincubated with propranolol or phentolamine for 2 min or with ouabain for 5 min and were then treated with acetylcholine or isoprenaline in the presence of the antagonist. Responses recorded as for Figure 1(a).

butyrylcholine and nicotine, and the three catecholamines, (–)-noradrenaline, (–)-adrenaline and (±)-isoprenaline all produced dose-dependent increases in tension in the denervated muscle. Figure 3 compares dose-response relations of denervated diaphragm muscles to the three catecholamines. The tension produced by each drug is plotted as a percentage of the maximum response. On a molar basis isoprenaline was one and a half times as potent as adrenaline which was in turn twice as potent as noradrenaline.

The increase in tension produced by adrenaline and isoprenaline was unaltered by (±)-tubocurarine (7 μM) and atropine (20 μM) in concentrations that blocked the acetylcholine response. The contractural responses of denervated muscles to isoprenaline and adrenaline were unaltered also by the α-adrenoceptor blocking agents phentolamine (Figure 4a) and thymoxamine (1 μM to 10 μM). However, catecholamine contractures of denervated diaphragm muscles were blocked by the β-adrenoceptor antagonists propranolol (Figure 4b), sotalol and methoxamine (0.01 μM to 10 μM) added either before or in the presence of the agonist. These results suggest that denervated mouse diaphragm muscle possesses β-adrenoceptors that are separate from cholinoreceptors.

The relationship of β-adrenoceptive effects to cyclicadenosine 3′,5′-monophosphate (cyclic AMP) levels is well documented (Robison, Butcher & Sutherland, 1971) and Bowman & Nott (1969) have suggested that cyclic AMP is involved in the adrenoceptive processes of skeletal muscle fibres. However, in the present experiments, neither cyclic AMP nor the dibutyryl ester of cyclic AMP (10 μM to 1 mM) produced any effect on the tension of denervated muscles or modified the contractures produced by either acetylcholine or catecholamines.

Catecholamines are known to stimulate glycogenolysis in skeletal muscle as in most tissues via a cyclic AMP-mediated process (Mayer & Stull, 1971). However, it is unlikely that this process is immediately essential for the catecholamine-induced contracture of denervated muscles because it was found that substitution of sucrose for glucose in the Ringer solution abolished isoprenaline-induced contractures within 15 minutes. On restoration of glucose, isoprenaline-induced contractures reappeared. Acetylcholine-induced contractures were not abolished by this treatment. If catecholamine-induced contractures were a direct result of increased glycogenolysis such immediate dependence on the external glucose would not be expected.

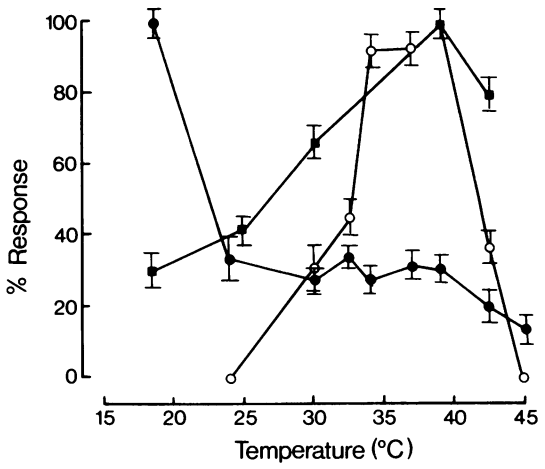

Figure 5 The effect of temperature on drug-induced contractural responses of denervated diaphragm muscle of mouse and on the fibrillatory activity of the muscle. (○) Tension developed in the presence of isoprenaline, 2.5 μM; (●) tension developed in the presence of acetylcholine, 3 μM; (■) amplitude of the fibrillatory activity of the muscle. Each point is the mean result from at least four muscles. Vertical lines show s.e. mean.

Effect of temperature, metabolic inhibitors, cardiac glycosides and lithium

Figure 5 shows the effect of temperature on the response of denervated muscle to acetylcholine and isoprenaline. It can be seen that the response to acetylcholine was greater at 18°C than at 37°C. Similar findings have been reported by Letley (1960) and Freeman & Turner (1969). On the other hand, the isoprenaline response showed a definite peak at 37°C as did the amplitude of the fibrillatory activity of the muscle. To investigate this further the response of denervated muscles to acetylcholine and isoprenaline was measured after treatment with metabolic inhibitors potassium cyanide (10 μM) and 2,4-dinitrophenol (10 μM). It was found that both metabolic inhibitors increased the resting tension and abolished the fibrillatory activity of the denervated muscle. In the presence of 2,4-dinitrophenol and potassium cyanide, isoprenaline-induced contractures of the denervated muscle were absent whereas acetylcholine contractures were unaffected or only partially inhibited. After removal of potassium cyanide from the muscle bath responses to isoprenaline reappeared after 15 minutes. However, the effects of 2,4-dinitrophenol were found to be irreversible.

The effects of glucose, 2,4-dinitrophenol, potassium cyanide and temperature suggested that the response of denervated muscle to isoprenaline was immediately dependent on oxidative metabolism. To test whether this dependence on oxidative metabolism was related to the action of the sodium pump of the muscle membrane, the effects of lithium and the cardiac glycosides ouabain and strophanthin K were investigated. Addition of ouabain (100 μM) or strophanthin K (100 μM) to the organ bath resulted in a rapid decrease (within 7 min) in the resting tension and fibrillatory activity of the denervated muscle. The introduction of lithium Ringer solution produced a gradual increase in the resting tension which reached its peak within 20–30 minutes. This increase in tension was accompanied by a decrease in the fibrillatory activity of the muscle. Preincubation of the denervated muscle with ouabain (Figure 4c) or strophanthin K for 15 min, or in lithium Ringer solution for 2 h abolished responses to isoprenaline but had little effect on acetylcholine contractures.

Effect of tetrodotoxin

Katz & Miledi (1967) found that depolarization of the extra-junctional membrane of denervated muscle produced by acetylcholine was insensitive to tetrodotoxin. On the other hand, the generation of action potentials in innervated muscle is blocked by tetrodotoxin (Narahashi, Deguchi, Urakawa & Ohkulo, 1960), which appears to inhibit specifically the inward sodium current (Kao, 1966). To see if the action of tetrodotoxin would provide a clue to the mechanism of the catecholamine-evoked contracture of denervated muscle, the effects of this drug were compared on both denervated and innervated diaphragm preparations. It was found that tetrodotoxin at a concentration of 3 μM completely blocked the innervated preparation to direct and indirect stimulation within 15 minutes. Similar doses of tetrodotoxin caused a decrease in resting tension of the denervated diaphragm and abolished both fibrillatory activity and isoprenaline-induced contractures. However, even after exposure for 60 min, tetrodotoxin (3 μM) was without effect on acetylcholine contractures.

The finding that the response to acetylcholine of denervated diaphragm muscle was totally unaffected by concentrations of tetrodotoxin that rapidly blocked the innervated preparation, provides confirmation for the view that the acetylcholine contracture of denervated skeletal muscle is the result of non-propagated depolarization of the muscle membrane (see Thesleff, 1960). However, the observation that isoprenaline contractures of the denervated muscle were absent in the presence of tetrodotoxin indicates that generation of the muscle fibre action potential is a prerequisite for the catecholamine response.

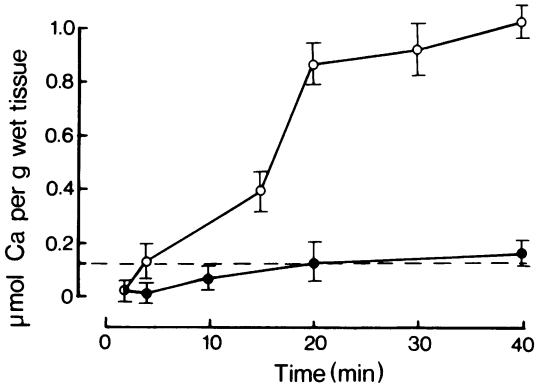

Figure 6 The time course of accumulation of calcium by denervated and innervated diaphragm muscles of mouse. Diaphragms were incubated in ^{45}Ca Ringer solution at 37°C for the intervals indicated and were then prepared for liquid scintillation counting as described in the Methods section. (○) Calcium accumulated in denervated muscles; (●) calcium accumulated in innervated muscles. Each point is the mean result from six muscles. Vertical lines show s.e. mean. The dashed line indicates the level of ^{45}Ca contained by the extracellular space.

Entry of calcium

Bhoola & Schachter (1961) reported that catecholamine-evoked contractures of denervated rat diaphragm muscle were dependent upon the presence of calcium in the Ringer solution. In the present experiments the dependence of isoprenaline contractures, acetylcholine contractures and contractions caused by direct electrical stimulation of muscles, on the calcium concentration of the Ringer solution was examined. After 45 min in calcium-free Ringer solution, the drug-induced contractures were absent but contractions caused by electrical stimulation were still evident after 2 hours. Thus the drug-induced contractures were specifically dependent on the presence of external calcium ions.

The influence of external calcium on the drug-induced contractures was investigated further by incubating muscles in Ringer containing $^{45}CaCl_2$ as a tracer. First, the resting entry of ^{45}Ca into denervated and innervated sides of diaphragm muscles was compared. To determine whether ^{45}Ca entered muscle fibres, the entry of ^{45}Ca was compared with the entry of $^{35}SO_4$. The entry of $^{35}SO_4$ into muscles gave a value for the extracellular space of the innervated sides of muscles of 0.25 ± 0.02, and 0.28 ± 0.02 s.e. mean ($n = 10$) for the denervated sides. These values were not significantly altered by drug treatment. The entry of ^{45}Ca into the innervated sides of muscles reached a plateau between 10 and 20 min after placing muscles in ^{45}Ca Ringer solution and this plateau level corresponded with the $^{35}SO_4$ space shown by the dashed line in Figure 6. However, on the denervated side there was a progressive entry of ^{45}Ca into muscle fibres (upper curve Figure 6).

Table 1 shows the effect of 4 and 20 min incubation with 2.5 µM isoprenaline on the entry of ^{45}Ca into paired segments of denervated hemidiaphragms. Treatment with isoprenaline for 4 min caused a threefold increase in the entry of ^{45}Ca into muscle fibres ($P < 0.001$). A similar effect was observed with adrenaline (2.7 µM). When denervated muscles were incubated in ^{45}Ca Ringer solution for 2 h and then placed in tracer-free Ringer solution, they lost 30% of the tracer in 4 minutes. Addition of isoprenaline (2.5 µM) to the washout solution had no significant effect on this loss of ^{45}Ca. Therefore, a net increase in the influx of calcium into the muscle fibres was caused by isoprenaline. The initial rate of influx of calcium caused by isoprenaline could not have been sustained because after 20 min the amount of ^{45}Ca in drug treated tissues was not significantly different from controls (Table 1).

Table 1 Effect of isoprenaline (2.5 µM) on accumulation of ^{45}Ca in denervated mouse diaphragm muscles

Incubation time (min)	*µmol Ca/g wet tissue* Control muscles	Treated muscles
4	0.15 ± 0.01	0.53 ± 0.06
20	0.87 ± 0.05	0.94 ± 0.06

Values are mean of 18 muscles ± s.e. mean.

In innervated muscles no catecholamine-induced influx of labelled calcium was observed either at the junctional region or in the non-innervated segments. Furthermore, the isoprenaline-induced influx of ^{45}Ca did not occur in muscles that had been denervated for 100 days, or in denervated muscles that were equilibrated with lithium Ringer solution, in the presence of ouabain (100 µM) or propranolol (1 µM), or at 18°C, all of these being experimental conditions in which the contractural response is absent.

Discussion

In the present study it was found that the increase in tension produced by the catecholamines in denervated mouse diaphragm muscles was accompanied by an increase in the fibrillatory activity of the muscles. Both the contractural response and increase in fibrillatory activity were related in terms of the relative potency of the catecholamines and sensitivity to antagonists. A catecholamine-induced increase in electrical activity associated with fibrillation of denervated muscle has been reported elsewhere (Bowman & Raper, 1965;

Smith & Thesleff, 1975). Bowman & Raper (1965) suggested that there was a causal relationship between the catecholamine-evoked increase in the frequency of fibrillatory potentials and contracture. The present evidence supports this view because the absence of isoprenaline-evoked contractures of denervated muscle in the presence of tetrodotoxin indicates very clearly the role of propagated action potentials in the increase in tension. The evidence from calcium flux measurements is supportive also in this respect because these show that the catecholamine-induced contracture of denervated diaphragm muscle is accompanied by an influx of calcium, which is known to enter the muscle fibres during activity (Bianchi & Shanes, 1959; Evans, 1974).

The 37°C optimum for the catecholamine response and the effects of 2,4-dinitrophenol, potassium cyanide, ouabain, strophanthin K and lithium could suggest a role for the sodium pump in the catecholamine-induced contracture of denervated muscle. This may be so, but Bowman & Raper (1964) showed that the frequency of fibrillatory potentials in denervated cat muscles was reduced by lowering the temperature and in the present study it was found that 2,4-dinitrophenol, potassium cyanide, ouabain, strophanthin K and lithium all blocked the catecholamine response and abolished fibrillatory activity. Therefore, it is likely that the important effect of these substances, in so far as the response to catecholamines is concerned, is the abolition of fibrillation.

Fibrillation of denervated muscles results from asynchronous contraction of muscle fibres and we have observed that only a fraction of denervated mouse diaphragm muscle fibres are contracting at any one time. Purves & Sakmann (1974) have estimated this fraction of active fibres to be about one-third for denervated rat diaphragm muscles. The results of Evans (1974) show that approximately 1 nmol Ca/mg dry muscle entered innervated mouse diaphragm muscles during a 15 min period of supramaximal tetanic stimulation. Thus if calcium entry per impulse is similar in denervated and innervated muscles then a value somewhat less than this would be expected for calcium entry into unstimulated denervated muscles. However, the present results (Figure 6) yield a value of 1.0 nmol Ca/mg dry unstimulated muscles per 15 min, and there was an initial fourfold increase in this level of calcium entry after treatment with isoprenaline. Therefore, it appears either that the resting permeability to calcium is higher in denervated fibres than in innervated fibres, and that this is increased still further by isoprenaline, or that denervated fibres have a much higher level of calcium entry per propagated impulse than occurs in innervated fibres.

At this stage it is possible to indicate some relationship between the increased tension of denervated muscles and the increased twitch tension of innervated muscles produced by the catecholamines (Bowman & Zaimis, 1955, 1958; Bowman *et al.*, 1962). In both cases muscle fibres have to be activated before any increase in tension can be shown. In the case of denervated muscle the activation is spontaneous but may be abolished by tetrodotoxin, which indicates the role of propagated action potentials in the increase in tension. In the case of innervated muscle there is no spontaneous activity and increased tension in the presence of catecholamines is only seen when muscles are stimulated electrically.

The present results indicate clearly that in denervated mouse diaphragm muscles the responses to catecholamines were not caused by reaction with cholinoceptors but with specific β-adrenoceptors, because they were produced most effectively by isoprenaline and were selectively blocked by β-receptor blocking drugs. This is comparable to the effect of catecholamines on denervated muscles of the cat, *in vivo* (Bowman & Raper, 1965). However, Paterson (1963) reported that in isolated denervated diaphragm of the rat the contracture evoked by adrenaline was blocked most effectively by α-receptor blocking drugs, while Yamada & Harigaya (1974) reported a biphasic contractural response to adrenaline elicited through both α- and β-receptors in denervated extensor digitorum longus and soleus muscles of the rat. We cannot explain the discrepancy between these results.

Comparison of the time course of development of acetylcholine- and isoprenaline-induced contractures in denervated mouse diaphragm muscles showed that after long periods of denervation, responses could be obtained to acetylcholine but not to isoprenaline. This again suggests that the sensitivity of denervated muscles to acetylcholine and the catecholamines are two separate receptive processes. The observation that foetal and neonatal tissues produced contractures in the presence of acetylcholine but not isoprenaline supports this view. In tissues denervated for long periods, in which the contractural response to isoprenaline was absent, and in the foetal and neonatal tissues studied, fibrillatory activity was undiscernible. This observation provides a further confirmation for the view that fibrillation is an integral part of the process involved in the catecholamine contracture of denervated skeletal muscle. However, the problem of the relationship between the β-adrenoceptor and the process which initiates spontaneous action potentials in denervated muscle fibres remains unsolved although the high resting influx of calcium in denervated muscles suggests the possibility that spontaneous inward calcium currents may play a trigger role.

This work was supported by the National Fund for Research into Crippling Diseases.

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(Received February 9, 1976. Revised April 5, 1976.)