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The Effects of Fructose 1,6-Diphosphate, Caffeine and Dantrolene Sodium on Suxamethonium-induced Contractures in Denervated Rat Skeletal Muscle

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Abstract—Previously unidentified forms of suxamethonium-induced contractures have been investigated in chronically denervated rat extensor digitorum longus (EDL) muscle at 20°C. Contractures were assigned to groups 1–6 on the basis of the peak tension (Tp_1) during 0–10 min exposure to the drug ($3{\cdot}0 \times 10^{-5}$M), ($7{\cdot}0 \times 10^{-6}$M), and ($3{\cdot}5 \times 10^{-6}$M) and the subsequent retention, increase, or decrease in tension (Tp_2), during the further 10 min. It is proposed that four stages exist in the development of contractile changes at 1–7, 8–35, 36–70 and 70–130 days after denervation (DPD) and that contractility is lost at 147 days after denervation. Initial changes, although present in EDL muscles in group 1 at 2·0 DPD s.d. ±1 (n=7) in response to the drug ($3{\cdot}0 \times 10^{-5}$M), were more apparent in EDL muscles in group 2 which were identified at 5·5 DPD s.d. ±1·6 (n=7) by an excessive contracture response (Tp_2) to the drug ($3{\cdot}0 \times 10^{-5}$M), 18·3 mN s.d. ±10·6. At 5·0 DPD s.d. ±2·7 (n=5) contracture tension (Tp_2) was commensurate with membrane depolarization, 13·1 mN/33·1 mV, but residual tension increased to 23·3 mN during the Krebs wash (80 min) whilst membrane depolarization decreased to 9·2 mV. Also, at 4·3 DPD s.d. ±2·3 (n=5) tension (Tp_2) increased significantly ($P \leq 0{\cdot}05$) in the presence of caffeine ($4{\cdot}1 \times 10^{-3}$M). Residual tension, which increased during a Krebs wash (30 min) at 0·42 mN min^{-1} after suxamethonium ($3{\cdot}0 \times 10^{-5}$M), was reduced by dantrolene sodium ($3{\cdot}8 \times 10^{-4}$M) at 0·2 mN min^{-1} after a latency of 36 min in muscles at 3·8 DPD s.d. ±1·1 (n=4). However, at 5 and 7 DPD (n=12) the latency of action of dantrolene sodium shortened from 30 to 6 min, and residual tension was reduced faster, 0·42 mN min^{-1} (cf. 0·2 mN min^{-1}). The second stage in the development of contractile changes was observed in EDL muscles at 8·4 DPD s.d. ±4·3 and 43·6 DPD s.d. ±25·3 (groups 3 and 4) which were characterized by a prominent peak in tension (Tp_1) in response to the drug ($3{\cdot}0 \times 10^{-5}$M), 16·5 mN s.d. ±6·6, c.f. 32·9 mN s.d. ±13·2, and a smaller second peak in tension (Tp_2), 8·4 mN s.d. ±4·3 c.f. 11·7 mN s.d. ±4·1, respectively. Further measurements, at 6·3 DPD s.d. ±1 (n=9) and 20 DPD s.d. ±9·7 (n=12), showed that although Tp_1 was commensurate with membrane depolarization, 21·2 mN/28·4 mV, and 30·5 mN/27·2 mV, in response to suxamethonium ($3{\cdot}0 \times 10^{-5}$M), Tp_2 was low with respect to membrane depolarization, 3·7 mN/38·2 mV and 9·7 mN/38·6 mV, respectively. The third stage in the development of contractile changes was observed in EDL muscles at 58·1 DPD s.d. ±22 (n=14) in group 5 which was typified by a sharp first peak in tension (Tp_1), 23·8 mN s.d. ±13·2 and by an absence of tension during 10–20 min exposure to the drug ($3{\cdot}0 \times 10^{-5}$M). In response to the drug ($3{\cdot}0 \times 10^{-5}$M) in the presence of fructose 1,6-diphosphate ($2{\cdot}3 \times 10^{-3}$M) or caffeine ($4{\cdot}1 \times 10^{-3}$M), the second peak in contracture tension (Tp_2) was recorded at 3 mN and 10·3 mN, respectively. In the fourth stage of contractile changes EDL muscles at 124·7 DPD s.d. ±2·3 (n=11), designated group 6, produced a minimal contractile response to suxamethonium ($3{\cdot}0 \times 10^{-5}$M), 6·4 mN (Tp_1) and 1·9 mN (Tp_2) in comparison with variable depolarization, 12·8 mV and 30·5 mV. Finally, at 147 DPD the drug-induced contracture response was absent (n=4) whilst membrane depolarization was less than normal control muscles, 7–11 mV (c.f. 10 13 mV). Contractures identified provide a basis for the study of the nature and sequence of changes in skeletal muscle after denervation.

Results of previous experiments showed that suxamethonium-induced membrane depolarization and contracture tension increased progressively in rat extensor digitorum longus muscle 1–28 days after denervation. Further experiments with tubocurarine and verapamil indicated that the influx of extracellular Ca^{2+} was not considered a significant factor in the development of contracture tension (Andrews 1984).

Investigation of morphological changes after denervation by Hogan et al (1965) and Hegab et al (1974) have shown that a rapid decrease in skeletal muscle mass and myofibrillar area occur after denervation. Hogan et al (1965) also observed a progressive loss of the glycogen content in fast twitch muscle and Srivastava & Das (1978) confirmed that the glycogen content of rat extensor digitorum longus (EDL) muscle was reduced after three days following sciatic nerve denervation. An increase in sarcotubular area, at three days following denervation, observed by Engel & Stonnington (1974), may have accounted for the prolonged twitch in fast and slow muscle after denervation in the cat (Lewis et al 1974), and in the rat (Lewis et al 1978). Furthermore, Finol et al (1981) proposed that the contractile changes following denervation occur in two phases in rat fast and slow twitch muscle, at 2–6 days, and 7–42 days after denervation. It was also proposed that changes occur in one or more elements in the excitation-contraction coupling mechanisms resulting in high levels of intracellular Ca^{2+}. Further evidence of changes in the sarcoplasmic reticulum have been found by Gutmann & Sandow (1965) in the caffeine-induced contracture response

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of rat EDL muscle, which commenced at 24 h and increased 3–6 days after denervation.

The present work was undertaken to clarify and analyse in detail the contracture response in fast twitch muscle to prolonged exposure to suxamethonium at specific periods after denervation.

Materials and Methods

Young adult female Wistar rats from a homogeneous group were divided into four sets. Rats in set 1, (217·8 g s.d. ± 32·4), were unilaterally denervated 1–147 days before experiments in the initial investigation into the contracture response of EDL muscle to 20 min exposure to suxamethonium at 20°C. In addition rats in set 2, (216·5g s.d. ± 32·4), were used to study the response of denervated muscle to the drug in the presence of fructose 1,6-diphosphate, or caffeine at 20°C. Right EDL muscles were denervated between 1–110 days before the experiment; contralateral EDL muscles were used as controls. Male rats in set 3, (214·6g s.d. ± 2·9·2) were used to study the effects of dantrolene sodium on residual contracture tension, six of these rats were kept as normal controls and the remainder were bilaterally denervated between 1–112 days before the experiments. 19 EDL muscles denervated between 1–7 days were selected from the larger sample to study the effect of dantrolene sodium on residual contracture tension induced by suxamethonium at 20°C. EDL muscles were selected when there was an observed residual tension increase during a 20 min Krebs wash.

Denervation procedure

Each rat was anaesthetized by intraperitoneal injection of pentobarbitone sodium (Sagatal, May & Baker) 40 mg kg^{-1}, diluted 1:6 with a sterile solution of sodium chloride (0·9% w/v), for optimum drug absorption. Operative procedures for denervation of the EDL muscle by section of the common peroneal nerve were carried out under aseptic conditions. The proximal end of the nerve was embedded in the connective tissue of superficial vastus lateralis muscle to prevent reinnervation of the EDL muscle and the wound closed with sutures. Residual loss of motor activity was limited to a loss of dorsiflexion of the ankle and toes, and lateral eversion of the foot. Animals were active and healthy during the specified periods after denervation.

Removal of the EDL for the in-vitro preparation

An incision was made to expose the muscles of the lower leg and foot in the anaesthetized rat. The in-vivo muscle length was measured with geometrical dividers before the muscle was removed and secured in the muscle bath in Krebs-Henseleit solution aerated with 95% O_2 and 5% CO_2. Muscle length was adjusted to that measured in-vivo.

Measurement of atrophy

Atrophy was measured at the end of the experiment by the percentage weight loss in chronically denervated EDL muscle compared with the normal contralateral muscle.

Measurement of contracture tension

Contracture tension was measured by a 2 oz strain gauge (Dynamometer U.F.1) and recorded on a Washington oscillograph (MD1 400). A 1 mV calibration was used to standardize the oscillograph record. Tension was measured at 15 s intervals during the first minute of the contracture, then at 2 min intervals during the 20 min exposure to the drug and during the Krebs-Henseleit wash until it returned to the baseline, or the experiment was terminated. Bath temperature was controlled at 20°C ± 0·5°C.

Measurement of membrane potential

Membrane potentials were recorded in batches of 10 taken in progressive 5 min sample blocks, through 20 min exposure to suxamethonium, from superficial fibres in the equatorial region of normal and denervated rat EDL muscles at 20°C. Measurements were made using glass microelectrodes, filled with 3 M KCl solution and of 10 M ohms resistance. Microelectrode input signals were amplified by a M 701 preamplifier (W.P. Instruments). A silver/silver chloride earth plate completed the circuit through a DC calibrator (Epil 116A) to a Tektronix 502 oscilloscope. A 10 mV pulse was used to standardize the oscilloscope record. Microelectrode placement in the isolated muscle fibres was monitored optically (magnification × 10 or × 20). Muscle fibres were illuminated by a halogen lamp through fibre optics to a lens mounted in the base of the muscle bath. The equipment was screened in the conventional manner and connected to a common earth. Membrane depolarization was calculated with reference to the baseline resting potential measurement.

Solutions and drug concentrations

The concentrations of suxamethonium used were derived from those found to cause neuromuscular block in normal rat isolated diaphragm muscle (Whittaker 1962a, b). A solution of suxamethonium chloride ($3·0 \times 10^{-5}$M), (BDH) was freshly prepared in Krebs-Henseleit solution (Perry 1968). Solutions of 7·0 and $3·5 \times 10^{-6}$M were made by serial dilution. Concentrations of fructose 1,6-diphosphate (BDH) and caffeine (BDH) were derived from those used by Bowman & Raper (1964) and were in Krebs-Henseleit solution as above. The sequence used was: suxamethonium alone ($3·0 \times 10^{-5}$M) with fructose 1,6-diphosphate ($2·3 \times 10^{-3}$M) or with caffeine ($4·1 \times 10^{-3}$M). The muscle was exposed to each drug, or combination of drugs for 20 min, after which a Krebs-Henseleit wash was applied for at least 30 min. If the residual contracture tension had not been reduced during the wash then dantrolene sodium ($3·8 \times 10^{-4}$M) was applied until tension returned to the baseline. The concentration of dantrolene (Eaton Laboratories) was derived from that used by Ellis & Carpenter (1972). A further Krebs-Henseleit wash was given before the next drug in the sequence was applied.

Results

Identification of suxamethonium-induced contracture categories

The effect of concentration on contracture type. Three concentrations of suxamethonium were used for comparison of the contracture response, ($3·5 \times 10^{-6}$M); ($7·0 \times 10^{-6}$M); ($3·0 \times 10^{-5}$M). The maximum isometric tension produced during each contracture was proportional to the concentration of the drug. Contracture responses in each category were

similar, but less pronounced, with lower concentrations of drug. The following results of initial experiments, 1–147 days following denervation (set 1) are therefore given in response to suxamethonium ($3{\cdot}0 \times 10^{-5}$M).

Contracture categories. Suxamethonium-induced contractures were absent in control normal EDL muscles (n = 82), in denervated muscles at 1·6 days after denervation s.d. ±0·7 (n = 11), or at 147 days after denervation (n = 4). Contractures were observed in chronically denervated contralateral muscles (n = 67) at variable periods after denervation and have been grouped 1–6. Examples of contractures measured in subsequent experiments are in Fig. 1 for comparison. The contractures were identified by the initial peak contracture tension (Tp_1), and the subsequent tension produced during the 20 min exposure to drug (Tp_2).

Group 1. Tension was sustained at a low level during 20 minutes exposure to suxamethonium at 5·1 mN s.d. ±3·2, in denervated muscles (n = 7). During the wash, tension reduced slowly and returned to the control level within 1 h.

Group 2. The slow development of tension, rising to 18·3 mN s.d ±10·6 at 20 min, typified this contracture. Tension continued to increase during the wash and did not return to the control level within 1 h. No change was recorded in the temperature or pH, therefore residual tension was considered as a specific response to suxamethonium in EDL muscles in this group (n = 7).

Group 3. The first peak tension in (Tp_1) was prominent, 16·5 mN s.d. ±6·6 during 15–30 s exposure to drug. Subsequently there was a reduction in tension followed by a small second peak tension, (Tp_2) at 20 min, 8·4 mN s.d ±6·6 (n = 6).

Group 4. The relatively large first peak tension 32·9 mN s.d. ±13·2 was measured within 8 min exposure to drug, subsequently, tension fell and then formed a second smaller peak (Tp_2) at 20 min, 11·7 mN s.d. ±14·1 (n = 22). During the wash tension returned to the control level within 1 h.

Group 5. A sharp peak tension (Tp_1), 23·8 mN s.d. ±13·2, was measured at 15 s of exposure to drug then tension rapidly declined. The second peak (Tp_2) was characteristically absent, and no tension was recorded in the wash. Muscles in this group (n = 14) required a 1·5 h wash before they would respond further to the drug. The long recovery time indicated a loss of sustained contractility.

Group 6. Minimal contracture tension was measured, 6·4 mN (Tp_1) and 1·9 mN (TP_2) whilst membrane depolarization was variable, 12·8 mV and 30·5 mV, at 124·7 days after denervation s.d. ±2·3 in EDL muscles in which 75·2% atrophy was the predominant feature (n = 11).

The effect of the duration of the denervation on the development of contractile changes. Contractures identified occurred in a sequence in EDL muscle groups 1–6 at 2 (s.d. ±1), 5·5 (s.d. ±1·6), 8·4 (s.d. ±4·3), 42·6 (s.d. ±25·3), 58·1 (s.d. ±22), 124·7 (s.d. ±2·3) days after denervation, respectively. Progressive muscle atrophy was also observed in which the % weight loss recorded in each group was 3·9 (s.d. ±6·4), 11·2 (s.d. ±6·3), 24·5 (s.d. ±13·5), 56 (s.d. ±11·2), 62·4 (s.d. ±1·2), 75·2 (s.d. ±4·7), respectively.

Comparison of simultaneous measurements of contracture tension and membrane depolarization in denervated EDL muscles. There was a sequential increase in contracture tension (Tp_1) in groups 1–5, in response to suxamethonium ($3{\cdot}0 \times 10^{-5}$M), 1·2, 5·9, 21·2, 30·5, 55·5, mN, respectively and a concomitant increase was recorded in membrane depolarization, 15·8, 24·3, 28·4, 27·2, 39·4 mV. However, contracture tension (Tp_2) was variable (Fig. 1) and did not correspond to

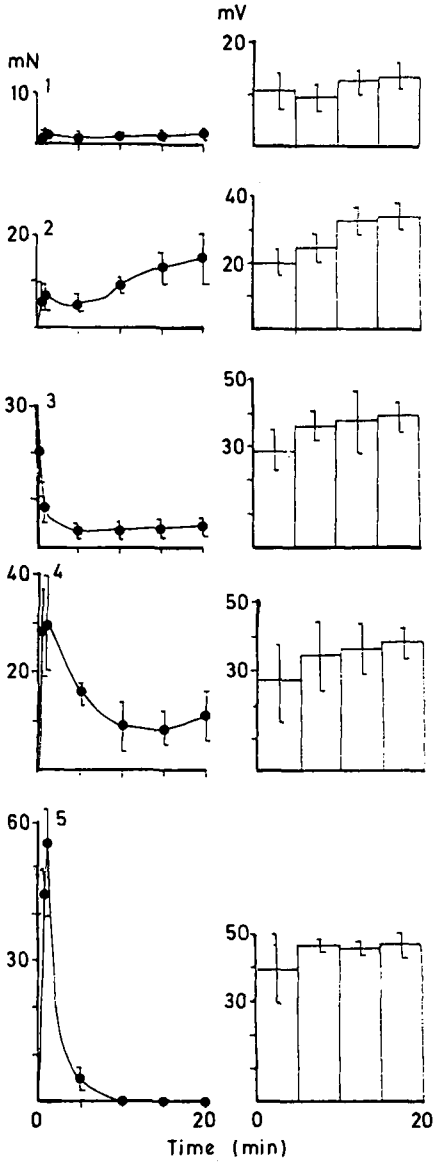

FIG. 1. Comparison of simultaneous measurements of contracture tension and membrane depolarization in denervated rat extensor digitorum longus muscle in response to suxamethonium ($3{\cdot}0 \times 10^{-5}$M) at 20°C. Simultaneous measurements of contracture tension and membrane depolarization in denervated muscle in response to 20 min exposure to suxamethonium ($3{\cdot}0 \times 10^{-5}$M). Denervated muscles were assigned to categories 1–5 on the basis of the peaks in contracture tension as described in the text. DPD = days post denervation (±s.d.) and were as follows: Group 1, 3·7 ±1·2 (n = 5); Group 2, 5·0 ±2·7 (n = 5); Group 3, 6·3 ±1·0 (n = 9); Group 4, 20 ±19·7 (n = 12); Group 5, 31·5 ±14·9 (n = 5).

the increase in membrane depolarization, except in contracture group 2, 13·1 mN/33·1 mV. Results indicated that the relatively low level of contracture response (Tp_2) in contracture groups 3, and 4, 3·7 mN/38·2 mV and 9·7 mN/38·6 mV, and the absence of (Tp_2) in group 5 when membrane depolarization remained high, 46·1 mV, required further investigation. Also the increase in residual tension and decrease in membrane depolarization, from 13·1 mN to 23·3 mN, and from 33·1 mV to 9·2 mV during the Krebs wash (80 min) in EDL muscles in group 2 required further study.

Investigation of intracellular changes after denervation

The initial investigation revealed an interesting feature of the second phase of suxamethonium-induced contractures during 20 min exposure to $3{\cdot}0 \times 10^{-5}$M. The second peak of contracture tension (Tp_2) was relatively high in contracture group 2, relatively low in groups 3 and 4, and absent in group 5. This variability was investigated using fructose 1,6-diphosphate, to provide additional intracellular energy for muscle contraction (Conn & Stumpf 1976); caffeine to induce Ca^{2+} release from the sarcoplasmic reticulum (Endo 1975a, b, 1977); and dantrolene sodium to reduce intracellular Ca^{2+} (Ellis & Carpenter 1974). EDL muscles (n = 35) in set 2, denervated 1–110 days before experiments, were subdivided into groups 1–5 according to the contracture responses to suxamethonium ($3{\cdot}0 \times 10^{-5}$M) identified in the initial experiments. Results of experiments during the development of contractile changes following denervation are presented in Fig. 2.

The response of normal EDL muscle to suxamethonium alone and with added fructose 1,6-diphosphate or caffeine. No contracture tension was found in normal muscles (n = 35) in response to the drug alone. Minimal contracture tension was recorded with fructose 1,6-diphosphate ($2{\cdot}3 \times 10^{-3}$M) or caffeine ($4{\cdot}1 \times 10^{-3}$M) present. Tension was maintained at a low level throughout contractures, 2 mN, and was not sustained during the wash.

The response of denervated EDL muscles to suxamethonium and fructose 1,6-diphosphate. Results (Fig. 2) show that added fructose 1,6-diphosphate produced a similar pattern of response to the drug alone but the tension was greater, especially in the second phase of contracture (Tp_2). Group 5, in particular, showed an interesting response in the production of a second peak of contracture tension (Tp_2). Results indicated that some glycolytic activity remained at 54·8 s.d. ± 10·1 days after denervation and that the absence of (Tp_2) in contracture group 5 with drug alone may be due to a lack of substrate, or a reduced glycolytic enzyme activity. A low response to fructose 1,6-diphosphate obtained with EDL muscles in group 6 indicated a substantial loss of contractility in the muscles in which weight loss was greater than 70%.

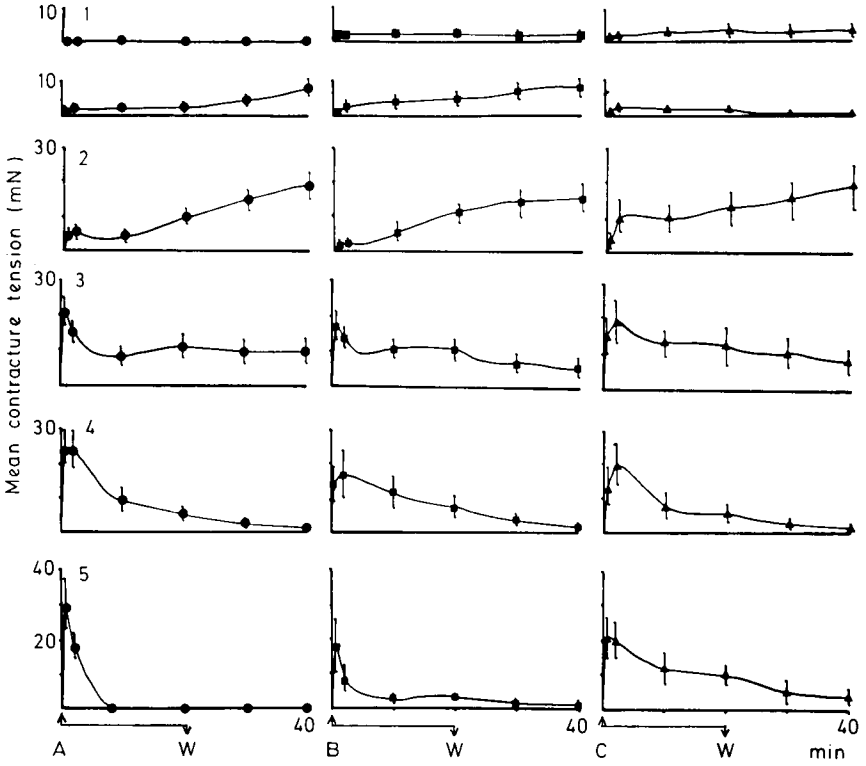

FIG. 2. Contractures produced by suxamethonium alone and with fructose 1,6-diphosphate, or with caffeine at 20°C. Rat EDL muscles (n = 35) were denervated at specific periods before in-vitro measurement of the contracture response to (A) suxamethonium ($3{\cdot}0 \times 10^{-5}$M) alone, or (B) with fructose 1,6-diphosphate ($2{\cdot}3 \times 10^{-3}$), or (C) with caffeine ($4{\cdot}1 \times 10^{-3}$M). Denervated muscles were assigned to groups 1–5 above according to the contracture response to suxamethonium. Minimal contracture tension was measured, but not recorded above, in a further 6 muscles, 83 days after denervation (s.d. ± 7), in which atrophy was a predominant feature. Normal contralateral muscles (n = 35) were used in control experiments. DPD = days post denervation (mean ± s.d.) and were as follows: Group 1, 2 ± 1 (n = 5); Group 2, 4·3 ± 2·3 (n = 5); Group 3, 9·8 ± 4·9 (n = 6); Group 4, 28·1 ± 10·5 (n = 8); Group 5, 54 ± 25·1 (n = 5). W = Krebs washout.

The effect of caffeine on suxamethonium-induced contracture tension in denervated muscles. Caffeine had a notable effect on the second phase of contracture tension (Tp_2) in groups 2 and 5 (Fig. 2) in which there was an increase of 3·2 and 10·3 mN compared with the absence of tension (Tp_2) recorded in response to suxamethonium alone. Comparison of tension measurements over 20 mins exposure time were analysed using the *t*-test and showed that the increase with caffeine was significant ($P < 0.05$). Caffeine had the most pronounced effect on muscles in group 2, which had relatively little atrophy, 7·1% weight loss s.d. ±2·17, and thus occurred 4·3 days after denervation s.d. ±2·3. The presence of the uncharacteristic second phase of contracture (Tp_2) in group 5 was also notable. Results indicated that caffeine caused Ca^{2+} release sufficient to produce a contractile response, even at a relatively late stage after denervation, 39·5 days s.d. ±0·7.

The action of dantrolene sodium on residual tension during the wash after the removal of suxamethonium alone or with caffeine or fructose 1,6-diphosphate. Results indicated that dantrolene sodium had the maximum effect on residual tension induced by suxamethonium ($3{\cdot}0 \times 10^{-5}$M) alone, followed by drug with fructose 1,6-diphosphate ($2{\cdot}3 \times 10^{-3}$M) and then with caffeine ($4{\cdot}1 \times 10^{-3}$M). Therefore, resistance to the action of dantrolene was indicated in the slow reduction of tension following suxamethonium and caffeine in comparison with suxamethonium alone (0·13 mN min^{-1} cf. 0·49 mN min^{-1}). In addition two phases of dantrolene action were noted, a latency and an active phase. The latency of action varied in this sample between 3 and 6 min.

Comparison of the latency of action of dantrolene on residual suxamethonium-induced contracture tension during the wash at variable periods after denervation. Results of the previous experiments, when dantrolene was used to reduce residual tension after the application of suxamethonium and caffeine, indicated that there might have been a relationship between the effectiveness of dantrolene and the duration of denervation. Dantrolene was used to reduce tension in EDL muscles (n = 16) denervated 1–7 days before exposure to suxamethonium ($3{\cdot}0 \times 10^{-5}$M). The remainder in which tension fell spontaneously, were used as controls (group T). Muscles were sub-divided into 4 groups (P-S) according to the latency of the action of dantrolene which was estimated by the length of time before the reduction of residual tension was measured. Results (Table 1) showed that there was a relationship between the duration of denervation and the latency of action of dantrolene. At 3·8 days after denervation s.d. ±1·1 the latency recorded was 36 mins whilst at 5 s.d. ±2·4, 6 s.d. ±1·2, and 7 days after denervation shorter latencies of 30, 20 and 6 min, respectively, were recorded. In addition, results indicated that the latency period was not one of total inactivity, but one in which dantrolene slowed the increase of residual tension.

Comparison of the action of dantrolene sodium on residual tension and membrane depolarization in denervated EDL muscles during a Krebs wash after 20 min exposure to suxamethonium. Results show that residual tension and membrane depolarization were proportional at the end of the Krebs wash, 15·9 mN/9 mV, 16·9 mN/9·9 mV and 27·57 mN/17·8 mV in EDL muscles at 3·6 s.d. ±1·1, 5 ±2·4 and 6 s.d. ±1·2 days after denervation (Table 2). In the presence of dantrolene residual tension continued to rise during the latency period to 24·7, 25·1, and 33·7 mN, whilst depolarization remained between 8–14 mV. Dantrolene reduced residual tension slowly during the active period, 23·9, 15 and 18·8 mN, whilst depolarization remained at the previous level. In comparison, residual tension and depolarization during the Krebs wash in muscles at 7 days after denervation was 13·9 mN/31·7 mV. During the latency period of the action of dantrolene, residual tension continued to rise to 19·4 mN without a change in membrane depolarization, but, during the active phase, membrane depolarization was reduced to 17·2 mV with a concomitant decrease in residual tension, 7·6 mN. Results indicated that membrane depolarization did not contribute to the reduction of residual tension

Table 1. The action of dantrolene on residual tension during the wash, after the removal of suxamethonium ($3{\cdot}0 \times 10^{-5}$M) at 20°C. Dantrolene sodium was used to reduce residual suxamethonium-induced contracture tension in rat EDL muscles (n = 16) denervated 1–7 days previously, designated group D. Residual contracture tension increase was recorded during 20 minutes Krebs wash. Group D was sub-divided into 4 group, P-S according to the latency of action of dantrolene sodium. Denervated EDL muscles in which no residual tension was measured were designated group T (n = 3).

	Rate of increase/decrease in tension (mN min^{-1})				
	Krebs wash 30 min		Dantrolene ($3{\cdot}8 \times 10^{-4}$M) 60 min		
Dantrolene latency (mean) (min)	30–15	15–0	Latency period	Active period	Duration of denervation (means ± s.d.) (days) EDL Groups
36	0·17↑	0·42↑	0·42↑	0·20↓	3·8 ± 1·1 P n = 4
30	0·17↑	0·57↑	0·26↑	0·47↓	5·0 ± 2·4 Q n = 4
20	0·06↑	0·29↑	0·02↑	0·38↓	6·0 ± 1·2 R n = 4
6	0·44↑	0·42↑	0·81↑	0·41↓	7·0 ± 0 S n = 4
—	0·02↑	0·02↑	—	—	3·7 ± 3·1 T n = 3

Table 2. Comparison of simultaneous measurements of residual contracture tension and membrane depolarization induced by suxamethonium ($3{\cdot}0 \times 10^{-5}$M) at 20°C in denervated rat EDL muscle during the Krebs wash followed by the addition of dantrolene sodium.

Duration of denervation (means ± s.d.) (days) EDL Groups	Mean membrane depolorization (mV) Mean contracture tension (mN)	Krebs wash (40 min) 5–10	20–25	35–40	Dantrolene sodium ($3{\cdot}8 \times 10^{-4}$M) 60 min 5–10	20–25	35–40	50–55
Group P	mV	10·5	10·0	9·0	11·7	7·4	9·7	8·3
3·6 ± 1·1	mN	8·8	11·3	15·9	21·8	23·6	24·7	23·9
n = 4								
Group Q	mV	10·0	11·9	9·9	9·1	8·2	8·0	7·2
5·0 ± 2·4	mN	14·7	17·7	16·9	22·8	25·1	23·8	15·0
n = 4								
Group R	mV	21·8	17·9	17·8	13·8	12·9	12·7	11·9
6·0 ± 2·3	mN	23·3	24·5	27·6	28·7	33·7	24·8	18·8
n = 4								
Group S	mV	38·2	34·1	31·7	31·5	18·6	22·2	17·2
7·0 ± 0·	mN	2·2	8·3	13·9	19·4	11·8	8·5	7·6
n = 4								
Group T	mV	21·7	21·0	18·0	19·0	18·9	17·0	17·5
3·7 ± 3·1	mN	1·0	0·	3·0	3·5	2·5	2·0	1·0
n = 3								

during exposure to dantrolene in EDL muscles 3–6 days after denervation but may have contributed to the reduction in tension at 7 days after denervation.

Discussion

Analysis of findings showed that contractures in denervated rat EDL muscle in response to suxamethonium ($3{\cdot}0 \times 10^{-5}$M) were variable at specific periods during 1–147 days after denervation and have been grouped 1–6. Records of cholinergic contractures in cat tibialis muscle, at 9 and 26 days after denervation (Zaimis 1951; Jewell & Zaimis 1954), would be classified according to the categories used in the present investigation as contractures 3 and 5, respectively.

The maximum contracture response to suxamethonium ($3{\cdot}0 \times 10^{-5}$M) measured in this investigation was observed within 15 s exposure to the drug at 26 days after common peroneal nerve section (s.d. ± 10·1). Contracture tension measured in rat EDL muscle after T6 spinal cord transection in response to suxamethonium (Carter et al 1981), and in the rat hemi-diaphragm in response to acetylcholine (Elmqvist & Thesleff 1960), did not permit direct comparison of the timed response because different preparations and contracture analysis were used. However, Brown (1937), Knowlton & Hines (1937), Emmelin & Malm (1965), and Carter et al (1981), have shown that peak contracture tension was proportional to the concentration of cholinergic agent used. Our results confirmed that observation.

Simultaneous records of contracture tension and membrane depolarization in denervated rat EDL muscle measured by us showed that during the first 10 min of exposure to the drug ($3{\cdot}0 \times 10^{-5}$M) there was a sequential increase in the first peak of contracture tension (Tp_1) and a commensurate increase in membrane depolarization in groups 1–5; 1·2 mN/15·3 mV, 5·9 mN/22·2 mV, 21·2 mN/32·3 mV, 30·5 mN/31 mV, 55·5 mN/42·8 mV. However, the second peak in contracture tension (Tp_2) during 10–2 min exposure to the drug showed that contracture tension did not correspond with the increase in membrane depolarization except in group 2.

However, EDL muscles in groups 1, 3, and 4 produced relatively little tension with respect to depolarization and in group 5 there was an absence of tension whilst membrane depolarization was high, 1·5 mN/19·7 mV, 13·1 mN/33·1 mV, 3·7 mN/38·2 mV, 9·7 mN/38·6 mV, 0 mN/46 mV. Although EDL muscles in group 2 produced a second peak in tension (Tp_2) commensurate with depolarization, residual tension increased during the Krebs wash whilst depolarization was reduced, 23·3 mN/9·2 mV. EDL muscles designated group 6 produced minimal tension during the 20 min exposure to suxamethonium although depolarization was above normal control records. These muscles were judged to be at the end of their viability as contractile tissue at 127 days after denervation s.d. ± 2·3, since the drug-induced contractures were absent at 147 days after denervation. Results of these experiments indicated that there was a progression of events after denervation which altered the contractile properties of rat EDL muscle and that the contracture groups identified mark the stages in contractile changes at selective periods during the following days after denervation, 2 s.d. ± 1 (group 1), 5·5 s.d. ± 1·6 (group 2), 8·4 s.d. ± 4·3 (group 3), 43·6 s.d. ± 25·3 (group 4), 58·1 ± 22 (group 5), and 127 s.d. ± 2·3 (group 6). In further experiments similarities in response to suxamethonium, caffeine and fructose 1,6-diphosphate indicated that there were not six stages but four stages in the development of contractile changes after denervation, in which groups 1 and 2 marked the first stage, groups 3 and 4 the second stage, group 5 the third stage and group 6 the final stage.

Finol et al (1981) proposed that the contractile changes occurred in 2 stages after denervation in rat skeletal muscle, namely 1–7 days and 7–42 days after denervation. Their observations coincide with the results of our investigation regarding the first two stages of contractile changes.

Those authors proposed that the early changes were a direct consequence of an alteration in excitation-contraction

coupling, due to a prolongation of the action potential which caused an increase in intracellular Ca^{2+} release (Finol & Lewis 1975), whilst the later stage was affected by myofibrillar atrophy. Excitation-contraction coupling changes in rat EDL muscle were indicated in the response to suxamethonium 3–7 days after denervation when the contracture tension response was excessive but not dependent on the influx of extracellular Ca^{2+} (Andrews 1984). The first stage of contractile changes fitted the observations made in our investigation regarding contracture group 2, which occurred 3–7 days after denervation. The effect of caffeine on suxamethonium contractures caused a significant increase in contracture tension during 10–20 min exposure to both agents together, compared with the response to suxamethonium alone. The role of caffeine in Ca^{2+} release has been established by Endo (1975 b, 1977) in his work on amphibian single skinned amphibian muscle fibres. Sensitivity to caffeine alone has also been recorded in rat skeletal muscle 1–6 days after denervation (Gutmann & Sandow 1965). Ashley & Lea (1978), and Ashley & Ridgeway (1970), have shown that Ca^{2+} has an intermediate role in the excitation-contraction process. Intracellular free Ca^{2+} differences rely on the sarcoplasmic reticulum (Ebashi 1972), which requires depolarization for Ca^{2+} release (Endo & Nakajima 1973). The results of experiments in our investigation indicate that the effect of excess Ca^{2+} release occurring in denervated rat EDL muscle prolongs the active state according to the model proposed by Lewis & Webb (1976).

Our results of experiments with dantrolene sodium also indicate that Ca^{2+} release is excessive, during the early stage of denervation. At 3·8 days after denervation s.d. $\pm$ 1·1 the longest latency of action of dantrolene sodium was observed in the reduction of residual contracture tension after exposure to suxamethonium, compared with the responses at 5–7 days after denervation. Ellis & Carpenter (1972) proposed that dantrolene interfered with Ca^{2+} release from the sarcoplasmic reticulum. This view was supported by Putney & Bianchi (1974) who proposed that dantrolene had an inhibitory effect on the triggering step of excitation-contraction coupling. An increase in sarcoplasmic reticulum surface area was measured by Stonnington & Engel (1973), at three days after denervation in rat skeletal muscle therefore an increase in Ca^{2+} release may be directly related to ultrastructure changes in myofibrils during the early period after denervation. Our measurements of muscle weight loss showed that EDL muscles in group 2 at 5·5 days after denervation s.d $\pm$ 1·6 had less than 10% atrophy and their responses to suxamethonium, caffeine and dantrolene indicated that contractile changes in those muscles may be related to ultrastructure changes identified by Stonnington & Engel (1973).

The results of this investigation confirm that, in the rat, the first stage of contractile changes occurs within the first week after denervation. However, Lewis et al (1978) found in the cat hind limb muscle that the changes occurred at 9 days after denervation, as opposed to 3–4 days after denervation in the rat. A species difference is indicated.

The effects of fructose 1,6-diphosphate on denervated EDL muscles which produced contracture (group 5), in response to suxamethonium, indicated that the absence of tension in the second phase of the drug-induced contracture (Tp_2) was caused by a lack of hexosphosphate substrate. The reduction in intracellular glycolytic enzyme activity measured according to Hogan et al (1965), was progressive throughout the period of denervation and that contracture 5, which occurred at 54 days after denervation s.d. $\pm$ 25·1 marks the point at which enzyme activity and substrate levels became critically low, and insufficient to maintain tension.

The relationship between muscle atrophy and enzyme changes enable atrophy to be used as a guide to metabolic changes in rat EDL muscle after denervation (Hogan et al 1965). Muscle atrophy, measured in the present investigation 0–147 days after denervation, was comparable with their results. Further evidence in support of the relationship between muscle atrophy and intracellular changes after denervation has been provided by Miledi & Slater (1968), Stonnington & Engel (1973), and Srivastava & Das (1978). In particular, Stonnington & Engel (1973) showed that muscle atrophy was proportional to myofibrillar mass loss, and that the decrease in fibre size and contractile elements were concomitant but not necessarily dependent.

It is proposed that 4 stages exist in the development of contractile changes after denervation in rat EDL muscle in response to prolonged exposure to suxamethonium. The initial stage of 1–7 days after denervation are characterized by an excessive contracture response Tp_2 to caffeine and a resistance to dantrolene sodium during the Krebs wash. In the second stage, a simultaneous increase in membrane depolarization and the initial peak contracture response (Tp_1) are the predominant features, 8–35 days after denervation. The third stage, 36–70 days following nerve section, is characterized by a progressive reduction in the contracture response, the absence of the second peak in contracture tension (Tp_2), and the final stage is characterized by a minimal contracture response to suxamethonium 70–130 days after denervation, and the eventual total loss of contractility at 147 days after peripheral nerve section in the rat.

Previously unidentified forms of suxamethonium-induced contractures assigned to categories 1–6 provide the basis for further study into the nature and sequence of contractile changes in skeletal muscle after denervation.

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