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THE ROLE OF EXTRACELLULAR CALCIUM IN THE CONTRACTIONS PRODUCED BY ACETYLCHOLINE IN CHRONICALLY DENERVATED MUSCLE

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1 Acetylcholine-induced contractions of the isolated chronically denervated soleus muscle of the mouse consist of two phases, but both phases are equivalent to the contracture phase seen *in vivo*.

2 Low $[Ca^{2+}]_o$ (0.5–1.5 mM) augmented peak tension, as well as the rate of relaxation, of the first phase, but inhibited the second phase. Ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) or La^{3+} (2 mM) also inhibited the second phase, but not the first.

3 It was concluded that the first phase requires Ca^{2+} release from the sarcoplasmic reticulum, and is terminated by inactivation of the contractile process. The second phase is caused by the entry of activator Ca^{2+} from the extracellular space.

4 Increasing $[Ca^{2+}]_o$ to 5 or 10 mM after the addition of acetylcholine caused a contraction, starting after a delay of about 50 seconds. EGTA or La^{3+} added during the second phase of the acetylcholine contraction caused relaxation after a much shorter lag time.

5 It is concluded that most of the Ca^{2+} entering from the extracellular fluid is taken up by the sarcoplasmic reticulum.

6 The acetylcholine second phase was augmented in low (25 mM) $[Na^+]_o$. It is concluded that Na^+ and Ca^{2+} compete for the acetylcholine controlled ionic channels.

7 Isolated chronically denervated diaphragm muscles were less sensitive to acetylcholine and the contraction usually consisted of a first phase only.

8 It is concluded that sequestration of Ca^{2+} entering from the extracellular fluid is more complete in the diaphragm.

Introduction

Chronically denervated skeletal muscle is supersensitive to close arterially injected acetylcholine (Brown, 1937; Axelsson & Thesleff, 1959). There is a double response which consists of a fast initial contraction associated with a burst of fibrillary potentials followed by a slow contracture and the abolition of fibrillation (Brown, 1937; Rosenblueth & Luco, 1937; Bowman & Raper, 1964). Chronically denervated muscle has usually been reported to produce single phase contractions when isolated (Elmqvist & Thesleff, 1960; Letley, 1960; Freeman & Turner, 1969; Lüllmann & Sunano, 1973), but biphasic responses have been obtained in the chronically denervated and isolated diaphragm (Preuner, 1971; Lüllman, Preuner & Schaube, 1974) and soleus muscle (Hall, Maleque & Wadsworth, 1975).

In this paper, we have compared the effect of changing $[Ca^{2+}]_o$ or of adding La^{3+} on the two phases of the acetylcholine contracture of mouse isolated denervated muscle. The experiments show that the second phase is dependent on an extracellular supply of calcium for activation but the first is not.

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Methods

Denervation

Muscles of adult male mice were denervated under ether anaesthesia using aseptic precautions. The animals were killed 7–12 days later by a blow on the head and the muscle on the operated side examined for fibrillations and removed. The soleus muscle was denervated by removing 1 cm of the sciatic nerve from the popliteal space of one leg. The diaphragm was denervated by evulsion of one phrenic nerve where it crosses the brachial plexus.

Table 1 Composition of physiological salt solutions used

	Gas	Concentration (mM)									
		Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	SO_4^{2-}	$H_2PO_4^-$	HCO_3^-	*tris*	*glucose*	*sucrose*
Krebs-Henseleit	5% CO_2	144	5.8	2.5	1.2	128	1.2	1.2	25	0	11.1	0
Tris-Krebs	O_2	118	5.8	2.5	1.2	121	1.2	0	0	25	11.1	0
0.5 mM Ca^{2+}-Krebs	5% CO_2	144	5.8	0.5	1.2	124	1.2	1.2	25	0	11.1	0
1 mM Ca^{2+}-Krebs	5% CO_2	144	5.8	1.0	1.2	125	1.2	1.2	25	0	11.1	0
1.5 mM Ca^{2+}-Krebs	5% CO_2	144	5.8	1.5	1.2	126	1.2	1.2	25	0	11.1	0
5 mM Ca^{2+}-Krebs	5% CO_2	144	5.8	5.0	1.2	133	1.2	1.2	25	0	11.1	0
10 mM Ca^{2+}-Krebs	O_2	118	5.8	10.0	1.2	136	1.2	0	0	25	11.1	0
Low Na^+-Krebs	5% CO_2	25	5.8	2.5	1.2	9.6	1.2	1.2	25	0	11.1	249

Organ bath procedure

The muscles were suspended in 5 ml baths under a resting tension of 0.5 g (soleus) or 2 g (diaphragm). Contractions were isometrically recorded with Grass FTO3C, Nihon-Kohden 58-1T or Ether strain gauges writing out on a Grass 7B or Washington 400 MD2 recorder. The physiological salt solutions, which were maintained at 37°C, had the compositions shown in Table 1.

In order to determine whether 25 mM Tris would affect the responses (Gillespie & McKnight, 1975) a comparison was made between acetylcholine responses in $HCO_3^-/H_2PO_4^-$ buffered and Tris buffered solutions. One comparison was made where $[Ca^{2+}]_o = 5$ mM ($n = 4$) and another where $[Ca^{2+}]_o = 10$ mM ($n = 4$). In both cases the tension and time course of acetylcholine responses were not affected by the choice of buffer.

Electrophysiology

Muscles were pinned out in a perspex chamber through which there was a continuous flow of Krebs-Henseleit solution (about 1 ml/minute). They were illuminated from below and viewed with an Olympus dissecting microscope. Muscle fibres were impaled with electrodes pulled from Pyrex glass tubing (o.d. 1.2 mm, wall thickness 0.3 mm) and filled with 3M KCl after which they had a d.c. resistance of 10–30 MΩ. Membrane potential was recorded with a Grass P16 preamplifier and displayed on an oscilloscope and pen recorder. Drugs were added to the perfusing solution or injected into the bath. In some experiments, less than half the muscle was pinned out and the other end was connected to a transducer mounted at an oblique angle for isometric tension recording.

The drugs used were acetylcholine chloride (Sigma), caffeine citrate (Evans), ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA, Sigma) and lanthanum chloride (Hopkin and Williams).

Statistics

The results are quoted as the mean ± the standard error of the mean. The significance of difference was assessed using the paired *t* test, taking $P < 0.05$ as significant.

Results

Soleus

Acetylcholine responses when $[Ca^{2+}]_o = 2.5$ mM. Contractions obtained with acetylcholine (5.5×10^{-6} M to 5.5×10^{-4}M) in Krebs-Henseleit solution contain-

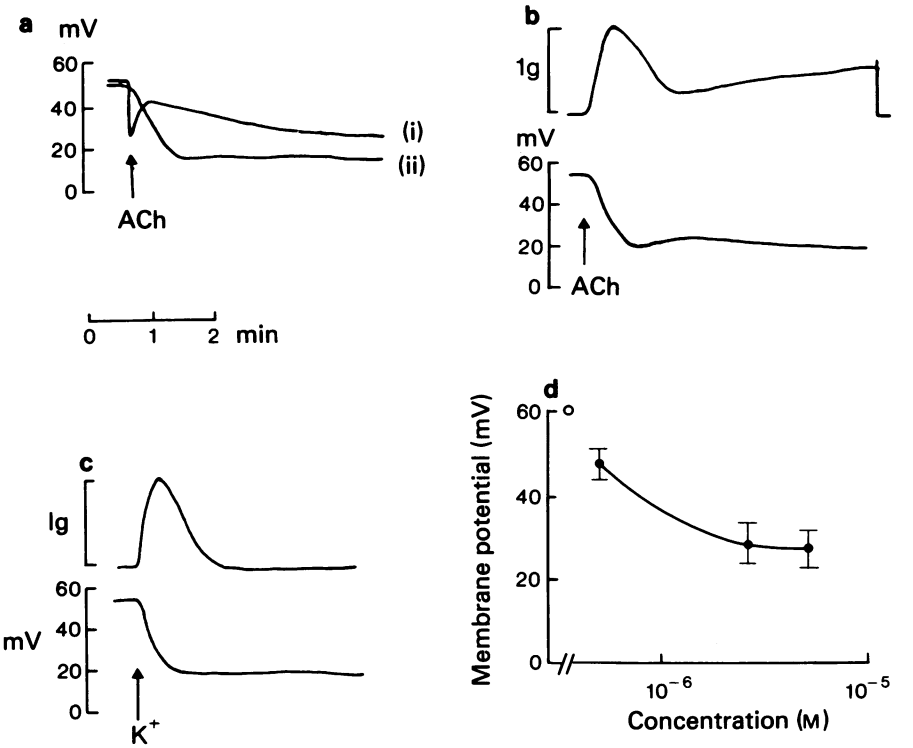


Figure 1 Denervated soleus preparations. (a) Depolarization produced by acetylcholine (ACh) (5.5×10^{-6} M) added to the perfusing solution by injection. The type of response marked (i) was recorded only when penetration was made in the region where the nerve entered the muscle, but (ii) was obtained in all parts of the fibre. (b) Effects of ACh (5.5×10^{-6} M) on tension and depolarization simultaneously recorded. ACh was added by syringe but the rate of addition was slower than that used in the organ bath experiments, resulting in a more gradual contractile response. (c) Effect of K^+ 50 mM on tension and membrane potential, recorded simultaneously. A second phase was never seen. (d) Concentration-response curve showing effect of ACh on membrane potential. (○) Resting membrane potential; (●) peak depolarization after changing perfusion solution to one containing ACh. Each point shows the mean of 12 experiments, the standard errors being indicated by the vertical bars.

ing 2.5 mM Ca^{2+} were normally biphasic (Figures 1b and 2, and Hall *et al.*, 1975). Fibrillatory activity was extinguished at the maximum depolarization which corresponded to the peak of the first phase. Thus both first and second phases (*in vitro*) are equivalent to the contracture phase seen *in vivo*. We observed no separate contraction accompanied by an increase of fibrillatory activity as occurs *in vivo* (Brown, 1937; Rosenblueth & Luco, 1937; Bowman & Raper, 1964).

The maximum of the first phase occurred in 10 s or less, while the maximum of the second phase was reached in 4 to 9 minutes. The first phase developed more slowly with lower concentrations of acetylcholine or when the rate of injection into the bath was more gradual. The time course and peak tension of the second phase was rather variable from one experiment to another. In a few experiments, a second phase could not be obtained with $[Ca^{2+}]_o = 2.5$ mM while in some others the first addition of acetylcholine (5.5×10^{-6} M) failed to produce a second phase although subsequent additions reproduceably gave biphasic responses. With lower concentrations of acetylcholine (5.5×10^{-7} M or 1.1×10^{-6} M) the second phase was seen occasionally, but the first phase was always present.

The mean resting membrane potential of chronically denervated mouse soleus fibres was 52 ± 1.3 mV, 75 impalements (in innervated soleus fibres it was 74 ± 2.3 mV, 75 impalements). Acetylcholine caused dose-dependent depolarization (Figure 1d); the time to peak depolarization was rather variable between experiments and appeared to depend on the rate of addition of the drug and on the position of impalement.

In innervated muscles, the endplate zone (defined as the region from which the miniature endplate potentials (m.e.p.ps.) could be recorded) was found to lie in

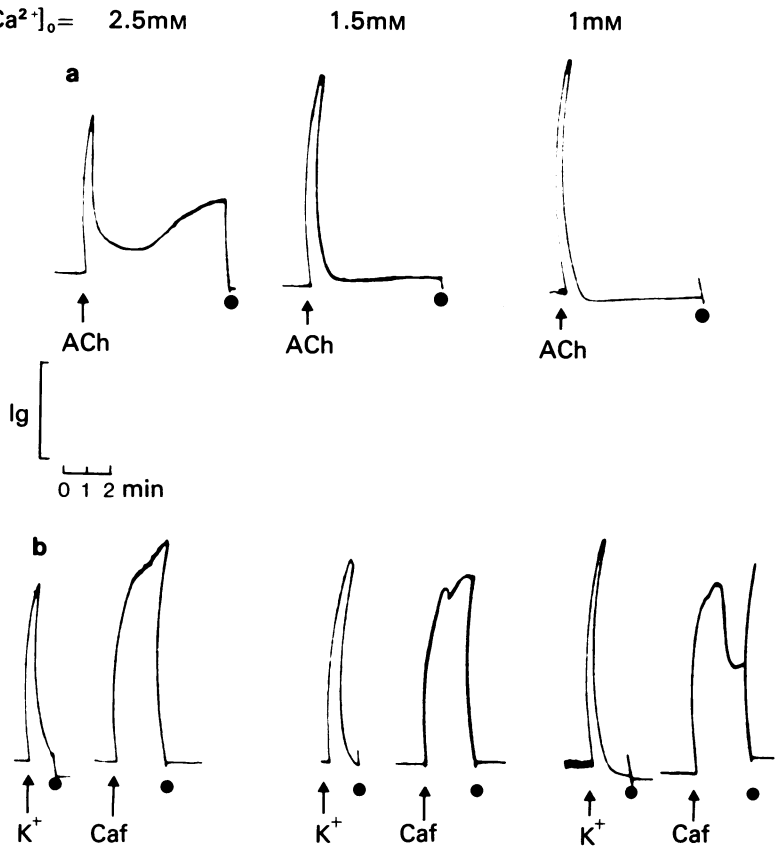


Figure 2 Contractions of denervated soleus muscles produced by acetylcholine (ACh), potassium and caffeine in different $[Ca^{2+}]_o$. (a) Responses to ACh 5.5 x 10^{-6} M. (b) Pairs of responses to KCl (K^+) 50 mM and caffeine (Caf) 13 mM recorded in Krebs solution containing $[Ca^{2+}]_o$ = 2.5, 1.5 and 1 mM (left, centre and right, respectively). Wash out is indicated by (●).

a band near the widest part of the muscle where it was penetrated by the nerve. This corresponds to the region that stains for cholinesterase (Albuquerque & McIsaac, 1970). Out of 50 penetrations made into this region of denervated fibres, 28% gave the type of response denoted (i) in Figure 1a, while the remainder gave a response similar to that indicated by (ii). In the absence of the nerve and of m.e.p.ps, it was not possible to say which of these penetrations were made close to the original endplates but it seems likely that the type (i) response is associated with the former endplate zone. In support of this interpretation, it was found that type (ii) responses were recorded from each of 9 penetrations made into peripheral parts of denervated fibres.

In the majority of penetrations, the acetylcholine effect consisted of a rapid fall in potential with an asymptotic tendency towards an equilibrium level that was maintained for at least 10 min (type (ii) response). The time to peak depolarization was shorter with syringe addition than that achieved by changing the perfusion fluid (Table 2). Lüllmann & Sunano (1973)

Table 2 Time to peak depolarization (s) from addition of acetylcholine (ACh) (5.5 x 10^{-6} M) and KCl (50 mM)

	ACh (5.5 x 10^{-6} M)	*KCl (50 mM)*
Type (ii) — perfusion	52 ± 6.7 s. (n = 10)	59 ± 7 s. (n = 12)
Type (ii) — syringe	25 ± 3.7 s. (n = 18)	32 ± 4 s. (n= 12)

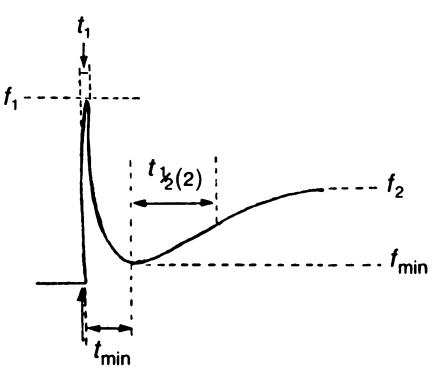
also reported prolonged depolarization produced by acetylcholine, although the contraction was transient.

KCl and caffeine response when $[Ca^{2+}]_o = 2.5$ mM. KCl caused a contraction that started to decay after a few seconds, tension returning to baseline within 1 minute. No second phase was ever observed. Depolarization was rapid, asymptotic and stable once equilibrium was reached. No repolarization was observed as long as $[K]_o$ remained elevated (Figure 1c). In six experiments where acetylcholine gave a type (i) response, KCl (50 mM) was tested while retaining the same penetration and in each case it produced a type (ii) response.

Caffeine caused a more slowly developing contracture that usually appeared to consist of two phases: an initial rapid contraction separated from a slow maintained contraction by an inflexion of variable prominence (Figures 2 and 4).

Effect of changing $[Ca^{2+}]_o$. In low $[Ca^{2+}]_o$ Krebs-Henseleit solution, the second phase of the acetylcholine contraction was reduced in amplitude. With 1.5 mM $[Ca^{2+}]_o$ the second phase was still present, but was only 12% of that observed in 2.5 mM $[Ca^{2+}]_o$. With 1.0 mM $[Ca^{2+}]_o$ the second phase was abolished and there was relaxation below the baseline (Figure 2). The maximum tension generated at the peak of the first phase was increased in 1.0 and 1.5 mM $[Ca^{2+}]_o$ and so was the rate of relaxation of this phase (Figure 3a), thus reducing t min in Table 3. When the Ca^{2+} concentration in the Krebs was reduced to 0.5 mM, the first phase was slightly reduced (to 86 ± 7% control). In some experiments, the deficiency of Ca^{2+} ($[Ca^{2+}]_o = 1$ mM) was made up by adding $MgSO_4$ to give $[Mg^{2+} + Ca^{2+}]_o = 3.7$ mM. Acetylcholine was still unable to produce a second phase in this solution. Caffeine and KCl-induced reponses were also slightly reduced in amplitude in 0.5 mM $[Ca^{2+}]_o$ but not in 1.0

Table 3 Tension and time-course of responses to acetycholine (5.5×10^{-6} M) in denervated soleus



	First phase		*Contraction minimum*		*Second phase*		n
	t_1 (s)	f_1 (g)	t_{min} (s)	f_{min} (g)	$t_{½(2)}$ (s)	f_2 (g)	
$[Ca^{2+}]_o$ 2.5 mM	5.4 ± 1.1	1.5 ± 0.1	55 ± 2	0.14 ± 0.06	100 ± 8	0.67 ± 0.11	10
$[Ca^{2+}]_o$ 0.5 mM	4.1 ± 0.8	1.3 ± 0.1	45 ± 2	−0.05 ± 0.01	—	—	12
$[Ca^{2+}]_o$ 1.0 mM	3.8 ± 0.7	1.9 ± 0.1	42 ± 2	0.07 ± 0.02	—	—	10
$[Ca^{2+}]_o$ 1.5 mM	4.4 ± 0.7	2.0 ± 0.2	50 ± 4	0.04 ± 0.01	79 ± 5	0.08 ± 0.03	8
$[Ca^{2+}]_o$ 5.0 mM	4.8 ± 1.2	1.6 ± 0.1	50 ± 3	0.44 ± 0.08	69 ± 5	1.29 ± 0.12	12
$[Ca^{2+}]_o$ 10.0 mM	4.1 ± 0.5	*1.5 ± 0.1	36 ± 7	0.95 ± 0.21	60 ± 10	1.8 ± 0.14	8
La^{3+} 2 mM		1.3 ± 0.1	58 ± 6	0.46 ± 0.08	—	0.41 ± 0.06	8
EGTA 2 mM		**1.8 ± 0.2	43 ± 5	0	—	—	6
$[Na]_o$ 25 mM		**2.0 ± 0.1	63 ± 4	1.0 ± 0.1	89 ± 9	1.6 ± 0.1	8

t = time; f = tension.

*10mM $[Ca^{2+}]_o$ actually reduced f_1. The group of muscles used for this experiment produced a slightly larger than normal mean f_1 tension in standard Krebs (1.97 ± 0.09g).

**Neither EGTA nor 25mM $[Na]_o$ significantly altered f_1. The groups of muscles used for these experiments produced slightly larger than normal mean f_1 tension in standard Krebs (1.7 ± 0.3g and 1.9 ± 0.1g respectively).

The control figures for all the other values were not different from controls in the original groups.

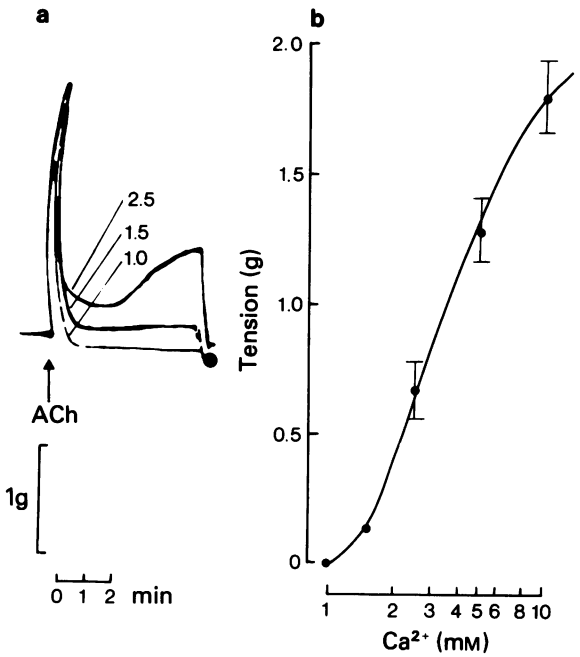


Figure 3 (a) Superimposition of contractions obtained with acetylcholine (ACh, 5.5 x 10^{-6} M) in 2.5, 1.5 and 1 mM $[Ca^{2+}]_o$ from Figure 2. Peak first phase tension and the rate of relaxation were greater in the low $[Ca^{2+}]_o$ solutions. (b) Dose-response curve of ACh (5.5 x 10^{-6} M) second phase in the denervated mouse soleus obtained with different $[Ca^{2+}]_o$. Each point is the mean of at least six experiments: vertical bars show s.e. mean.

or 1.5 mM $[Ca^{2+}]_o$ (Figure 2). When $[Ca^{2+}]_o$ was lowered to 1.0 or 0.5 mM, relaxation during K^+ depolarization occurred more rapidly (Figure 2). However, when Mg^{2+} was substituted for Ca^{2+}, no change in relaxation rate was seen.

The second phase of the contraction to acetylcholine was augmented when the extracellular $[Ca^{2+}]$ was increased (Figure 3b). It was found also that the second phase started earlier with higher extracellular Ca^{2+} concentrations (Table 3). With Krebs solution containing 5 mM Ca^{2+} the second phase was almost equal in size to the first, while at 10 mM there was little relaxation between phases and the second exceeded the first in amplitude (Figure 4). The first phase peak tension was reduced where $[Ca^{2+}]_o = 10$ mM (Table 3). The KCl and caffeine-induced responses were not consistently affected by increasing $[Ca^{2+}]_o$ to 5 mM. In 10 mM $[Ca^{2+}]_o$ solution, the KCl-induced contraction was reduced but caffeine gave contractions of control size. Even when $[Ca^{2+}]_o$ was raised, no second phase to the KCl contraction was observed (10 experiments).

Depolarization produced by acetylcholine was studied with $[Ca^{2+}]_o = 1$, 2.5 and 5.0 mM. The form of reduced in amplitude in 0.5 mM $[Ca^{2+}]_o$ but not in 1.0 or 1.5 mM $[Ca^{2+}]_o$ (Figure 2). When $[Ca^{2+}]_o$ was lowered to 1.0 or 0.5 mM, relaxation during K^+ the response as well as the maximum change in the membrane potential was unaffected by variation of the $[Ca^{2+}]_o$.

EGTA. The addition of 2 mM EGTA to normal Krebs-Henseleit solution (containing 2.5 mM Ca^{2+}) 2 min before acetylcholine virtually abolished the second phase while having no effect on the first phase peak tension (Figure 5). However, the rate of relaxation from the first phase was accelerated (Table 3). KCl-induced contractions were not affected, while those of caffeine were slightly reduced. The depolarization produced by acetylcholine was unaffected by EGTA.

Lanthanum. Acetylcholine-induced contractions in Tris-buffered Krebs were identical to those in normal Krebs-Henseleit solution. The addition of $LaCl_3$ (2 mM) to this solution reduced but did not abolish the second phase of the acetylcholine contraction (Figure 5). Increasing the concentration of La^{3+} to 4 mM had no additional effect. $LaCl_3$ (2 mM) slightly reduced the acetylcholine first phase, and the KCl and caffeine contractions. The change in membrane potential produced by acetylcholine was not altered by $LaCl_3$ (2 mM).

Time-course of the effects of changing $[Ca^{2+}]_o$. In the presence of low $[Ca^{2+}]_o$ (1 mM), acetylcholine produced a first phase contraction only. But when $[Ca^{2+}]_o$ was raised to 5 or 10 mM after 5 min (the normal time of the second phase) there was a rise in tension (Figure 6). The peak tension in these experiments (Table 4) was very similar to that previously observed (Table 3) for the acetylcholine second phase in 5 and 10 mM $[Ca^{2+}]_o$. The half time for development of this contraction in 5 or 10 mM $[Ca^{2+}]_o$ was similar to that shown in Table 3 at the same extracellular Ca^{2+} concentration. When the Ca^{2+} concentration of the Krebs solution was increased in the continued presence of acetylcholine, tension development started after a lag time that is not significantly different from the time of the contraction minimum (t_{min}) observed when the order of addition was reversed.

The addition of EGTA (2 mM) at the peak of the acetylcholine second phase recorded in $[Ca^{2+}]_o = 5$ mM produced relaxation sometimes preceded by a transient rise in tension (Figure 6). The lag time was less than that seen after increasing $[Ca^{2+}]_o$ in the presence of acetylcholine, but the half times of contraction and of relaxation were similar in these two experiments (Tables 4 and 5). Addition of lanthanum (2 mM) also caused relaxation of the second phase of the acetylcholine contraction (recorded in tris-buffered Krebs). This effect was slower than that seen with

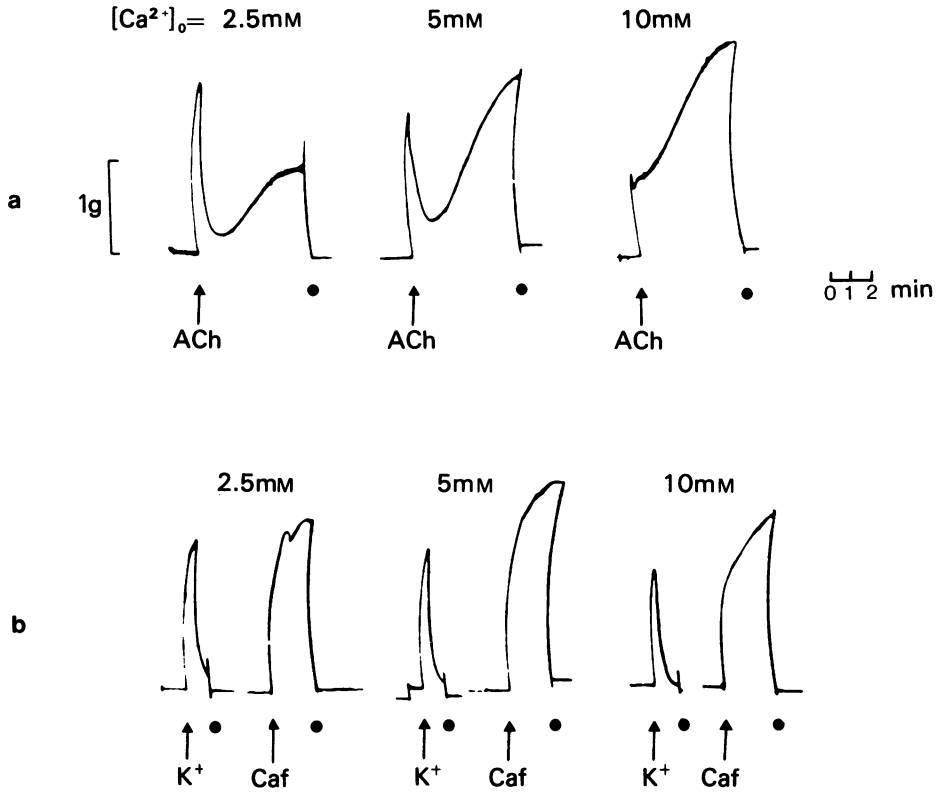
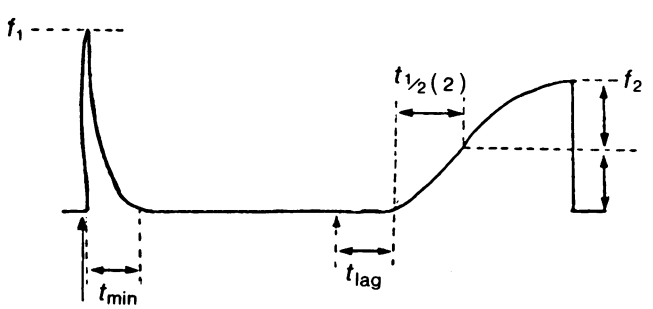


Figure 4 Effect of increasing $[Ca^{2+}]_o$ on contractions obtained with acetylcholine (ACh) 5.5×10^{-6} M, potassium chloride (K^+) 50 mM and caffeine (Caf) 13 mM in denervated soleus preparations. (a) Responses to acetylcholine recorded in $[Ca^{2+}]_o$ = 2.5, 5 or 10 mM; (b) pairs of responses to potassium and caffeine recorded at the same three extracellular Ca^{2+} concentrations.

Table 4 Tension and time-course of the effect of changing $[Ca^{2+}]_o$ in the presence of acetylcholine (5.5×10^{-6} M)



	First phase			*Ca^{2+} addition*		n
	f_1 (g)	t_{min} (s)	t_{lag} (s)	f_2 (g)	$t_{\frac{1}{2}(2)}$ (s)	
$[Ca^{2+}]_o$ 5 mM	1.6 ± 0.2	54 ± 2	48 ± 3	1.4 ± 0.2	72 ± 13	6
$[Ca^{2+}]_o$ 10 mM	1.5 ± 0.2	54 ± 2	45 ± 6	1.8 ± 0.2	71 ± 14	8

t = time; f = tension.

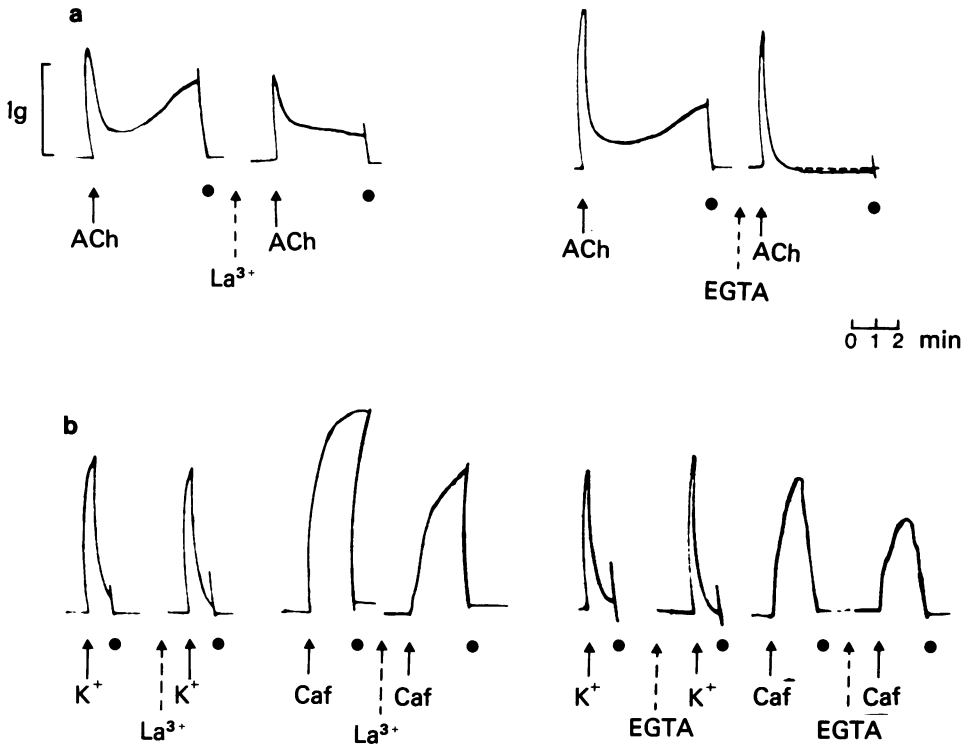
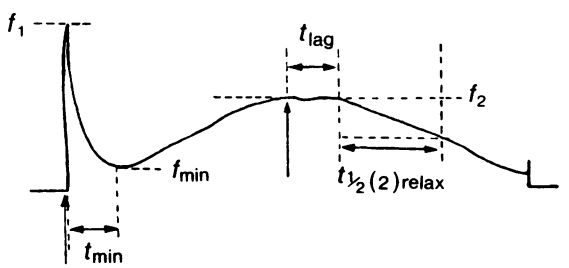


Figure 5 Effect of lanthanum and EGTA on contractions induced by acetylcholine (ACh) 5.5 × 10^{-6} M, potassium (K^+) 50 mM and caffeine (Caf) 13 mM in the denervated soleus. (a) Contractions to ACh recorded before and after lanthanum 2 mM and EGTA 2 mM. After acetylcholine in the presence of EGTA there is relaxation below the baseline, indicated by the dashed line. (b) Control responses and those obtained in the presence of lanthanum or EGTA.

Table 5 Tension and time-course of effects of EGTA and lanthanum on responses to acetylcholine (5.5 × 10^{-6} M) recorded in $[Ca^{2+}]_o = 5$ mM



	First phase	*Contraction minimum*		*Second phase*	*La^{3+} or EGTA*		
	f_1 (g)	t_{min} (s)	f_{min} (g)	f_2 (g)	t_{lag} (s)	$t_{\frac{1}{2}\,relax}$ (s)	n
La^{3+} (2 mM)	1.7 ± 0.1	52 ± 4	0.52 ± 0.13	0.86 ± 0.23	21 ± 13	96 ± 15	5
EGTA (2 mM)	1.3 ± 0.06	46 ± 5	0.28 ± 0.06	0.54 ± 0.11	20 ± 4	77 ± 19	6

t = time; f = tension.

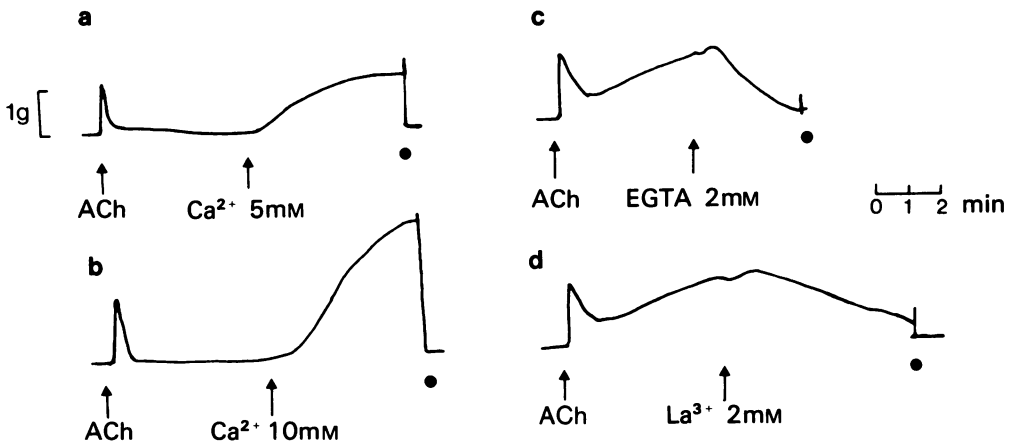


Figure 6 (a & b) Contractions to acetylcholine (ACh) 5.5 × 10^{-6} M of denervated soleus muscles were initially obtained in Krebs solution containing 1 mM [Ca^{2+}] where a first phase only was produced. The subsequent addition of $CaCl_2$ to give $[Ca^{2+}]_o$ = 5 mM or 10 mM produced a slow increase in tension. (c & d) Contractions to ACh were obtained in Krebs solution containing 5 mM [Ca^{2+}] to give a large second phase. The addition of EGTA (2 mM) or La^{3+} (2 mM) produced a transient contraction followed by slow relaxation.

EGTA and the rate of relaxation was more gradual (Table 5 and Figure 6).

Low $[Na]_o$. The second phase of the acetylcholine-induced contraction was augmented in Krebs containing reduced Na^+ concentration (25 mM) but the first phase peak tension was not affected (Table 3). Relaxation from the first phase was less and slightly delayed resulting in a small increase in t min. The rate of contraction in the second phase was faster, so that the half maximum time $t\frac{1}{2}$ (2) was shorter. The maximum tension developed in the second phase (f_2) was much greater than in controls. Thus, the overall pattern of the second phase response resembled that in a high concentration of calcium (10 mM).

Diaphragm

The chronically denervated mouse diaphragm muscle was less sensitive than the soleus to acetylcholine, but had a similar sensitivity to KCl. The EC_{50} in the soleus was 7.6 × 10^{-7} M and in the diaphragm it was 1.3 × 10^{-4} M.

In contrast to the soleus, the diaphragm usually produced no second phase with acetylcholine in the presence of $[Ca^{2+}]_o$ = 2.5 mM. Even when $[Ca^{2+}]_o$ was raised to 5 or 10 mM, there was very little or no second phase except in some deteriorated preparations. In these experiments the second phase was not reduced by EGTA. In agreement with these results it was found that increasing $[Ca^{2+}]_o$ from 1 mM to 11 mM after addition of acetylcholine (5.5 × 10^{-4} M) caused much less and slower development of second phase tension than in soleus preparations.

Discussion

Acetylcholine first phase

Both acetylcholine and K^+ produce persistent depolarization; this is accompanied by a contraction which, however, subsides within 1 minute. A second phase of the tension response was observed with acetylcholine only. In single frog twitch fibres, K^+-induced contractures are complete in about 5 s despite continued depolarization (Hodgkin & Horowicz, 1960) and this is thought to be due to 'inactivation' of the calcium release mechanism that occurs spontaneously with time (Lüttgau, 1963; Curtis, 1964; Milligan & Edwards, 1965; Caputo, 1972; Stuesse, Lindley & Kirby, 1974; Stuesse & Lindley, 1975; Chandler, Rakowski & Schneider, 1976).

It seems likely that decay of the acetylcholine first phase is due to contractile inactivation. Both K^+ and acetylcholine-induced contractions spontaneously relax following a similar time course after reaching their maximum at about the time of peak depolarization. Further evidence for this comes from the observation that, in low $[Ca^{2+}]_o$, relaxation was more rapid resulting in a shortened t min. The value for t min was also shortened in solutions containing high calcium concentrations, not because relaxation was more rapid, but because phase 2 developed more rapidly. In

single frog muscle fibres contractile inactivation is increased when $[Ca^{2+}]_o$ is reduced (Lüttgau, 1963; Caputo & Giminez, 1967), but not if Mg^{2+} is substituted for Ca^{2+} (Lüttgau, 1963). Since the acetylcholine first phase was also shortened in low $[Ca^{2+}]_o$ (except when Mg^{2+} was substituted for Ca^{2+}), we conclude that it is initiated by depolarization of the membrane and terminated by contractile inactivation.

Acetylcholine second phase

The second phase of acetylcholine contraction, unlike the first, was reduced by La^{3+} or when $[Ca^{2+}]_o$ was lowered and was augmented when $[Ca^{2+}]_o$ was raised. Since this part of the acetylcholine contraction is completely dependent on extracellular calcium, we conclude that calcium ions enter the myoplasm from the extracellular space and directly activate the myofibrils. It is to be expected that some Ca^{2+} would enter through the acetylcholine controlled ionic channels since acetylcholine increases endplate permeability to Ca^{2+}, as well as to Na^+ and K^+ (Takeuchi, 1963; Katz & Miledi, 1969; Evans, 1974). Furthermore, it has been shown that sufficient calcium ions can enter by this route to initiate directly a contraction both in denervated mammalian muscle (Jenkinson & Nicholls, 1961) and in frog muscle (Manthey, 1974).

The second phase was abolished when $[Ca^{2+}]_o$ was reduced to 1 mM, although there must have been a considerable concentration gradient favouring Ca^{2+} entry since $[Ca^{2+}]_i$ during relaxation is $<10^{-6}$ M (Ebashi & Endo, 1968). This could be explained if Ca^{2+} influx was balanced by sequestration into the sarcoplasmic reticulum, as suggested for K^+ contractures by Caputo (1972). The delay before initiation of the acetylcholine second phase probably represents the time necessary for saturation of the sarcoplasmic reticulum uptake process, since only when this is complete can Ca^{2+} entering from the extracellular space lead to the development of tension. This delay was similar whether the muscle was first equilibrated with acetylcholine or with $[Ca^{2+}]_o = 2.5$ mM. Thus, the time taken for the starting of the second phase in normal Krebs (about 50 s) broadly corresponds to the time required for the start of the second phase when $[Ca^{2+}]_o$ was raised in the presence of acetylcholine. On the other hand, additions of EGTA or La^{3+} during the second phase produced inhibition of this phase after a much shorter lag time, probably representing the time required for diffusion through the bath to the superficial muscle fibres. The half time ($t_{\frac{1}{2}(2)}$) for development of the second phase tension in control Krebs was similar to the half maximum relaxation time ($t_{\frac{1}{2}\,relax}$) on addition of EGTA or La^{3+} (70–90 seconds). This presumably is a measure of the speed of diffusion through the thickness of the muscle.

Although both La^{3+} and EGTA depressed the second phase, their effects were not identical. La^{3+} reduced the second phase but did not abolish it completely even when the concentration of La^{3+} was doubled. Since La^{3+} postpones spontaneous relaxation of K^+ contractures (Dörscheidt-Käffer & Lüttgau, 1974) by delaying contractile inactivation (Andersson & Edman, 1974) it is likely that the 'second phase' tension in this experiment was in fact the first phase, greatly prolonged.

Low (25 mM) Na^+ solution had an effect on the second phase resembling that of high (5 or 10 mM) Ca^{2+}. This was presumably because sodium and calcium ions compete for the ionic channels opened by acetylcholine. In fact, it has been shown by Lorković (1972) that in depolarized denervated rat muscles acetylcholine contracture tension is a function of $[Ca]/[Na]^2$.

In our experiments, the first phase peak tension was increased and relaxation occurred more quickly in low $[Ca^{2+}]_o$, while in high $[Ca^{2+}]_o$ relaxation of the first phase was delayed. These results confirm those of Freeman & Turner (1969) using the denervated diaphragm and of Gordon (1976) using the denervated rat soleus. Gordon (1976) did not observe a second phase because the contact time was short: the acetylcholine was washed out soon after maximal tension had occurred and before a second phase would have had time to develop.

Depolarization

The two types of depolarization observed in the denervated mouse soleus (Figure 1) probably correspond to the responses of the former endplate receptors (diphasic) and the newly developed extrajunctional receptors (monophasic). Albuquerque & McIsaac (1970) have shown that, although after denervation, acetylcholine sensitivity increases along the whole length of the extensor digitorum longus and soleus muscles, sensitivity remained maximal at the former endplate. There are also differences between the two types of receptors in terms of their sensitivity to antagonists (Beránek & Vyskočil, 1967) and in their electrophoretic mobility (Brockes & Hall, 1975). In denervated frog fibres, single channel conductance is lower and average lifetime longer (Katz & Miledi, 1972; Dreyer, Walther & Peper, 1975; Neher & Sakmann, 1975) but the reversal potential is similar for endplate and denervated receptors (Trautmann & Zilber-Gachelin, 1976). It is not clear which, if any, of these differences account for the different types of depolarization recorded at endplate and extrajunctional sites. However, the organ bath studies clearly point to the differences between the two phases of the acetylcholine contraction being at the level of excitation-contraction coupling.

Diaphragm

Responses of the denervated diaphragm differed from responses of the denervated soleus in two respects. A higher concentration of acetylcholine was required and a second phase was seldom seen. However, two phase contractions were obtained by Preuner (1971) and by Lüllmann *et al.* (1974) when using isotonic but not when using isometric recording. In the depolarized denervated rat diaphragm, the acetylcholine contraction is reduced in low $[Ca^{2+}]_o$ (Jenkinson & Nicholls, 1961). Thus it is probably equivalent to a 'second phase', especially since a first phase could not have occurred as the Ca^{2+} release mechanism would have been inactivated by depolarization. Similar results are obtained using other denervated rat muscles in a depolarizing solution (Lorković, 1972). Jenkinson & Nicholls (1961) reported that in the depolarized denervated diaphragm, acetylcholine contractions did not occur if the temperature was above 30°C. Perhaps our conditions (37°C, isometric recording) were therefore not optimal for the development of the second phase. Nevertheless, the difference between the soleus and the diaphragm, observed under the same conditions, was striking. It seems possible that this difference might be due to a difference in receptor numbers or in the intracellular Ca^{2+} sequestration.

Following denervation, the Ca^{2+} uptake activity of the sarcoplasmic reticulum increases both in the diaphragm (Howell, Fairhurst & Jenden, 1960) and in the soleus (Margreth, Salviati, di Mauro & Turati, 1972). We have not been able to trace any reference to a direct comparison of the Ca^{2+} uptake between denervated soleus and denervated diaphragm muscles. However, Ca^{2+} uptake by fragmented sarcoplasmic reticulum was found by Sreter (1970) to be about 5-fold less in the denervated rat soleus than in the denervated rat gastrocnemius, while Howell *et al.* (1960) found that denervated rat diaphragm sarcoplasmic reticulum had 0.62 of the Ca^{2+} uptake activity of denervated rat gastrocnemius. In other words, Ca^{2+} accumulation occurs in the order gastrocnemius > diaphragm $\gg$ soleus.

The denervated rat soleus was found to bind 99.3 fmol/mg of [^{125}I]-α-bungarotoxin (Almon, Andrew & Appel, 1974) while the denervated rat diaphragm bound only 44.5 fmol/mg (Colquhoun, Rang & Ritchie, 1974). Thus, in the rat, it seems that the receptor density in the denervated soleus is more than twice that in the denervated diaphragm.

Taking these points together, it seems possible that denervated diaphragm fibres, having less acetylcholine receptors, allow less Ca^{2+} to enter from the extracellular space and, having a more active sarcoplasmic reticulum, a smaller proportion of this Ca^{2+} is available to the myofibrils. These factors may explain why acetylcholine produces a pronounced second phase in the denervated soleus, but in the denervated diaphragm it is small or absent.

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References

ALBUQUERQUE, E.X. & McISAAC, R.J. (1970). Fast and slow mammalian muscle after denervation. *Expl. Neurol.*, **26**, 183–202.

ALMON, R.R., ANDREW, C.G. & APPEL, S.H. (1974). Acetylcholine receptor in normal and denervated slow and fast muscle. *Biochemistry*, **13**, 5522–5528.

ANDERSSON, K.E. & EDMAN, K.A.P. (1974). Effects of lanthanum on potassium contractures of isolated twitch muscle fibres of the frog. *Acta physiol. scand.*, **90**, 124–131.

AXELSSON, J. & THESLEFF, S. (1959). A study of supersensitivity in denervated mammalian skeletal muscle. *J. Physiol., Lond.*, **147**, 178–193.

BERÁNEK, R. & VYSKOČIL, F. (1967). The action of tubocurarine and atropine on the normal and denervated rat diaphragm. *J. Physiol., Lond.*, **188**, 53–66.

BOWMAN, W.C. & RAPER, C. (1964). Spontaneous fibrillary activity of denervated muscle. *Nature, Lond.*, **201**, 160–162.

BROCKES, J.P. & HALL, Z.W. (1975). Acetylcholine receptors in normal and denervated rat diaphragm muscle II. Comparison of junctional and extrajunctional receptors. *Biochemistry*, **14**, 2100–2106.

BROWN, G.L. (1937). The action of acetylcholine on denervated mammalian and frog muscle. *J. Physiol., Lond.*, **89**, 438–461.

CAPUTO, C. (1972). The time course of potassium contractures of single muscle fibres. *J. Physiol., Lond.*, **223**, 483–505.

CAPUTO, C. & GIMINEZ, M. (1967). Effects of external calcium deprivation on single muscle fibres. *J. gen. Physiol.*, **50**, 2177–2195.

CHANDLER, W.K., RAKOWSKI, R.F. & SCHNEIDER, M.F. (1976). Effects of glycerol treatment and maintained depolarization on charge movement in skeletal muscle. *J. Physiol., Lond.*, **254**, 285–316.

COLQUHOUN, D., RANG, H.P. & RITCHIE, J.M. (1974). The binding of tetrodotoxin and α-bungarotoxin to normal and denervated mammalian muscle. *J. Physiol., Lond.*, **240**, 199–226.

CURTIS, B.A. (1964). The recovery of contractile ability following a contracture in skeletal muscle. *J. gen. Physiol.*, **47**, 953–964.

DÖRRSCHEIDT-KÄFER, M. & LÜTTGAU, H.C. (1974). The effect of lanthanum ions on mechanical threshold and potassium contractures in frog skeletal muscle fibres. *J. Physiol., Lond.*, **242**, 101–102P.

DREYER, F., WALTHER, C. & PEPER, K. (1975). Receptors in the postsynaptic membrane of normal and denervated frog skeletal muscle fibres: their reactions to ACh and other cholinergic agonists. *Eur. J. Physiol.*, **359**, R71.

EBASHI, S. & ENDO, M. (1968). Calcium ion and muscle contraction. *Prog. Biophys. Mol. Biol.*, **18**, 123–183.

ELMQVIST, D. & THESLEFF, S. (1960). A study of acetylcholine induced contractures in denervated mammalian muscle. *Acta pharmac. tox.*, **17**, 84–93.

EVANS, R.H. (1974). The entry of labelled calcium into the innervated region of the mouse diaphragm. *J. Physiol., Lond.*, **240**, 517–533.

FREEMAN, S.E. & TURNER, K.J. (1969). Ionic interactions in acetylcholine contraction of the denervated rat diaphragm. *Br. J. Pharmac.*, **36**, 510–522.

GILLESPIE, J.S. & McKNIGHT, A.T. (1975). The effect of tris on the response of various isolated, mammalian preparations to nerve and drug stimulation. *J. Physiol., Lond.*, **248**, 12–13P.

GORDON, T. (1976). The effects of external calcium and magnesium ions on the response of denervated muscle to acetylcholine. *J. Physiol., Lond.*, **255**, 575–586.

HALL, M.T., MALEQUE, M.A. & WADSWORTH, R.M. (1975). Desensitization in the innervated and in the chronically denervated soleus muscle of the mouse. *Br. J. Pharmac.*, **55**, 125–131.

HODGKIN, A.L. & HOROWICZ, P. (1960). Potassium contractures in single muscle fibres. *J. Physiol., Lond.*, **153**, 386–403.

HOWELL, J.N., FAIRHURST, A.S. & JENDEN, D.J. (1960). Alterations of the Ca accumulating ability of striated muscle following denervation. *Life Sci.*, **5**, 439–446.

JENKINSON, D.H. & NICHOLLS, J.G. (1961). Contractures and permeability changes produced by acetylcholine in depolarised denervated muscle. *J. Physiol., Lond.*, **159**, 111–127.

KATZ, B. & MILEDI, R. (1969). Spontaneous and evoked activity of motor nerve endings in calcium ringer. *J. Physiol., Lond.*, **203**, 689–706.

KATZ., B. & MILEDI, R. (1972). The statistical nature of the acetylcholine potential and its molecular components. *J. Physiol., Lond.*, **224**, 665–699.

LETLEY, E. (1960). The effect of temperature on the direct muscle twitch response and the action of drugs on the isolated denervated rat diaphragm. *Br. J. Pharmac.*, **15**, 345–350.

LORKOVIĆ, H. (1972). Antagonism between calcium and monovalent cations in depolarised denervated muscles. *Am. J. Physiol.*, **222**, 1427–1434.

LÜLLMANN, H., PREUNER, J. & SCHAUBE, H. (1974). A kinetic approach for an interpretation of the acetylcholine-d-tubocurarine interaction on chronically denervated skeletal muscle. *Naunyn-Schmiedeberg's Arch. Pharmac.*, **281**, 415–426.

LÜLLMAN, H. & SUNANO, S. (1973). Acetylcholine contracture and excitation—contraction coupling in denervated rat diaphragm muscle. *Eur. J. Physiol.*, **342**, 271–282.

LÜTTGAU, H.C. (1963). The action of calcium ions on potassium contractures of single muscle fibres. *J. Physiol., Lond.*, **168**, 679–697.

MANTHEY, A.A. (1974). Changes in Ca permeability of muscle fibres during desensitization to carbamylcholine. *Am. J. Physiol.*, **226**, 481–489.

MARGRETH, A., SALVIATI, G., DI MAURO, S. & TURATI, G. (1972). Early biochemical consequences of denervation in fast and slow skeletal muscles and their relationship to neural control over muscle differentiation. *Biochem. J.*, **126**, 1099–1110.

MILLIGAN, J.V. & EDWARDS, C. (1965). Some factors affecting the time course of the recovery of contracture ability following a potassium contracture in frog striated muscle. *J. gen. Physiol.*, **48**, 975–983.

NEHER, E. & SAKMANN, B. (1975). Noise analysis of voltage clamp currents induced by different cholinergic agonists in normal and denervated frog muscle fibres. *Eur. J. Physiol.*, **355**, R63.

PREUNER, J. (1971). Characterization of a biphasic response of the chronically denervated diaphragm of the rat to the same cholinergic agonist. *Naunyn-Schmiedebergs Arch. Pharmac.*, **270**, 169–179.

ROSENBLUETH, A. & LUCO, J.V. (1937). A study of denervated mammalian skeletal muscle. *Am. J. Physiol.*, **120**, 781–797.

SRETER, F.A. (1970). Effect of denervation on fragmented sarcoplasmic reticulum of white and red muscle. *Expl. Neurol.*, **29**, 52–64.

STUESSE, S.C. & LINDLEY, B.D. (1975). Contractile inactivation in frog single denervated muscle fibres. *Am. J. Physiol.*, **229**, 1492–1497.

STUESSE, S.C., LINDLEY, B.D. & KIRBY, A.C. (1974). Potassium contractures of frog single denervated muscle fibres: time course and central spread. *Am. J. Physiol.*, **227**, 200–208.

TAKEUCHI, N. (1963). Some properties of conductance changes at the end plate membrane during the action of acetylcholine. *J. Physiol., Lond.*, **167**, 128–140.

TRAUTMANN, A. & ZILBER-GACHELIN, N.F. (1976). Further investigations on the effect of denervation and pH on the conductance change at the neuro-muscular junction of the frog. *Eur. J. Physiol.*, **364**, 53–58.

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