Br. J. Pharmac. (1978), **62**, 107–113

THE EFFECTS OF GLYCEROL DETUBULATION, OF MANGANESE IONS, OF DANTROLENE AND OF NITRATE IONS ON THE RESPONSES OF ISOLATED CHRONICALLY DENERVATED SOLEUS MUSCLES OF THE MOUSE TO ACETYLCHOLINE

MOIRA T. HALL, M.A. MALEQUE[1] & R.M. WADSWORTH

Department of Physiology and Pharmacology, University of Strathclyde, Glasgow, G1 1XW

1 In the isolated chronically denervated soleus muscle of the mouse acetylcholine produced a biphasic contraction.

2 In muscles detubulated by glycerol treatment, the first phase of the acetylcholine contraction was almost abolished, but the second was only slightly reduced or was unaffected.

3 Manganese ions (10 mM) reduced the first phase of the acetylcholine response, but enhanced the second.

4 Dantrolene sodium (5.94×10^{-5} M) reduced the first phase of the acetylcholine response, but not the second.

5 Nitrate ions (118 mM) augmented the first phase but not the second.

6 It was concluded that the first phase requires the excitation-contraction-coupling sequence: membrane depolarization-T-tubules-Ca^{2+} release from sarcoplasmic reticulum. In contrast, the second phase appears to occur independently of these processes.

Introduction

The addition of acetylcholine to isolated chronically denervated soleus muscles results in biphasic contractions (Hall, Maleque & Wadsworth, 1975). The second phase of tension is reduced when the extracellular calcium concentration is lowered and increased when $[Ca^{2+}]_0$ is raised. The first phase peak tension, however, is slightly increased in low $[Ca^{2+}]_0$ and is probably terminated by a process of contractile inactivation. It was therefore concluded that activator calcium for the second phase comes from a source in equilibrium with the extracellular fluid, while activator calcium for the first phase is derived from an intracellular source (Hall, Maleque & Wadsworth, 1977). It was thought likely that this would be the sarcoplasmic reticulum, and we have attempted to investigate this by using agents thought to have a selective action on calcium release from the sarcoplasmic reticulum (nitrate and dantrolene) or on the T-tubules (manganese and glycerol).

[1] Present address: Department of Physiology, Meharry Medical College, Nashville, TN 37208, U.S.A.

Methods

The method of denervation, the organ bath procedure and electrophysiological techniques have been described by Hall *et al.* (1977). Acetylcholine and K^+ in some experiments produced a rapid and in others a more gradual depolarization (type a and type b responses; Hall *et al.*, 1977) but since similar results were obtained with both types of responses, no distinction has been made in the text. The muscles used for electrophysiological experiments were fibrillating, but the pen recorder records showed spontaneous fluctuations of membrane potential in a few cases only. In our experiments we did not prevent muscle contraction; we relied on limiting muscle movement by pinning out the preparation securely. It was therefore difficult to retain a penetration in a spontaneously active fibre. This may be the reason why records showing membrane potential for several minutes, or showing the effects of acetylcholine or K^+, do not show action potentials. The salt solutions used had the composition given in Table 1. Similar responses were obtained with Tris-buffered or HCO_3^-/$H_2PO_4^-$ buffered solutions.

Glycerol detubulation

The T-Tubules were selectively destroyed according to the method described by Eisenberg & Eisenberg (1968) and by Howell (1969). After obtaining control responses, the Krebs-Henseleit solution was made hypertonic by the addition of glycerol 410 mM and the muscles were soaked in this solution for 1 to 1.5 hours. The muscles were then allowed to recover in standard Krebs-Henseleit solution for a further 1 h, after which their sensitivity to caffeine was normal, indicating that the calcium-releasing function of the sarcoplasmic reticulum was unimpaired.

The drugs used were acetylcholine chloride (Sigma), caffeine citrate (Evans) and dantrolene sodium (Eaton). Dantrolene sodium was dissolved in propylene glycol. The solvent itself was without effect at the concentrations used.

Results are quoted as the mean ± s.e. mean. The significance of differences was assessed at the 0.05 level by the Wilcoxon matched-pairs signed-ranks test.

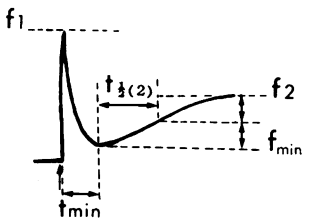
Results

In the chronically denervated soleus muscle of the mouse, acetylcholine produces a biphasic contraction, the height of the second phase being reduced in low but raised in high $[Ca^{2+}]_o$, while the first phase is not similarly affected by abrupt changes in the extracellular Ca^{2+} concentration (Hall *et al.*, 1977). We

Table 1 Composition of physiological salt solutions used

		Concentration (mM)										
	Gas	*Na^+*	*K^+*	*Ca^{2+}*	*Mg^{2+}*	*Cl^-*	*SO_4^{2-}*	*$H_2PO_4^-$*	*HCO_3^-*	*Tris*	*Glucose*	*NO_3^-*
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
Nitrate-Krebs	5% CO_2	144	5.8	2.5	1.2	9.6	1.2	1.2	25	0	11.1	118

Table 2 Tension and time-course of acetylcholine (5.5×10^{-6} M)-induced contractions in the denervated soleus; effect of procedures having a selective action on the first phase



	First phase	*Contraction minimum*		*Second Phase*		
	f_1 (g)	*t_{min} (s)*	*f_{min} (g)*	*$t_{½(2)}$ (s)*	*f_2 (g)*	n
Control	1.70 ± 0.1	55 ± 4	0.18 ± 0.08	104 ± 3	0.40 ± 0.1	
Glycerol 410 mM	0.12 ± 0.01*	—	—	127 ± 18	0.47 ± 0.1	7
Control	1.80 ± 0.09	43 ± 4	0.11 ± 0.01	105 ± 6	0.48 ± 0.06	
Mn^{2+} 10 mM	0.64 ± 0.16*	34 ± 5	0.20 ± 0.05	74 ± 14	0.90 ± 0.25*	9
Control	1.58 ± 0.08	60 ± 4	0.06 ± 0.03	100 ± 6	0.22 ± 0.06	
Dantrolene 5.94×10^{-5} M	0.87 ± 0.08*	36 ± 2*	0.13 ± 0.05	87 ± 9	0.55 ± 0.06	6
Control	1.51 ± 0.13	60 ± 11	0.22 ± 0.09	105 ± 23	0.75 ± 0.23	
NO_3^- 118 mM	1.90 ± 0.13*	85 ± 9	0.55 ± 0.18	100 ± 35	0.49 ± 0.14	8

* Significantly different from control value ($P < 0.05$) using Wilcoxon matched-pairs signed-ranks test.

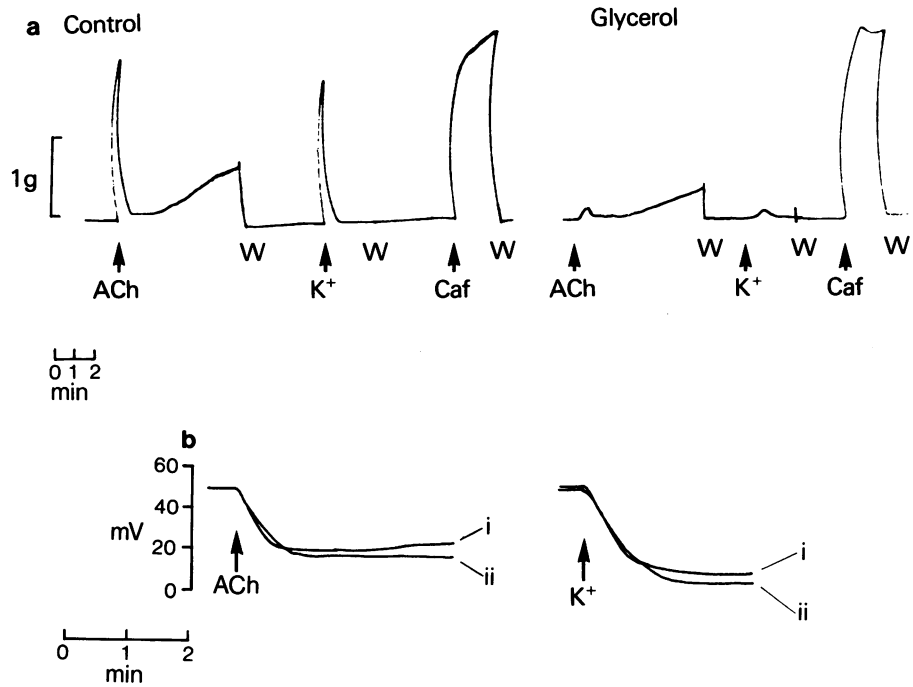


Figure 1 The effect of glycerol detubulation on contraction and membrane potential of the chronically denervated soleus muscle of the mouse. (a) On the left are responses obtained in control Krebs solution to acetylcholine 5.5×10^{-6} M (ACh), potassium 50 mM (K^+) and caffeine 13 mM (Caf). Glycerol was then added to the bath at a final concentration of 410 mM and the muscle was incubated for 1 h in this solution and then returned to normal Krebs. Acetylcholine now produced a contraction with a much reduced first phase and slightly delayed second phase, K^+ contracture was also inhibited, but not caffeine. (b) Depolarization produced by acetylcholine or potassium: (i), control; (ii), after detubulation with glycerol. In this and subsequent figures, washout is marked W.

Table 3 Peak tension of potassium (50 mM) and caffeine (13 mM) contractures in the chronically denervated mouse soleus

	KCl (50 mM)		*Caffeine (13 mM)*	
	Tension (g)	n	*Tension (g)*	n
Control	1.62 ± 0.16		1.75 ± 0.06	
Glycerol 410 mM	0.09 ± 0.05*	10	1.78 ± 0.10	18
Control	1.63 ± 0.21		2.50 ± 0.12	
Mn^{2+} 10 mM	0.02 ± 0.02*	6	2.08 ± 0.10*	6
Control	1.72 ± 0.18		3.23 ± 0.32	
Dantrolene 5.94×10^{-5} M	0.62 ± 0.14*	6	2.73 ± 0.34*	9
Control	1.41 ± 0.19		2.48 ± 0.30	
NO_3^- 118 mM	2.05 ± 0.38*	6	1.95 ± 0.20	7

* Significantly different from control value ($P < 0.05$).

have recorded these contractions after one of the four treatments (glycerol detubulation, Mn^{2+}, dantrolene or NO_3^-) thought to act selectively on excitation-contraction coupling. Since the acetylcholine response was reproducible in a given muscle, we first recorded control contractions, and then repeated these after one of the four treatments. The test and control responses were then compared for each muscle (Table 2). The effects of NO_3^- and Mn^{2+} are reversible, and it was possible to reproduce the control responses by returning the muscle to the standard Krebs-Henseleit solution, though this was not done routinely. The effects of glycerol and dantrolene are irreversible. To assess the specificity of the four treatments employed, we used potassium and caffeine, because they produce reproducible contractures and because they act at known sites. Control K^+ and caffeine contractures were first obtained in Krebs-Henseleit solution (along with acetylcholine contractions) and all three were then repeated after one of the four treatments. The effects on K^+ and caffeine responses are presented in Table 3.

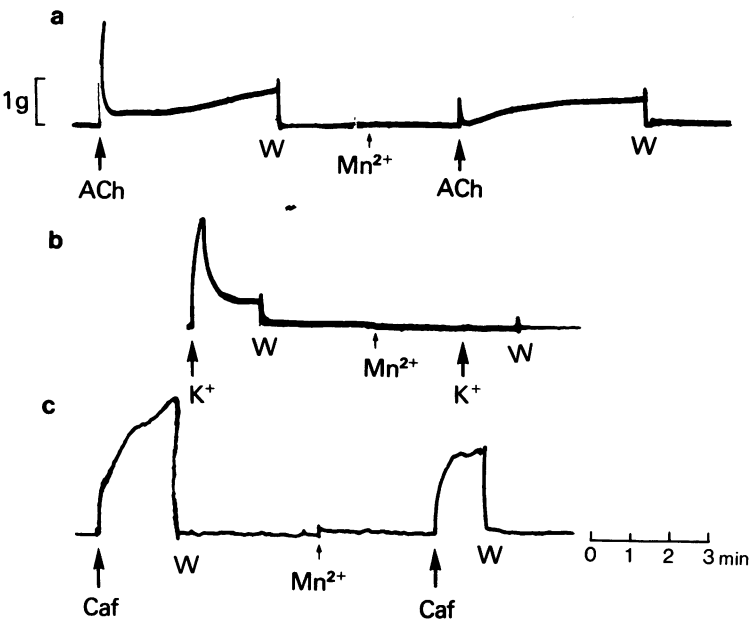


Figure 2 The effect of manganese on responses of the chronically denervated soleus. Control responses, recorded in Tris-buffered Krebs, are shown on the left; on the right these are shown repeated in the presence of $MnCl_2$ 10 mM. (a) Acetylcholine, 5.5×10^{-6} M (ACh). The first phase, but not the second, was reduced by Mn^{2+}. (b) The potassium 50 mM (K^+) contracture was completely inhibited. (c) The caffeine 13 mM (Caf) contracture was slightly reduced.

Glycerol detubulation

Glycerol-treated muscles were unresponsive to K^+, but the sensitivity to caffeine was normal (Figure 1) indicating selective destruction of the T-tubules as first shown by Eisenberg & Eisenberg (1968) and by Howell (1969) in frog sartorius muscles. After detubulation the first phase of acetylcholine contractions was almost abolished, but the second phase was still present. In most experiments, the peak tension of the second phase was increased after detubulation; in a few it was reduced, but never to the same extent as the reduction in the first phase (Figure 1 and Table 2).

Depolarization produced by acetylcholine or KCl was unaffected by detubulation (Figure 1).

Manganese

$MnCl_2$ (10 mM) added to Tris-buffered Krebs-Henseleit solution caused a slight reduction (16 ± 3%) in the peak tension of caffeine contractions, while the K^+ response was nearly always abolished (Table 3). Selective inhibition of submaximal K^+ responses with little or no effect on caffeine agrees with the results of Oota, Takauji & Nagai (1972), Chiarandini & Stefani (1973) and Sakai, Kurihara & Yoshioka (1974) in frog muscles, where Mn^{2+} is thought to inhibit excitation-contraction coupling by an action on the T-tubules. In the presence of Mn^{2+} (10 mM), the first phase of the acetylcholine response was reduced to approximately one third, and relaxation occurred more rapidly. The second phase, on the other hand, was enhanced and started to develop earlier (Figure 2 and Table 2).

Dantrolene

Dantrolene sodium caused a dose-dependent inhibition of directly elicited twitches of denervated soleus muscles (Figure 3). The dose-response curve was shallow, and even at a concentration of 5.94×10^{-5}M there was only 25 ± 3% inhibition. Higher concentrations were not used because the solvent (propylene glycol) caused twitch depression in concentrations exceeding 6%. Dantrolene sodium (5.94×10^{-5}M) also inhibited K^+ contractures (by 51 ± 10%) and caffeine contractures (by 16 ± 4%, Table 3). These results are in agreement with the actions of dantrolene as reported by Putney & Bianchi (1974), by Takauji, Takahashi & Nagai (1975) and by Moulds (1977) except for caffeine contractures (see Discussion). Dantrolene is thought to prevent trigger Ca^{2+} release (Putney & Bianchi, 1974) or to inhibit Ca^{2+} release

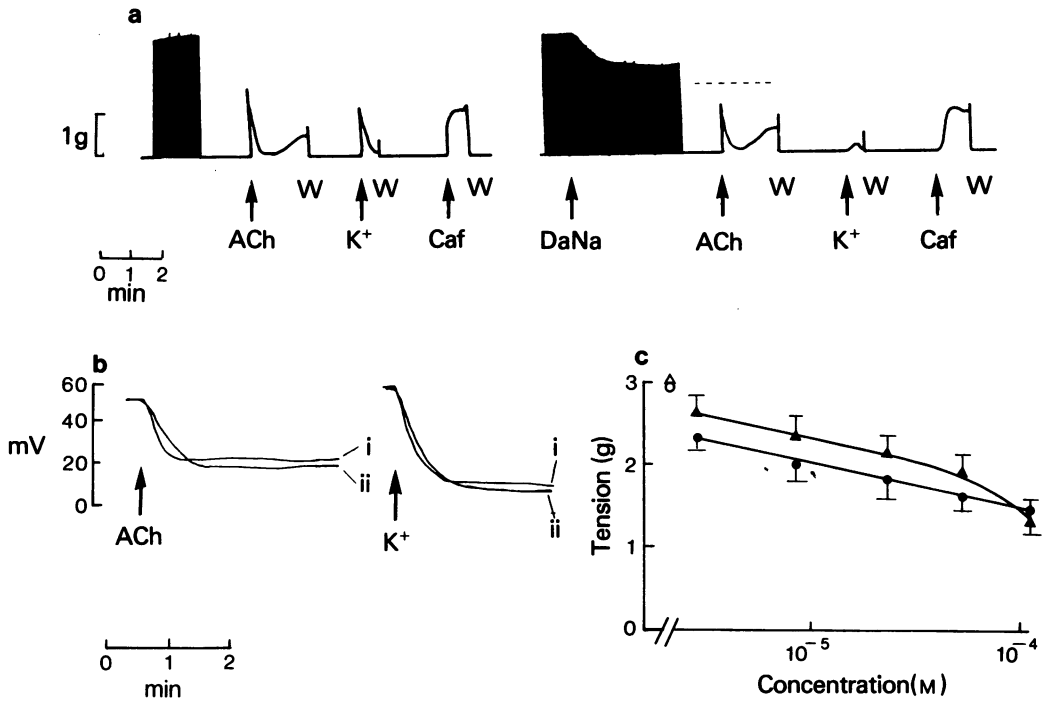


Figure 3 The effects of dantrolene sodium on the chronically denervated soleus muscle of the mouse. (a) Directly elicited twitches and responses to acetylcholine 5.5 × 10^{-6} M (ACh), potassium 50 mM (K^+) and caffeine 13 mM (Caf) were obtained before and after dantrolene 5.94 × 10^{-5} M (DaNa). The peak tension of the first phase in the control acetylcholine response is indicated by the dashed line. (b) Effect of dantrolene 5.94 × 10^{-5} M on depolarization produced by acetylcholine and potassium in denervated soleus muscle. (i) control, (ii) after dantrolene 5.94 × 10^{-5} M. (c) Dose-response curve for dantrolene: control tension of twitches obtained using ring electrodes with innervated (○) and chronically denervated (△) muscles and in the presence of dantrolene (●, ▲). Each point is the mean of 8 results, the vertical bars represent the standard errors.

from the sarcoplasmic reticulum (Desmedt & Hainaut, 1977). Dantrolene sodium (5.94 × 10^{-5} M) reduced the first phase of the acetylcholine contraction by 44 ± 5%. On the other hand, the mean tension of the second phase was enhanced although this did not reach statistical significance (Figure 3 and Table 2). The rate of relaxation of the first phase, as well as the rate of contraction of the second, were increased by dantrolene.

Acetylcholine or KCl depolarization was not affected by dantrolene (Figure 3).

Nitrate

Nitrate augments twitches, and potassium and caffeine contractures in amphibian muscle (Hodgkin & Horowicz, 1960; Matsushima, Fujino & Nagai, 1962; Foulks, Perry & Sanders, 1971). In the chronically denervated mouse soleus muscle, 118 mM NO_3^- augmented directly elicited twitches by 32 ± 10%. K^+ contractures were not augmented in every experiment, but the mean tension was increased. However, caffeine contractures were increased by nitrate in only 2 experiments out of 7 (Table 3). The peak tension of the acetylcholine first phase was significantly augmented by (31 ± 16%) and the rate of relaxation was usually decreased (Table 2 and Figure 4). However, the acetylcholine second phase was reduced in most experiments.

Discussion

Dantrolene is thought to inhibit trigger calcium (Putney & Bianchi, 1974) or to directly inhibit Ca^{2+} release from intracellular storage sites (Desmedt & Hainaut, 1977). Glycerol and manganese both interrupt excitation-contraction coupling by an action on the T-tubules: glycerol by breaking up the T-tubules into a series of vacuoles, and manganese probably

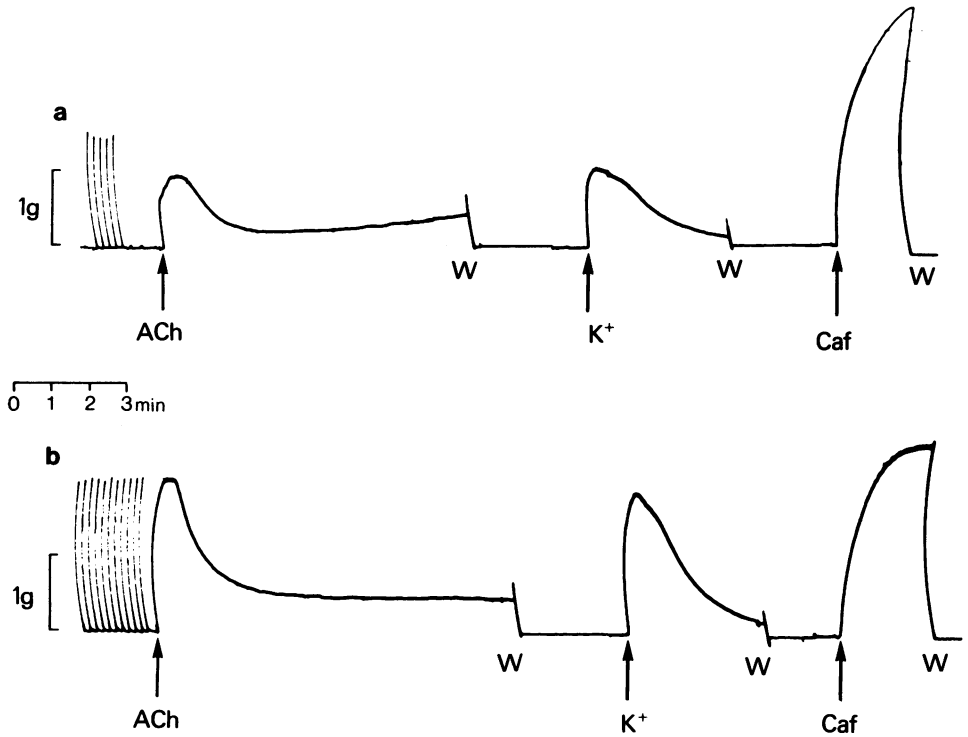


Figure 4 The effects of nitrate on the chronically denervated soleus. (a) Recording made in the standard Krebs solution. (b) After changing to 118 mM NO_3^- Krebs. The twitches were obtained by applying rectangular pulses of 30 ms, 1 Hz and supramaximal voltage to ring electrodes surrounding the muscle. Contractions were obtained with acetylcholine 5.5×10^{-6} M (ACh), potassium 50 mM (K^+) and caffeine 13 mM (Caf). Nitrate augmented the first phase of the acetylcholine contraction, but not the second.

by preventing the entry of trigger Ca^{2+}. These three procedures all inhibited the acetylcholine first phase while the second phase was either not affected or enhanced. Taken together these results suggest that the first phase requires the T-tubules and sarcoplasmic reticulum, while the second phase does not. This conclusion is supported by the experiments with NO_3^- which augmented the acetylcholine first phase, but not the second. Since NO_3^- increases the effectiveness of the depolarization-release coupling (Ebashi & Endo, 1968), and also inhibits sarcoplasmic Ca^{2+} uptake (Ebashi & Endo, 1968; Carvalho, 1968; The & Hasselbach, 1975), this result confirms the conclusion that the T-tubules and sarcoplasmic reticulum are important in the first phase and not in the second. It is interesting that in most experiments in the presence of nitrate, relaxation from the first phase was delayed and the minimum interphase tension (f_{min} in Table 2) was increased both of which would be expected from an agent that inhibits intracellular Ca^{2+} sequestration.

In chronically denervated rat diaphragm muscles, Freeman & Turner (1969) found that thiocyanate 12 mM (which acts in the same way as NO_3^-) augmented and prolonged the acetylcholine contracture, and that manganese (4 mM) reduced it. Lüllmann & Sunano (1973) confirmed that the acetylcholine contracture was decreased by manganese (5 mM) and also found it was inhibited by glycerol detubulation. Both groups of authors observed monophasic contractions in their experiments, and our results (for the first part of the acetylcholine contraction in the mouse soleus) are in agreement with theirs.

In denervated mouse soleus muscles, we found with dantrolene a slight decrease in the peak tension of caffeine contractures in agreement with the results of Moulds (1977) who used innervated mouse soleus. In frog muscle, however, caffeine contractures are not reduced by dantrolene (Ellis & Carpenter, 1971; Putney & Bianchi, 1974; Takauji, *et al.*, 1975), which perhaps suggests a difference between mammalian and amphibian muscles. In the chick biventer cervicis the caffeine contracture is biphasic and only the second phase is reduced by dantrolene (Khan, 1976).

The effects, on the acetylcholine response, of the four procedures used all point to the same conclusion.

The first phase of the acetylcholine contraction was decreased by glycerol, by manganese and by dantrolene, and was increased by nitrate. These actions were selective on the first phase whereas the second phase was in every case either unaltered or oppositely affected. It is striking that the procedures used altered potassium contractures in a similar way to their effect on the acetylcholine first phase, and it therefore seems likely that the first phase is generated by a depolarization-dependent mechanism similar to that responsible for K^+ contractures. On the other hand, the second phase must involve a different mechanism, and evidence was presented by Hall, *et al.* (1977) to show that Ca^{2+} enters from the extracellular fluid through the acetylcholine controlled ionic channels to activate the contractile mechanism directly.

M.A.M. was supported by an academic staff scholarship from the Association of Commonwealth Universities. Dantrolene sodium was a gift from Eaton Laboratories.

References

CARVALHO, A.P. (1968). Effects of potentiators of muscular contraction on binding of cations by sarcoplasmic reticulum. *J. gen. Physiol.*, **51**, 427–442.

CHIARANDINI, D.J. & STEFANI, E. (1973). Effects of manganese on the electrical and mechanical properties of frog skeletal muscle fibres *J. Physiol.*, **232**, 129–147.

DESMEDT, J.E. & HAINAUT, K. (1977). Inhibition of the intracellular release of calcium by dantrolene in barnacle giant muscle fibres. *J. Physiol.*, **265**, 565–585.

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

EISENBERG, B. & EISENBERG, R.S. (1968). Selective disruption of the sarcotubular system in frog sartorius muscle. *J. cell Biol.*, **39**, 451–467.

ELLIS, K.O. & CARPENTER, J.F. (1971). The effects of dantrolene sodium on skeletal muscle. *Fedn Proc.*, **30**, 670.

FOULKS, J.G. PERRY, F.A. & SANDERS, H.D. (1971) Augmentation of caffeine-contracture tension by twitch-potentiating agents in frog toe muscle. *Can. J. Physiol. Pharmac.*, **49**, 889–900.

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

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.

HALL, M.T., MALEQUE, M.A. & WADSWORTH, R.M. (1977). The role of extracellular calcium in the contractions produced by acetylcholine in chronically denervated muscle. *Br. J. Pharmac.* (in press).

HODGKIN, A.L. & HOROWICZ, P. (1960). The effect of nitrate and other anions on the mechanical response of single muscle fibres. *J. Physiol.*, **153**, 404–412.

HOWELL, J.N. (1969). A lesion of the transverse tubules of skeletal muscle. *J. Physiol.*, **201**, 515–533.

KHAN, H.H. (1976). Pharmacological studies on dantrolene sodium. *Ph.D. thesis, Strathclyde University.*

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

MATSUSHIMA, T., FUJINO, M. & NAGAI, T. (1962). Effects of anomalous anions on the caffeine contracture. *Jap. J. Physiol.*, **12**, 106–112.

MOULDS, R.F.W. (1977). A comparison of the effects of sodium thiocyanate and dantrolene sodium on a mammalian isolated skeletal muscle. *Br. J. Pharmac.*, **59**, 129–133.

OOTA, I., TAKAUJI, M. & NAGAI, T. (1972). Effect of manganese ions on excitation-contraction coupling in frog sartorius muscle. *Jap. J. Physiol.*, **22**, 379–392.

PUTNEY, J.W. & BIANCHI, C.P. (1974). Site of action of dantrolene in frog sartorius muscle. *J. Pharmac. exp. Ther.*, **189**, 202–212.

SAKAI, T., KURIHARA, S. & YOSHIOKA, T. (1974). Action of manganese ions on excitation-contraction coupling of frog skeletal muscle fibres. *Jap. J. Physiol.*, **24**, 513–530.

TAKAUJI, M., TAKAHASHI, N. & NAGAI, T. (1975). The effect of dantrolene sodium on excitation-contraction coupling in frog skeletal muscle. *Jap. J. Physiol.*, **25**, 747–758.

THE, R. & HASSELBACH, W. (1975). The action of chaotropic anions on the sarcoplasmic calcium pump. *Eur. J. Biochem.*, **53**, 105–113.

(Received May 26, 1977.
Revised August 1, 1977.)