J. Physiol. (1973), **232**, *pp.* 129–147
With 8 text-figures
Printed in Great Britain

EFFECTS OF MANGANESE ON THE ELECTRICAL AND MECHANICAL PROPERTIES OF FROG SKELETAL MUSCLE FIBRES

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(*Received 3 November 1972*)

SUMMARY

1. The effects of Mn on the electrical and mechanical properties of frog muscle fibres have been studied.

2. In normal saline 10 or 20 mM-Mn hyperpolarized the fibres and had no effect on the membrane resistance. In isotonic K_2SO_4 saline, Mn increased the membrane resistance indicating that this agent reduced the conductance to K.

3. The action potential is prolonged by Mn while the overshoot amplitude is unaffected. The threshold of the action potential is shifted to more positive values of membrane potential.

4. The isometric twitch is reduced by 45% in 10 mM-Mn; this effect is observed within 8 sec of the application.

5. Mn (10 mM) reduced K contractures induced by 40 or 75 mM-K (constant [K].[Cl] product) and shifted to the right in a parallel manner the curve tension *vs.* log K concentration. The calculated mechanical threshold for K contractures was shifted from -48 to -33 mV.

6. Caffeine contractures (3–4 mM) and supramaximal K contractures (190 mM-K) were unaffected by 10 mM indicating that contractile proteins and the ability of the sarcoplasmic reticulum to release Ca are not impaired.

7. It is concluded that Mn is mainly affecting the excitation-contraction coupling by altering the mechanical threshold. Since Mn reduces the permeability to Ca in several excitable membranes, it is suggested that the mechanical threshold depends on the entry of Ca to the muscle.

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INTRODUCTION

The essential role of Ca in muscle contraction was definitely established by Heilbrunn & Wiercinski (1947) with the demonstration that this cation induced localized tension when injected intracellularly in frog single muscle fibres. These authors suggested that contraction may result from an entry of Ca from the extracellular fluid during the excitation of the muscle. This idea was further extended by Bianchi & Shanes (1959) after their measurements of Ca uptake during potassium induced contractures. However, this suggested mechanism has been discounted on the basis of the quantitative analysis of these last results (Sandow, 1965). Thus, external Ca seems to be related to contraction only as a factor for maintaining electrical excitability of the muscle membrane.

Mn ions have been shown to reduce or block the movements of Ca across a variety of excitable membranes: those of barnacle and crayfish muscle (Hagiwara & Nakajima, 1966; Takeda, 1967; Chiarandini, Reuben, Girardier, Katz & Grundfest, 1970), heart muscle (Rougier, Vassort, Garnier, Gargouil & Coraboeuf, 1969) and squid giant axon (Katz & Miledi, 1969; Baker, Meves & Ridgeway, 1971).

In the present study, this pharmacological property of Mn was used to explore a possible participation of Ca movements across the sarcolemma during muscle contraction. A preliminary report of some of these results has been published (Chiarandini & Stefani, 1971).

METHODS

Experiments were performed at room temperature (20–25 °C) on sartorius and tibialis anticus longus (T.A.L.) muscles of the South American frog *Leptodactylus ocellatus*. Frogs were kept at room temperature. The experiments were carried out between August 1970 and May 1971.

Intracellular recording and stimulation. The membrane potential was differentially measured between two micropipettes filled with 3 M-KCl, one placed intracellularly and the other in the bath. The micropipettes were connected via 3 M-KCl bridges to calomel half-cells and hence to the two input grids of a differential cathode follower with an input capacity of about 1 pF. The resistance of the selected recording micropipettes was 15–30 MΩ, and their tip potentials −5 mV or less. The reference micropipette had its tip broken under microscopic control to avoid any tip potential.

For measuring the effective resistance between the inside and the outside of the fibre, a second micropipette was placed intracellularly 50–100 μm from the recording one. The cells were impaled in the central region of the muscle. DC and/or rectangular pulses of current were passed through this second micropipette, which was connected, via a chlorided silver wire, to a 50 or 100 MΩ resistance, leading to a square pulse generator in parallel to a DC voltage source.

The injected current was measured with an Ag-AgCl wire placed in the bath, connected to earth via a high input impedance operational amplifier in the ammeter

configuration. With a 1 MΩ resistor in the feed-back, a current of 1 nA gave an output of 1 mV. The signals were displayed on a dual beam oscilloscope and photographed.

Dissection of single fibres and mechanical recording. Twitch fibres were dissected from the internal head of the T.A.L. muscle. This muscle is convenient for isolating fibres because it has a comparatively small amount of connective tissue, and the fibres are shorter than those of other muscles commonly used. Flat bundles having tendons at each end were removed from the muscle with fine scissors and usually left resting for 2 hr at room temperature, or kept overnight at 4° C, before starting the dissection. The fibres were visualized under a dissecting microscope and dark-field illumination; at least two types of fibres could be distinguished by their optical appearance: transparent and grainy fibres. Transparent fibres were usually large and almost all of the single fibres used belonged to this group. A fine stainless-steel stimulating electrode was used to select fibres with propagated twitches for dissection. Fibres were isolated using fine forceps and knives made from stainless-steel razor blades and transferred to the experimental chamber with a stainless steel scoop.

The experimental chamber was 5 mm deep, 5 mm wide, and 20 mm long, and had an inlet to flush solutions with syringes, and an outlet to drain the chamber by gravity. The change of solutions was usually done by flushing 5 ml in 3–4 sec. A preliminary test with a dye solution showed that 1·5 sec after starting the flushing about 95 % of the solution in the chamber was replaced and that after flushing the whole volume, 98 % of the original solution was removed.

The fibres were horizontally mounted and had one tendon tied to an extension attached to the strain gauge (model VC-2 Statham) while the other was fixed to the bottom of the chamber. The fibres were stretched about 10 % of slack length and tension was isometrically recorded. The interval between K contractures was at least 10 min. The mechanical output was registered with a curvilinear pen recorder or oscilloscope.

Electrical stimulation and recording of the extracellular action potential. In most cases, fibres were stimulated along their entire length with two Pt wires connected to a stimulus isolation unit. The stimulus duration was 0·3 msec and the voltage was adjusted as needed for threshold and suprathreshold stimulation. When the extracellular action potential was recorded, the fibres were stimulated focally with two thin nichrome wires, insulated except at the tip. Extracellular action potentials were recorded with two nichrome wires. One was placed very close to the surface of the muscle fibre, opposite to the stimulated end, and the other, critically placed in the bath so as to reduce the stimulus artifact.

Solutions. The normal saline had the following composition (mM): 115 NaCl, 2·5 KCl, 1·8 $CaCl_2$. Solutions with a K concentration, $[K]_0$, above normal were prepared as described by Hodgkin & Horowicz (1959) with a constant [K].[Cl] product: 300 mM^2; they will be referred to as K solutions. On occasions, the NaCl of the normal saline was isotonically replaced by KCl (KCl solutions). Mn was added to Cl solutions as $MnCl_2$ and as $MnSO_4$ to the Cl-free and to the K solutions. The dissociation constant of $MnSO_4$ is $5{\cdot}2 \times 10^{-3}$ M (Robinson & Stokes, 1965) and a solution containing 95 mM-SO_4 and 10 mM-$MnSO_4$ has only about 1 mM of ionized Mn. Solutions were buffered to pH 7·3 with 2 mM Tris (hydroxymethyl) aminomethane chloride or sulphate according to the main anion of the fluid. The pH was controlled before each experiment.

RESULTS

Effects of Mn on electrical properties of muscle fibres

A. *Resting potential and effective resistance.* The addition of Mn to the saline increased the resting potential (E_{rp}) of the muscle fibres. The E_{rp} in the T.A.L. was $82{\cdot}6 \pm 0{\cdot}8$ mV (thirteen fibres, mean ± S.E.) and after equilibrating the muscle for 30 min in a saline with 10 mM-Mn, the E_{rp} was $-90{\cdot}0 \pm 0{\cdot}7$ mV in thirteen other fibres. This hyperpolarization is significant ($P < 0{\cdot}01$). Similarly, in sartorius muscles, the E_{rp} of $-93{\cdot}7 \pm 0{\cdot}5$ mV (twenty-three fibres) was changed to $-100{\cdot}2 \pm 0{\cdot}5$ mV in the presence of 20 mM-Mn (twenty fibres). This difference is highly significant ($P < 0{\cdot}001$).

The effective resistance (R_{eff}) was evaluated from small voltage deflexions when pulses of inward (hyperpolarizing) current of about 10 nA and 100 msec were passed through the cell membrane. The R_{eff} was measured in all fibres at a membrane potential fixed to -95 mV by passing DC current. The R_{eff} of twenty-four fibres in three T.A.L. muscles was 502 ± 41 kΩ. A second measurement, made 30 min after soaking the preparation in a saline with 10 mM-Mn, gave a value of 551 ± 63 kΩ for another twenty-four fibres. The difference between the two values is not statistically significant.

B. *K-induced depolarization.* The first step of the excitation-contraction (E–C) coupling involves a membrane depolarization (Kuffler, 1946; Sandow, 1965); therefore it was important to study whether Mn alters the ability of potassium to depolarize the membrane.

For these experiments small bundles of fibres from T.A.L. muscles were used. The muscle fibres were exposed initially to solutions with different [K] and a constant [K].[Cl] product, and the E_{rp} measured about 90 sec after the change of solutions. After each measurement the preparation was soaked in normal saline for 10 min before another K solution was applied. Once these measurements were completed, the bundle was soaked for 10 min in a normal saline with 10 mM-Mn added. Thereafter, K salines with 10 mM-Mn were applied and the E_{rp} again recorded. Table 1 clearly shows that Mn did not modify the magnitude of the depolarizations induced with elevated $[K]_o$.

In other experiments in which the fibres were depolarized by increasing $[K]_o$ but keeping constant the [Cl] of the saline, it was found that Mn reduced the K-induced depolarizations. The E_{rp} in presence of 117·5 mM-KCl was $-21{\cdot}2 \times 1{\cdot}8$ mV (five fibres). After the fibres were repolarized in normal saline, the KCl solution with 10 mM-Mn added was applied. The E_{rp} recorded in the same fibres was $-29{\cdot}8 \pm 2{\cdot}8$ mV. The difference of $-8{\cdot}6 \pm 2{\cdot}0$ mV is significant for paired data ($P < 0{\cdot}01$). In this last

experiment the E_{rp} depends on the membrane conductances to K (g_K) and to Cl (g_{Cl}) (Hodgkin & Horowicz, 1959) and the effect of Mn could be explained by assuming that Mn reduces g_K, increases g_{Cl}, or affects both.

C. *Potassium conductance.* The possibility that Mn indeed reduces g_K is shown by the increase in membrane resistance produced by Mn in two sartorius muscles equilibrated in isotonic K_2SO_4. In this ionic condition the R_{eff} is an estimate of g_K (Katz, 1949). The R_{eff} after soaking the muscles in 95 mM-K_2SO_4 (solution H, Hodgkin & Horowicz, 1959), for 45 min was 319 ± 12 kΩ (thirty-five fibres) and increased to 468 ± 24 kΩ (thirty-five fibres) after adding 10 mM-Mn and allowing 30 min for equilibration. The difference between the values is significant ($P < 0{\cdot}001$).

TABLE 1. Effect of 10 mM-Mn on K-induced depolarization with solutions having a constant [K].[Cl] product of 300 mM²

External K concentration (mM)	Resting potential (mV)	
	Control	10 mM-Mn added
2·5	−87·2 ± 0·8 (8)	−91·5 ± 0·5 (8)
10	−61·9 ± 0·4 (4)	−62·4 ± 0·9 (4)
20	−48·4 ± 0·6 (4)	−50·0 ± 0·4 (4)
30	−34·7 ± 0·7 (8)	−36·8 ± 0·9 (7)
75	−13·7 ± 0·6 (8)	−13·2 ± 0·3 (8)

Data from two bundles from two T.A.L. muscles. Values are given as mean ± S.E.; figure between parentheses is number of examined fibres. The difference between the paired sets of values is significant only when $[K]_0 = 2{\cdot}5$ mM.

D. *Action potential.* The twitch amplitude depends on the amplitude and duration of the action potential (Sandow, Taylor & Preiser, 1965), therefore it became necessary to analyse possible actions of Mn on the action potentials of the muscle fibres. Fig. 1 shows intracellularly recorded action potentials from two sartorius muscles (records *A* to *D* and *D* and *F*). The action potentials shown in *A* and *E* were obtained in normal saline, in *B*, *C* and *D* with 10 mM-Mn added, and in *F* with 20 mM-Mn added. It can be seen that the afterpotential is prolonged and increased by Mn, starting at a more positive membrane potential. The records at faster sweep speed obtained in the second muscle (*E* and *F*) show that the duration of the spike is slightly prolonged by Mn. Note that the amplitude of the overshoot is unaffected.

The electrical threshold for the action potential was shifted to a more positive voltage by Mn. This was determined with stepwise depolarizations of the fibres with intracellular current pulses until an action potential was elicited. The voltage at the inflexion point where the action potential started was considered as the electrical threshold. The effects of Mn on

threshold, overshoot, duration of the spike, and value of membrane potential at the beginning of the negative after-potential are summarized in Table 2.

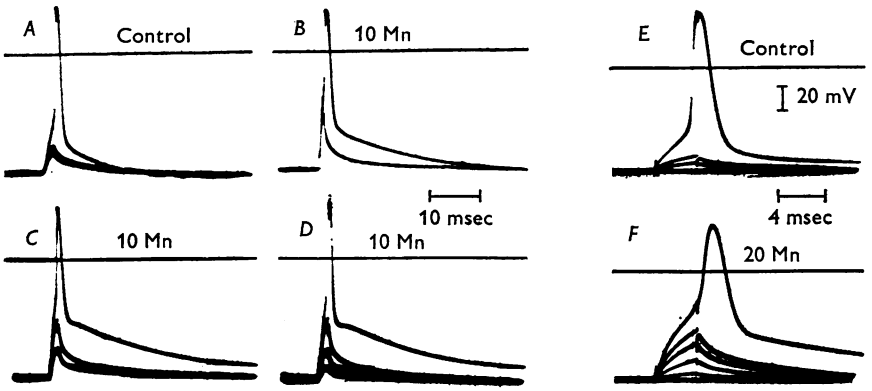

Fig. 1. Effects of Mn on intracellularly recorded action potentials. Depolarizing current pulses were delivered intracellularly until an action potential was elicited. *A*, control action potential. *B*, *C* and *D*, action potentials in the presence of 10 mM-Mn in three other fibres from the same sartorius. *E*, control action potential at a faster sweep speed in another sartorius. *F*, action potential in another fibre of the same muscle, under 20 mM-Mn. Notice the marked increase and prolongation of the negative after-potential and the increase in electrical threshold (*F*).

TABLE 2. Effects of Mn^{2+} on the action potential

	Threshold (mV)	Overshoot (mV)	Duration at 0 mV membrane potential (msec)	Voltage at the beginning of the negative after-potential (mV)
Control	$-49{\cdot}0 \pm 0{\cdot}7$ (17)	$41{\cdot}0 \pm 1{\cdot}3$ (21)	$0{\cdot}95 \pm 0{\cdot}02$ (10)	$-64{\cdot}0 \pm 0{\cdot}8$ (21)
10 mM-Mn	$-39{\cdot}0 \pm 1{\cdot}8$ (9)	$37{\cdot}0 \pm 1{\cdot}4$ (21)	$1{\cdot}10 \pm 0{\cdot}03$ (8)	$-50{\cdot}0 \pm 0{\cdot}8$ (19)
	$P < 0{\cdot}001$	$P > 0{\cdot}05$	$P < 0{\cdot}01$	$P < 0{\cdot}001$

Data from two sartorius muscles. Values are given as mean ± S.E.; figure between parentheses is number of examined fibres.

Effects on mechanical responses

A. *Twitch and tetanus.* Mn at concentration of 5 mM or above reduced the twitch amplitude. Since 10 mM-Mn produced an evident effect, this concentration was preferred. In twelve single muscle fibres, 10 mM-Mn reduced the twitch amplitude by $45{\cdot}0 \pm 6{\cdot}7$ % (mean ± S.E.). This inhibition of the twitch amplitude is not related to the increase in the osmolarity of the saline due to the addition of the $MnCl_2$. In five fibres a control was

performed finding that 20 or 30 mM-NaCl added to the saline had no effect on the amplitude of the twitch.

Fig. 2 shows the effect and velocity of action of 7·5 and 10 mM-Mn tested on the same single muscle fibre. For both concentrations of Mn the reduction of the twitch amplitude was maximal within less than 8 sec after the application of the agent. The depressant action of Mn is reversed quickly after returning to normal saline.

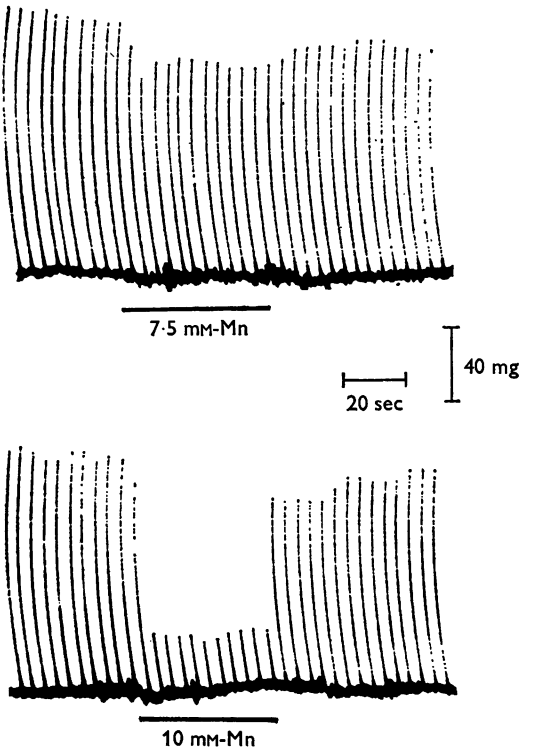

Fig. 2. Action of Mn on twitches in a single muscle fibre. Twitches evoked by extracellular stimulation at a frequency of 1 every 4 sec. *Upper record*: the application of a saline with 7·5 mM-Mn (continuous line) somewhat reduced the amplitude of the twitch. *Bottom record*: same fibre with 10 mM-Mn (continuous line), the twitch was markedly reduced in about 8 sec. Upon returning the fibre to control saline the twitch recovered the amplitude.

To have direct evidence that modifications of the action potential were not the basis of the effect of Mn, mechanical and electrical events were simultaneously recorded in few single fibres. Propagated extracellular spikes were recorded at one end of the fibre, while the cell was stimulated at the other end. Fig. 3 illustrates the results of one of these experiments.

The left row shows the twitch in control saline (*a*), in the presence of 10 mM Mn (*b*), and after washing out the Mn (*c*). The right row shows the extracellular spikes in the same fibre in control saline (*a*), with 10 mM-Mn added (*b*), and after washing out (*c*). It can be seen that Mn reduces the twitch without appreciably changing its time course.

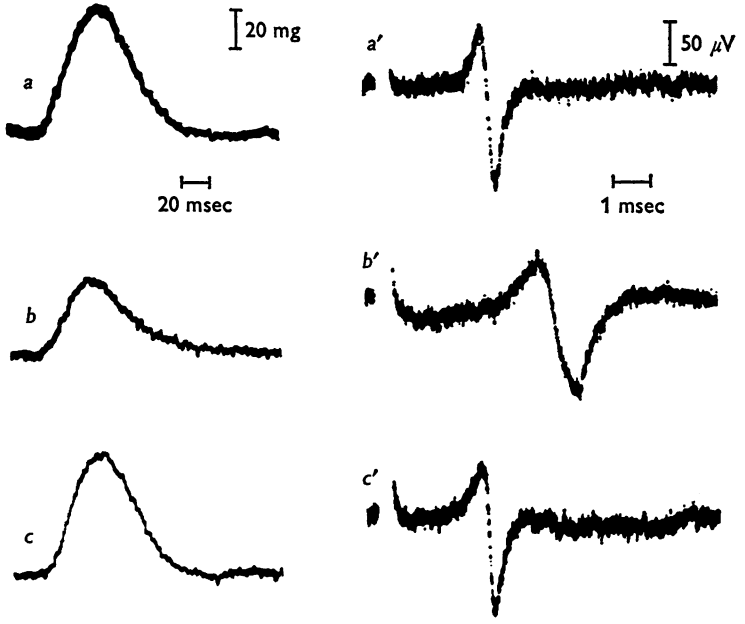

Fig. 3. Extracellular action potentials and twitches in a single muscle fibre. *Left row*: oscilloscope recording of the twitches: *a*: control; *b*: 10 mM-Mn added and *c*: recovery in control saline. *Right row*: extracellular record of propagated action potentials: *a'*: control; *b'*: 10 mM Mn and *c'*: after returning to control saline. The effect of Mn was first studied on the twitches then the sequence was repeated recording the action potentials extracellularly, and monitoring the twitch amplitude with a pen recorder.

The delay between the stimulus artifact and the spike is prolonged by 30–40 %. The extracellular spike duration is also increased while the positive (upward) and negative deflexions are somewhat reduced. Similar results were obtained in another two single fibres.

The effect of 10 mM-Mn on tetanus amplitude was studied in six single fibres. Stimulation frequencies of 50, 75 and 125 Hz were used during 0·5 sec. In two cells no change was detected on the tetanus amplitude, while the twitch tension was reduced by 37 %. In the other four fibres 10 mM-Mn reduced the tetanus amplitude by 10, 16, 16 and 42 %.

B. *K-induced contractures.* The effect of Mn on K contractures was studied in ten single fibres. Contractures were consistently depressed or

blocked. However, the blocking effect of Mn could be overcome by increasing the K concentration. Fig. 4 illustrates contractures induced by 40, 75 and 190 mM-K, in the absence and presence of 10 mM-Mn in the same single muscle fibre. Mn was added to the control saline about 1 min before flushing the K solution which also contained Mn. The control and test contractures were obtained in a random sequence. It can be seen that

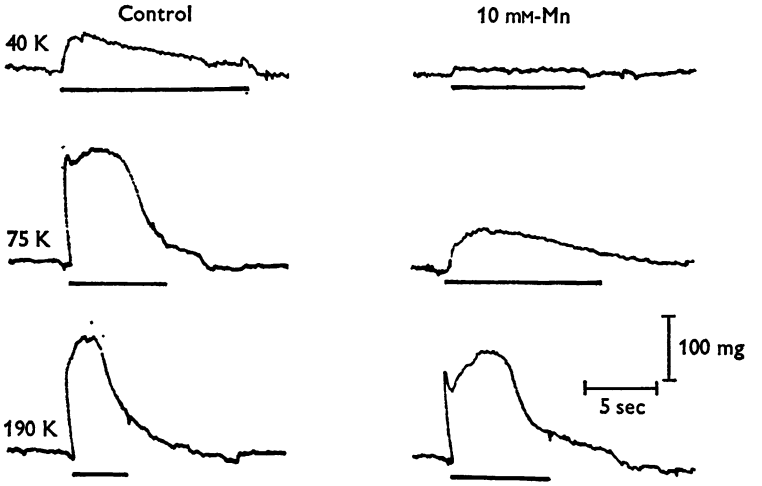

Fig. 4. Effect of Mn on K contractures in a single muscle fibre (diameter 89 μm). K contractures were induced with solutions having a constant [K].[Cl] product. A saline with 10 mM-Mn was flushed one minute before eliciting tension. The fibre was alternately exposed for 10–15 sec (continous lines) to the different K salines without and with 10 mM-Mn. The interval between contractures was 10–15 min. Mn reduces 40 and 75 mM-K tensions and slows down the contractures induced with 190 mM-K. The effect was reversible.

40 and 75 mM-K contractures were reduced; the effect was more pronounced with the lowest concentration. In five single fibres, 75 mM-K contractures were reduced by 73 ± 4 % (mean $\pm$ S.E.) with 10 mM-Mn. The amplitude of the 190 K contractures was practically not affected (Figs. 4–6). It should be noted that the real concentration of ionized Mn during the K contractures is uncertain. The K solutions with 10 mM-Mn have only about 1mM ionized Mn (see Methods). Therefore, during the contracture the concentration of ionized Mn at the external surface of the fibre should be between 1 and 10 mM while inside the T-system it should be close to 10 mM, and would decay with the filling of the tubules with the K saline.

Besides reducing the amplitude of the contractures, Mn changed their time course. This is evident in the contractures elicited with 190 mM-K

shown in Figs. 4 and 5, where the time courses can be compared since their amplitudes were practically identical.

The relationship between tension amplitude and different $[K]_o$ was studied in three single fibres in control saline and with 10 mM-Mn added. Fig. 6 illustrates the results of two fibres. Mn shifted the curve tension *vs.* log $[K]_o$ towards the right, changing the minimal $[K]_o$ to produce a detectable tension from 20 mM to about 35 mM. According to the E_{rp} values reported in Table 1, this represents a change of the mechanical threshold from -48 to -33 mV.

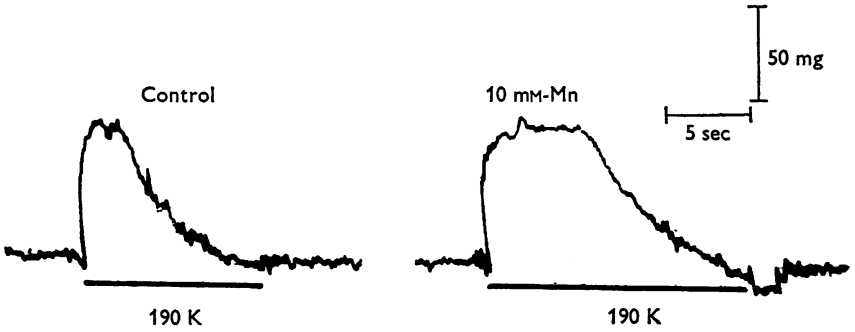

Fig. 5. Modification on the time course of a supramaximal K contracture by Mn fibre (diameter 72 μm). Left record: control 190 mM-K contracture. Right record: 15 min later the fibre was exposed for 1 min to 10 mM-Mn and then to 190 mM-K solution wth 10 mM-Mn. The amplitude of the tension was similar to the control but subsided more slowly.

The reported data shows that 10 mM-Mn is less effective on the twitch than on the submaximal K contractures. As indicated, twitches and 75 mM-K contractures were diminished by 45 and 73 % respectively. To analyse this point further, the blocking action of different Mn concentrations on the amplitudes of the twitch and of the 75 mM-K contracture was studied in the same fibre. In Fig. 7 it can be seen that 2·5 mM-Mn reduced the K contracture to a great extent, 20 mM-Mn practically abolished the K tension but the twitch was reduced by 35 %. Similar findings were obtained in a second fibre.

C. *Caffeine-induced contractures.* The results reported in previous sections showed that Mn reduced the tension output in fibres electrically activated by action potential or K depolarization. In both cases, Mn may be acting at various levels. For example, it could be reducing the efficiency of coupling between the membrane depolarization and the increase of myoplasmic Ca concentration, responsible for tension development (Heilbrunn & Wiercinski, 1947; Niedergerke, 1955). Mn could be reducing as well the release of Ca from the sarcoplasmic reticulum (SR) or the sensiti-

vity of contractile proteins towards Ca. To analyse these possibilities, we examined the effects of Mn on caffeine contractures.

There is good evidence that this alkaloid induces tension by a movement of Ca from the SR into the myoplasm (Weber & Herz, 1968) without

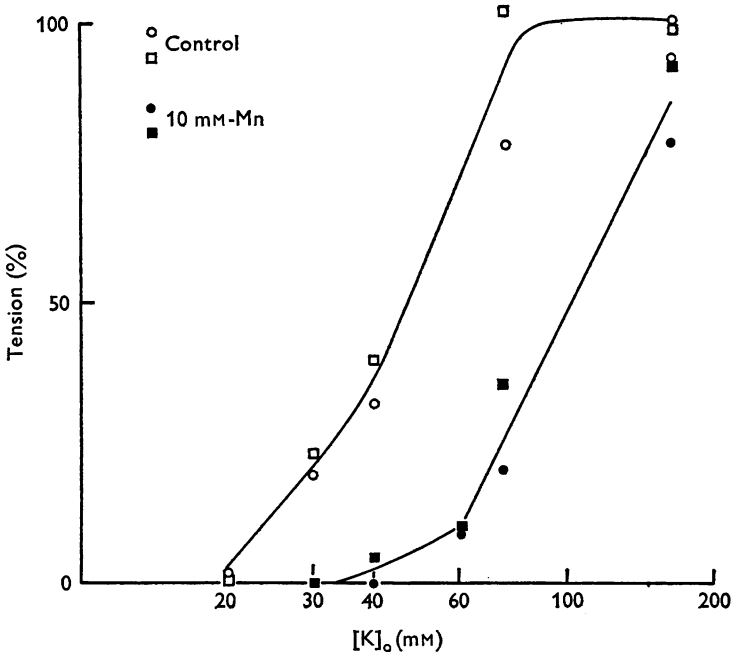

Fig. 6. Effect of Mn on the curve relating tension output *vs.* logarithm of $[K]_o$. Results from two fibres. Tensions were induced with solutions having a constant [K].[Cl] product. The fibre was alternately exposed to solutions with different K concentrations and without or with 10 mM-Mn. Mn was added one minute before each contracture. *Open symbols:* control contractures. *Filled symbols*: effect of 10 mM-Mn. Mn shifts the curve towards the right increasing the mechanical threshold. Control 190 mM-K tensions were about 2·5 kg/cm^2 in both fibres.

involving the E–C coupling step (Axelsson & Thesleff, 1958; Lüttgau & Oetliker, 1968). In three fibres, 10 mM-Mn did not affect submaximal caffeine contractures (3–4 mM) even after pre-soaking the fibres for 17 min in the solution with Mn (Fig. 8).

These results show that Mn is not modifying the mobilization of Ca from the SR or the sensitivity of contractile proteins towards Ca, and that probably Mn is exerting its action on the E–C coupling step. These results are in keeping with the data obtained in crayfish muscle fibres (Zachar & Zacharova, 1968; Chiarandini *et al.* 1970).

DISCUSSION

Effects of Mn on the electrical properties of muscle fibres

In general, the effects of Mn on the electrical properties of frog muscle fibres are similar to those reported when the extracellular concentration of Ca, $[Ca]_o$, is increased. Mn hyperpolarized the fibres to E_{rp} values close

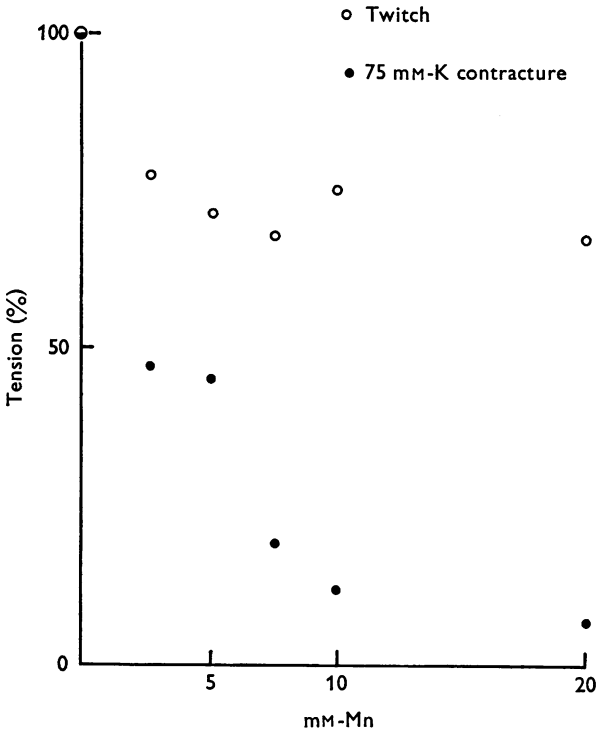

Fig. 7. Effect of different Mn concentrations on twitch and 75 mM-K contracture in the same single fibre. Twitch tension: 45 mg; K contracture: 115 mg. The blocking effect of Mn is more evident on the K contracture. It should be remembered that the ionized Mn concentrations in the K salines are lower than those indicated in the plot, as explained in Methods.

to the equilibrium potential for K (Adrian, 1956; Hodgkin & Horowicz, 1959). A similar effect on the E_{rp} has been found when raising $[Ca]_o$ (Jenerick & Gerard, 1953). This last effect is due to a reduction of the resting permeability to Na (Adrian & Freygang, 1962) and very likely, Mn acts in a similar way.

Mn increases R_{eff} when the muscle is soaked in isotonic K_2SO_4 solution, a condition in which practically all the current across the membrane is carried by potassium, and R_{eff} gives an estimate of g_K (Katz, 1949).

However, in muscles bathed with control saline, no significant increase in R_{eff} was detected in the presence of Mn. This fact is probably related to the marked variability of R_{eff} from fibre to fibre and to the existence of the g_{Cl} shunt in the muscle membrane that might mask a decrease in g_K (Hutter & Noble, 1960). For instance, if it is assumed that in normal saline the reduction of g_K by Mn is about 50 %, as happened in the experiments performed in isotonic K_2SO_4 solution, and that g_K accounts for

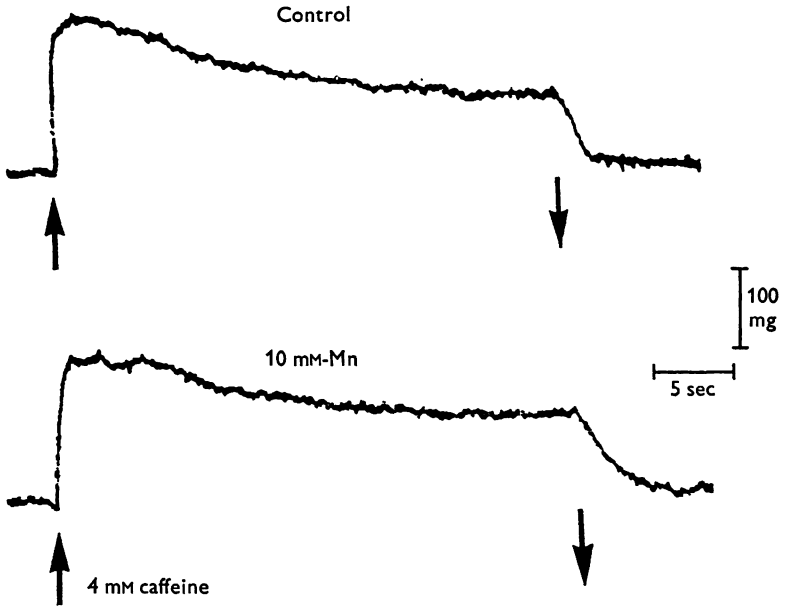

Fig. 8. Action of Mn on caffeine contractures in a single muscle fibre (diameter 67 μm). *Upper record*: control tension evoked by 4 mM caffeine. *Bottom record*: the fibre was exposed before the contracture to 10 mM-Mn for 17 min. Mn did not affect the tension induced by the submaximal concentration of caffeine.

50 % of the membrane conductance, the reduction of g_K due to Mn would be reflected by an increase of R_{eff} of about 15 %. Such an increment is not easily detected with the technique employed in the present study.

The reduction of g_K by Mn can also explain the reduced effectiveness of high K to depolarize the muscle fibres when the Cl concentration was kept constant. In this case the E_{rp}, immediately after the solution change, depends on the ratio g_K/g_{Cl} (Hodgkin & Horowicz, 1959) and Mn, by altering this ratio, would make the membrane potential less sensitive to changes in $[K]_o$.

The action potential is prolonged by about 15 % and the electrical threshold is markedly increased by Mn. Increasing $[Ca]_o$ has similar

effects in muscle and squid giant axon (Ishiko & Sato, 1957; Frankenhaeuser & Hodgkin, 1957; Frankenhaeuser & Lännergren, 1967).

Mn greatly enhances the amplitude of the negative after-potential. Recent experiments have shown that in the beginning of the negative after-potential the ratio P_{Na}/P_K is 1/30 (Adrian, Chandler & Hodgkin, 1970). The results obtained with Mn could be explained by a modification of this permeability ratio or by a delay and reduction of the potassium activation during the action potential. This could be correlated with the well known action of Ca on g_K in the squid axon (Frankenhaeuser & Hodgkin, 1957).

Effects of Mn on the mechanical responses

Mn reduces both K contractures and twitch responses in frog muscle. A similar effect on K or current-induced tension was observed in crustacean muscle (Orkand, 1962; Zachar & Zacharova, 1968). The effects on the twitch are not related to modifications of the action potential. A possible effect of Mn related to the slower conduction velocity of the action potential is discarded since in most experiments the fibres were stimulated along the whole length avoiding propagation delays.

Near maximal K contractures are more sensitive to Mn than are twitches. Contractures elicited by 75 mM-K, which gives an almost maximal tension are reduced by 10 mM-Mn by about 75 %, while the twitches are reduced only by about 45 %. This difference is even more evident in the experiment of Fig. 7, and it is even more pronounced considering that the K solutions have a lower concentration of ionized Mn (see Methods). One possible explanation for the discrepancy is that the blocking effect on the twitch is counteracted by the prolonged spike which should increase the mechanical output (Sandow *et al.* 1965; Taylor *et al.* 1969), However, the possibility cannot be ruled out that the processes underlying the two mechanical responses are partially different, and that that of the K contracture is more sensitive to Mn.

Mn very rapidly affected the mechanical responses of the fibres. The effect was maximal in less than 8 sec suggesting that Mn acts on the surface and transverse tubular membranes and not intracellularly. This is also supported by the fact that the depressant effect of Mn is rapidly reversed when the agent is washed out. The effect of increasing $[Ca]_o$ on K contractures and twitches has a similar time course (Lüttgau, 1963; Frankenhaeuser & Lännergren, 1967).

The insensitivity of caffeine contractures to Mn clearly indicates that Mn is not altering the contractile proteins, or the Ca release from the SR, and suggests that Mn acts at some early stage of the process of contraction, probably at the coupling between the electrical events and the

release of Ca from the SR (see Sandow, 1965, 1970). This point of view is supported by the fact that the mechanical threshold measured for K contractures was shifted to a more depolarized level, and by the observation that the maximal activations of muscle (tetanus and 190 mM-K contracture) were slightly reduced or unchanged by the agent. The change of the threshold could explain the reduction of the mechanical responses since they depend on the mechanical threshold (Taylor *et al.* 1969). Similar effects on the mechanical threshold have been reported with elevated $[Ca]_o$ in twitch fibres (Luttgau, 1963; Costantin, 1968) and in the presence of Mn for K contractures in frog rectus muscle (Edwards & Lorković, 1967).

A possible mode of action of Mn

The reported effects on tension development may be explained by a possible 'stabilizing' action of Mn on the muscle membrane. Mn, being a divalent cation, could be adsorbed to the walls of the T-system and muscle surface and by neutralizing negative fixed charges, set up a local positive potential. This would increase the electric field within the membrane, and result in a shift of the mechanical threshold that could account for the observed reduction of twitches and K contractures.

Another possibility is that the effects of Mn are related to a reduction or block of the membrane permeability to Ca. Mn has been shown to reduce the permeability to Ca in a large variety of excitable cells. Its blocking action on Ca permeability is indicated by the reduction of the inward Ca current in barnacle muscle (Hagiwara, 1966), as well as by the reduction of the spike overshoot in snail neurones (Meves, 1968) and in barnacle and crayfish muscle (Hagiwara & Nakajima, 1966; Takeda, 1967). The voltage clamp data in barnacle muscle show that, in that preparation, the effect of Mn is specifically on the permeability to Ca (Hagiwara, 1966). In crayfish striated muscle, Mn seems to also reduce a resting membrane permeability to Ca, related to the supply of Ca to the SR (Chiarandini *et al.* 1970). The inward Ca current during the action potential in frog atrial muscle is also reduced by Mn (Rougier *et al.* 1969). Moreover, in rabbit atrial muscle Mn reduces the uptake of ^{45}Ca (Sabatini-Smith & Holland, 1969). Furthermore, the Ca-dependent depolarizing response in the presynaptic terminal of the giant synapse of the squid and the Ca entry associated with K depolarization in squid axon are blocked by Mn (Katz & Miledi, 1969; Baker *et al.* 1971).

The foregoing data indicate that Mn might be acting on frog muscle also as a blocker of Ca permeability and suggest that the observed modification of the mechanical threshold may be due to this pharmacological property of Mn. Frog muscle is permeable to Ca. A Ca uptake has been

shown to exist at rest and to be enhanced during twitch and K contractures (Bianchi & Shanes, 1959; Winegrad, 1970). If the mechanical threshold depends on a certain Ca permeability, there should be a relationship between mechanical threshold and Ca entry. Nitrate has been shown to increase the ^{45}Ca uptake in frog muscle (Bianchi & Shanes, 1959; Weiss & Bianchi, 1965) and to reduce the mechanical threshold (Hodgkin & Horowicz 1960). Caffeine at subthreshold concentrations increases the ^{45}Ca uptake (Bianchi, 1961) and reduces the mechanical threshold (Sandow, Taylor, Isaacson & Seguin, 1964; Heistracher & Hunt, 1969). The reduction of ionic strength also increases Ca entry and lowers the mechanical threshold (Lorković, 1967). In keeping with our hypothesis, tetracaine reduces Ca uptake and increases the threshold for mechanical activation (Feinstein, 1963; Lüttgau & Oetliker, 1968). In conclusion, these observations support our suggestion that the mechanical threshold and the magnitude of the Ca entry are closely related. Mn, a Ca permeability blocker, would shift the threshold to more depolarized levels of membrane potential by reducing the ability of Ca to move across the sarcolemma.

There is evidence that membrane depolarization in excitable cells leads to an enhanced calcium entry that most likely reflects a voltage-dependent increase of calcium permeability (Hodgkin & Keynes, 1957; Fatt & Ginsborg, 1958; Bianchi & Shanes, 1959; Kusano, Livengood & Werman, 1967; Katz & Miledi, 1969; Baker, Hodgkin & Ridgway, 1970; Baker *et al.* 1971). Mn may act on the mechanical threshold by shifting a hypothetical curve relating Ca permeability to membrane potential, towards more positive values of membrane potential. The effect of raising $[Ca]_o$ on the mechanical threshold (Lüttgau, 1963; Constantin, 1968) could be explained by this hypothesis.

Bianchi & Bolton (1967) proposed a scheme for the E–C coupling in which a Ca release from the transverse tubules during the activation of the muscle will trigger a secondary release of Ca from the SR. The Ca thus released would be the activator of the contractile proteins. The concept of a secondary release of Ca is supported by recent evidence. Electrophysiological experiments have shown that the kinetics of activation of the contraction can be explained by postulating a regenerative system (Adrian *et al.* 1969). Ford & Podolsky (1970) and Endo, Tanaka & Ogawa (1970) have reported in frog skinned muscle fibres that the free myoplasmic Ca itself induced the release of Ca from the SR. A similar regenerative mechanism may exist in crayfish muscle fibres since intracellular injections of Ca produce local contractures, enhanced by caffeine and that could propagate (Reuben, Brandt, Katz & Grundfest, 1970).

The possible physiological role of a Ca influx triggering a secondary release of Ca from the SR has been emphasized recently by Ford &

Podolsky (1972). It is supported by the finding that a $[Ca]_o$ of 10^{-9} M, a condition that will decrease the Ca entry, is capable of reducing considerably the amplitude of the K contractures in fibres whose E_{rp} was kept normal by the addition of Mg (E. Stefani & D. J. Chiarandini, unpublished results). However, a recent report by Armstrong, Bezanilla & Horowicz (1972) shows that with a similar low $[Ca]_o$ frog muscle fibres are capable of twitching. Some reasons for this divergent result will be discussed in a future publication.

The possible role of a Ca entry in E–C coupling and the relationship between mechanical threshold and the magnitude of the Ca entry suggest an explanation for the effect of Mn. Mn might reduce the Ca entry during the activation of the muscle by the action potential or by increasing $[K]_o$. This decrease in Ca entry, reflected as a change in the mechanical threshold, would in turn reduce the hypothetical secondary release of Ca from the SR and, as a consequence, the mechanical output.

The authors thank Dr Orentlicher for his helpful comments on the manuscript. Dr Chiarandini and Dr Stefani are Investigators of the Consejo Nacional de Investigaciones Científicas y Técnicas de Argentina (CONICET). The work was supported by grants from the CONICET and from the USPHS (ROI-NS 06953-03 NEUA).

While this paper was in preparation, a study by Ota, I, Tahanji, M. & Magai, T. (1972), *Effects of manganese ions on excitation-contraction coupling in frog sartorius muscle*, *Jap. J. Physiol.* **22**, 379–392, was published.

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