Effects of cobalt, magnesium, and cadmium on contraction of rat soleus muscle

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ABSTRACT The effects on isometric tension of three divalent ions that block calcium channels, magnesium, cobalt, and cadmium, were tested in small bundles of rat soleus fibers. Cobalt, at a concentration of 2 or 6 mM, reversibly depressed twitch and tetanic tension and the depression was much greater in solutions containing no added calcium ions. Magnesium caused much less depression of tension than cobalt. The depression of tension was not accompanied by membrane depolarization or a reduction in the amplitude of action potentials. A reduction caused by 6 mM cobalt in the amplitude of 40 or 80 mM potassium contractures was not accompanied by a comparable reduction in tension during 200 mM potassium contractures, and could be explained by a shift in the potassium contracture tension-voltage curve to more positive potentials (by +7 mV on average). Similar effects were not seen with 2 or 6 mM magnesium. At a concentration of 20 mM, both cobalt and magnesium depressed twitch and tetanic tension, cobalt having greater effect than magnesium. Both ions shifted the potassium contracture tension-voltage curve to the right by +5 to +10 mV, caused a small depression of maximum tension, and slowed the time course of potassium contractures. Cadmium (3 mM) depressed twitch, tetanic, and potassium contracture tension by more than 6 mM cobalt, but experiments were complicated by the gradual appearance of large contractures that became even larger, and sometimes oscillatory, when the solution containing cadmium was washed out. It was concluded that divalent cations affect both activation and inactivation of tension in a manner that cannot be completely explained by a change in surface charge.

INTRODUCTION

The nature of the signal linking depolarization of the membrane of the transverse (T-) tubules in skeletal muscle with calcium release from the sarcoplasmic reticulum is unknown. Recently evidence has been accumulating in favor of the idea that a calcium channel–like protein in the T-tubule membrane may act as the voltage sensor for contraction (Hui et al., 1984; Eisenberg et al., 1983; Lamb, 1985; Rios et al., 1986; Luttgau et al., 1987; Lamb and Walsh, 1987; Rios and Brum, 1987; Dulhunty and Gage, 1988; Brum et al., 1988; Tanabe et al., 1988). The T-tubule membrane is rich in dihydropyridine binding sites (Schwartz et al., 1985), many of which can form functional calcium channels (Flockerzi et al., 1986) that carry the T-tubule calcium current (Sanchez and Stefani, 1978; Almers et al., 1985). This calcium current is not itself essential for mechanical activation because contraction continues when the extracellular solution contains no calcium ions (Armstrong et al., 1972; Miledi et al., 1984) or when calcium currents across the surface membrane are blocked by divalent cations or dihydropyridines (Almers and Palade, 1981; Caputo, 1981; Lorkovic and Rudel, 1983; McClesky, 1985; Gallant and Goettl, 1985; Lamb, 1986; Luttgau et al., 1987; Rakowski et al., 1987; Dulhunty and Gage, 1988). However dihydropyridines, which are thought to bind specifically to calcium channels, can modify contraction (Gallant and Goettl, 1985; Alvira-Sakar et al., 1986; Rakowski et al., 1987; Dulhunty and Gage, 1988). Furthermore, changes in extracellular calcium ion concentration can also influence excitation-contraction coupling (Luttgau and Spiecker, 1979; Cota and Stefani, 1981; Dulhunty and Gage, 1988). It was therefore of interest to investigate whether other divalent cations which interact with calcium channels in muscle (Almers and Palade, 1981) can alter excitation-contraction coupling.

Some previous studies have indicated that in amphibian muscle divalent cations alter the voltage dependence of tension by neutralizing fixed negative charges on the extracellular surface of the cell membrane (Caputo, 1981; Lorkovic and Rudel, 1983; Dorrscheidt-Kafer, 1976; Bolanos et al., 1986) and Kostias et al. (1986) have reported that contraction in mammalian muscle is depressed by cobalt ions. We have extended these studies by examining the effects of magnesium, cobalt, and cadmium on contraction in rat soleus muscle.

METHODS

Preparation

Soleus muscles were dissected from 250–300-g male Wistar rats, pinned out in a petri dish lined with Sylgard (Dow Corning Corp., Midland, MI) and dissected into small bundles containing 5–10 fibers.

0006-3495/89/07/1/14 $2.00

Stimulation and recording

The methods for eliciting and recording isometric twitches, tetanic contraction, and potassium contractures have been described in detail previously (Dulhunty and Gage, 1985). Briefly, a bundle of fibers was mounted in a small volume, rapid flow bath with a solution changeover time of <0.5 s. The fibers were electrically stimulated via massive platinum electrodes which extended along either side of the preparation. Twitches were elicited at 0.1 Hz with supramaximal 0.5-ms pulses. Tetanic contractions were produced with trains of stimuli at 100 Hz for the time necessary to establish a clear tension plateau, usually 0.8–1.5 s. Potassium contractures were produced by rapidly flowing through solutions containing raised potassium concentrations. Tension was continuously recorded via an Akers semiconductor transducer (model AE 875, SensoNor a.s., Horten, Norway) on a chart recorder (model 7402A, Hewlett-Packard Co., Palo Alto, CA) and selected twitches, tetanic contractions, or potassium contractures were stored on magnetic disk for later computer analysis. The frequency response of the chart recorder was 55 Hz for full-scale deflections and 125 Hz for 10-mm deflections, the usual amplitude of twitch responses. The experiments were done at 22.5 ± 0.5°C.

Solutions

All experiments, except where otherwise state, were done in solutions containing a low chloride concentration. This is necessary to allow a rapid change in membrane potential following a change in potassium concentration (Hodgkin and Horowicz, 1960; Dulhunty and Gage, 1985). The anion substitute used for chloride was sulphate because we have accumulated an extensive database using this anion. Where necessary, sucrose was used as an osmotic substitute to maintain a constant osmotic pressure. The compositions of the variety of solutions used are given in Table 1A–C. The free concentrations of the divalent cations have been calculated and listed in Table 1D. Solutions with various divalent cation concentrations are named in the text and in Table 1 according to the amount of the divalent cation to be tested that was added to the control solution.

Membrane potential and action potentials

Membrane potentials were recorded using glass microelectrodes (filled with 2.5 M KCl, with resistances of 4–7 MΩ) with conventional intracellular recording techniques. Small volume baths with rapid exchange times were used to change extracellular solutions during continuous recording and multiple penetration experiments. Intracellular action potentials were recorded with the voltage follower capacity neutralization adjusted for optimal response times. Depolarizing current pulses were injected into the fiber through a second microelectrode located several hundred micrometers from the recording electrode, using a constant current generator.

Resting membrane potentials were measured in control and high-potassium solutions in the absence and presence of magnesium, cobalt, and cadmium. Membrane potentials in 200 mM potassium solutions were usually between −3 and −4 mV. This is very close to the potential of −2.5 mV calculated using the Goldman, Hodgkin, and Katz equation for solutions with ion concentrations listed in Table 1 and other parameters previously determined for mammalian skeletal muscle (Dulhunty, 1979), i.e., $P_{Na}/P_K = 0.06$, $P_{Cl}/P_K = 4.5$ $[K]_i = 150$ mM, $[Na]_i = 40$ mM, and $[Cl]_i = 0.7$ mM (where P_x is the membrane permeability and $[x]_i$ is the internal concentration of ion x). The internal chloride concentration was calculated assuming (a) that chloride was in equilibrium with the resting membrane potential of −80 mV, because the effects of the chloride pump (Dulhunty, 1979) have not been established

TABLE 1A Composition of most commonly used control and magnesium- and cobalt-containing solutions

	Na_2SO_4	NaCl	K_2SO_4	KCl	$CaSO_4$	$MgSO_4$	$CoCl_2$	$CoSO_4$	Sucrose
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*
Low chloride series									
A Control	32.25	16	1.75	0	7.6	1	0	0	170
B Low Na	0	0	1.75	0	7.6	1	0	0	286
C 40K	80	0	12	16	7.6	1	0	0	0
D 200K	0	0	92	16	7.6	1	0	0	0
Calcium-free low chloride series									
E 6Mg	32.25	16	1.75	0	0	7	0	0	170
E1 6Mg 40K	80	0	12	16	0	7	0	0	0
E2 6Mg 200K	0	0	92	16	0	7	0	0	0
F 6Co	32.25	16	1.75	0	0	1	6	0	170
F1 6Co 40K	80	0	12	16	0	1	6	0	0
F2 6Co 200K	0	0	92	16	0	7	0	0	0
Calcium-containing low chloride series									
G 20Mg	32.25	16	1.75	0	7.6	20	0	0	130
G1 20Mg 40K	80	0	12	16	7.6	20	0	0	0
G2 20Mg 200K	0	0	92	16	7.6	20	0	0	0
H 20Co	23.25	16	1.75	0	7.6	0	0	20	130
H1 20Co 40K	80	0	12	16	7.6	0	0	20	0
H2 20Co 200K	0	0	92	16	7.6	0	0	20	0

Concentrations are in millimolar. N.B. 1. All solutions contained 11 mM glucose and 2mM TES ((*N*-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid) buffer adjusted to pH 7.4 with NaOH, unless specifically stated otherwise. N.B. 2. Low-sodium solutions with magnesium or cobalt were made in the same way as solution B, but with appropriate changes in cobalt and magnesium concentrations.

TABLE 1B Composition of solutions used less commonly than those listed in Table 1A

	Na_2SO_4	NaCl	K_2SO_4	KCl	$CaSO_4$	$CaCl_2$	$MgSO_4$	$MgCl_2$	$CoCl_2$	$CoSO_4$	Sucrose	TES	CaEGTA
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*
Low chloride, calcium-containing solution													
I 2Co	32.25	16	1.75	0	7.6	0	1	0	2	0	170	2	0
Low chloride, low calcium solution													
J Control	32.25	16	1.75	0	0	0	10	0	0	0	100	10	20
K 2Co	32.25	16	1.75	0	0	0	10	0	2	0	100	10	20
Chloride-containing solutions													
L Control	0	150	0	3.5	0	2.5	0	8	0	0	0	2	0
M 6Co	0	150	0	3.5	0	2.5	0	2	0	6	0	2	0
Chloride-containing, calcium-free solutions													
N Control	0	150	0	3.5	0	0	0	10.5	0	0	0	2	0
O 6Co	0	150	0	3.5	0	0	0	4.5	0	6	0	2	0

in low external chloride situations and (*b*) that the internal chloride concentration remains at that level at brief times after changing to a high-potassium solution.

RESULTS

Effects of cobalt and magnesium

Most experiments described here were done in low-chloride solutions (sulphate substituted for chloride) so that a rapid change in membrane potential could be obtained when extracellular potassium concentration was raised during potassium contractures (see Methods). Addition of cobalt sulphate to give total concentrations from 2 to 20 mM depressed twitch and tetanic tension within several minutes. The depression of twitches and tetanic contractions produced in a preparation by 2 mM cobalt sulphate (solution I) is illustrated in Fig. 1 *A*.

The depression of tension caused by 2 mM cobalt sulphate was even greater in solutions which contained no added calcium ions (solution K), as illustrated in Fig. 1 *B*. In this experiment, the 2.5 mM calcium sulphate was replaced with 10 mM magnesium sulphate (solution J) to prevent spontaneous twitching in the absence of calcium. This was found to cause a small, ~5% depression of tension.

These effects were not dependent on the presence of sulphate rather than chloride ions in solutions. Depression of tension caused by 6 mM cobalt in a chloride-containing solution (solution M) is illustrated in Fig. 1 *C*. Again, the depression of tension caused by the cobalt was even greater in a calcium-free solution (solution O) (Fig. 1 *D*).

The rate of depression of tension caused by cobalt was very rapid, reaching a steady level within 3 min. Furthermore, recovery of tension following washout of cobalt was also very rapid and not slower following longer exposures. In one preparation in which the absolute tension in normal solution remained constant (±5%), the rate of recovery of tension after exposure to 20 mM cobalt

TABLE 1C Composition of cadmium-containing solutions

	Na_2SO_4	NaCl	K_2SO_4	KCl	$CaCl_2$	$MgSO_4$	$MgCl_2$	$CdCl_2$	Sucrose
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*
Low chloride, calcium-free solutions									
P Control	32.25	16	1.75	0	0	9	0	0	170
Q 3Cd	32.25	16	1.75	0	0	4	0	3	170
R 200K	0	0	92	16	0	9	0	0	0
S 3Cd 200K	0	0	92	16	0	4	0	3	0
Chloride- and calcium containing solutions									
T Control	0	150	0	2	2.5	0	5	0	0
U 3Cd	0	150	0	2	2.5	0	2	3	0
Chloride-containing, calcium free solutions									
V Control	0	150	0	2	0	0	7.5	0	0
W 3Cd	0	150	0	2	0	0	4.5	3	0

TABLE 1D Calculated free concentrations of divalent cations in sulphate solutions

Solution	Ca^{2+}	Mg^{2+}	Co^{2+}	Cd^{2+}
	mM	*mM*	*mM*	*mM*
A	1.20	0.19	—	—
B	3.50	0.50	—	—
C	0.68	0.10	—	—
D	0.75	0.12	—	—
E	—	1.30	—	—
F	—	0.22	0.94	—
G	1.13	3.46	—	—
H	1.15	—	2.49	—
I	1.26	0.19	0.27	—
J	—	1.85	—	—
K	—	1.92	0.27	—
P	—	1.67	—	—
Q	—	0.81	—	0.39
R	—	1.04	—	—
S	—	0.48	—	0.22

Stability constants used were: $CaSO_4$, 204 M^{-1}; $MgSO_4$, 170 M^{-1}; $CoSO_4$, 256 M^{-1}; $CdSO_4$, 288 M^{-1}; K_2SO_4, 7 M^{-1}; Na_2SO_4, 5 M^{-1} (Martell and Smith, 1976; Seys and Monk, 1965).

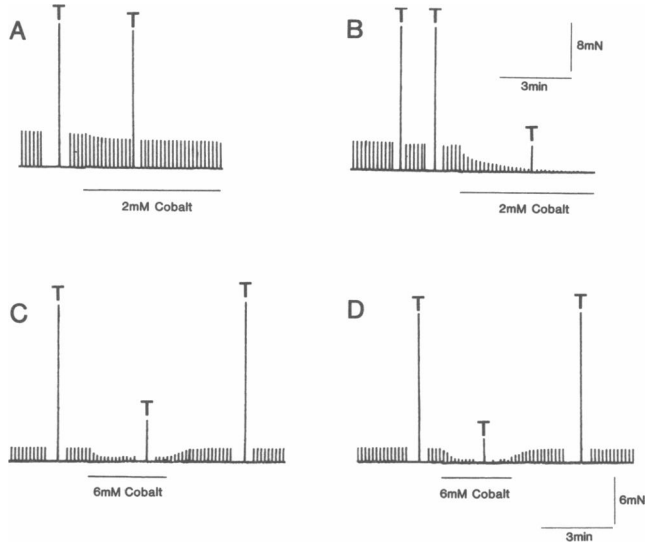

FIGURE 1 Depression of twitches (small vertical deflections) and tetanic contractions (large vertical deflections, *T*) by 2 mM (*A* and *B*) and 6 mM (*C* and *D*) cobalt. (*A*) 2 mM cobalt in a low-chloride solution with 7.6 mM calcium. (*B*) 2 mM cobalt in a low-chloride, low-calcium solution. (*C*) 6 mM cobalt in a chloride containing, 2.5 mM calcium, solution. (*D*) 6 mM cobalt in chloride-containing, calcium-free solution. The period of exposure to solutions containing cobalt is indicated by a horizontal line below the trace. In *A*, the solution was changed from control solution (solution A, Table 1A) to control solution plus 2 mM cobalt (solution I, Table 1B). In *B*, the solution was changed from a low-calcium control solution (solution J, Table 1B) to control solution plus 2 mM cobalt (solution K, Table 1B). In *C*, the solution was changed from a chloride-containing control solution (solution L, Table 1B) to a similar solution containing 6 mM cobalt (solution M, Table 1B). In *D*, the solution was changed from a chloride-containing, calcium-free solution (solution N, Table 1B) to a similar solution containing 6 mM cobalt (solution O, Table 1B). The horizontal calibrations show 3 min. Vertical calibrations show 8 mN for *A* and *B* and 6 mN for *C* and *D*.

(solution H) was identical after exposures of 3, 6, and 25 min. Recovery of tension (newtons) was 90% complete within 1.5 min of the start of the washout procedure in all three cases. It seems likely, therefore, that the effects of cobalt were exerted at the surface membrane rather than at an intracellular site.

The depression of twitch and tetanic tension caused by cobalt could have been due to a decrease in the amplitude of action potentials. However, when action potentials were recorded directly with intracellular microelectrodes in solutions containing 2 or 6 mM cobalt (solutions I and F), no depression of amplitude was seen. Action potentials recorded before (*A*) and following exposure to a solution containing 6 mM cobalt (*B*) are shown in Fig. 2. Although the decay of the action potential was prolonged in the presence of cobalt, the rise time and amplitude were not altered. The time to 80% of the peak of the action potential measured from the beginning of the stimulus artifact was 4.35 ms in both records in Fig. 2 and the time to peak was 4.5 ms in the control action potential (*A*) and 4.6 ms in cobalt (*B*). The amplitude of the action potential was 100.5 mV in control (*A*) and 101.3 mV in the cobalt solution (*B*). Similar observations were recorded in 10 fibers exposed to 6 mM cobalt sulphate: overshoots ranged from +25 to +30 mV.

It was concluded that the depression of tension caused by cobalt was not secondary to changes in action potential amplitude. This conclusion was reinforced by the observation that cobalt could also depress potassium contractures, as illustrated in Fig. 3, *A* and *B*. It can be seen in Fig. 3 *B* that 6 mM cobalt sulfate (solution F) reversibly depressed contractures produced by 40 mM (Fig. 3 *A*) and 80 mM (Fig. 3 *B*) potassium. As tension during potassium contractures does not depend on the generation of action potentials, the depression of potassium contracture tension cannot have been due to changes in action potentials.

Contraction might be depressed if cobalt caused membrane depolarization and consequent inactivation of tension generation. However, no significant change in membrane potential was seen when fibers were exposed to solutions containing 6 mM cobalt sulphate (solution F), 7 mM magnesium sulphate (solution E), or 7.6 mM calcium sulphate (solution A), as can be seen in Table 2. Furthermore, the presence of cobalt did not significantly affect the depolarization caused by solutions containing 40 mM potassium (Table 2).

It is well recognized that divalent cations can have a

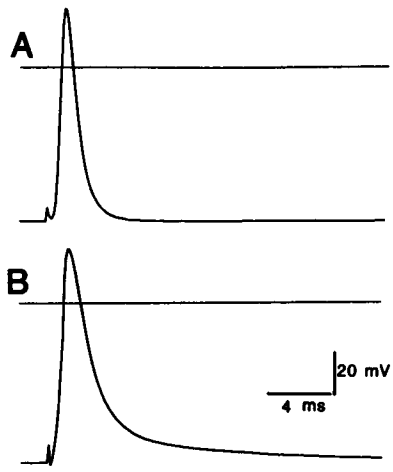

FIGURE 2 (*A*) An action potential in control solution (solution A, Table 1A). (*B*) An action potential in the 6 mM cobalt solution (solution F, Table 1A). Horizontal lines show 0 mV level. Horizontal calibration denotes 4 ms and the vertical calibration 20 mV. Note afterdepolarization in *B*.

local influence on membrane field by screening negative fixed charges on the surface of membranes. If some of these charges influenced the electrical field seen by a voltage sensor involved in excitation-contraction coupling, addition of divalent ions such as cobalt could have an effect similar to membrane hyperpolarization and depress tension by making the membrane field more negative during action potentials or during potassium contractures. Such an effect would not be seen as a change in membrane potential and should not depend on the species of divalent cation. The results given in Table 3 do not support this explanation for the depression of tension caused by the divalent cations. At the same concentration, magnesium was less effective than cobalt in depressing tension. To compare the effects of magnesium and cobalt, each preparation was exposed to a series of solutions; first control solution (solution A), then a solution containing 7 mM magnesium sulphate (solution E), then to a solution containing 6 mM cobalt sulphate (solution F), then back to 7 mM magnesium sulphate (solution E), and finally in control solution (solution A). The measurements were obtained by averaging tension recorded before and after exposure to the 6 mM cobalt sulphate so that any possibility of effects due to "run-down" during an experiment could be avoided. It can be seen that the depressant effect of adding 6 mM cobalt sulphate to solutions was much greater than that of adding 6 mM magnesium sulphate (Table 2). Because any screening of negative fixed charges would be expected to be very similar whether solutions contained cobalt or

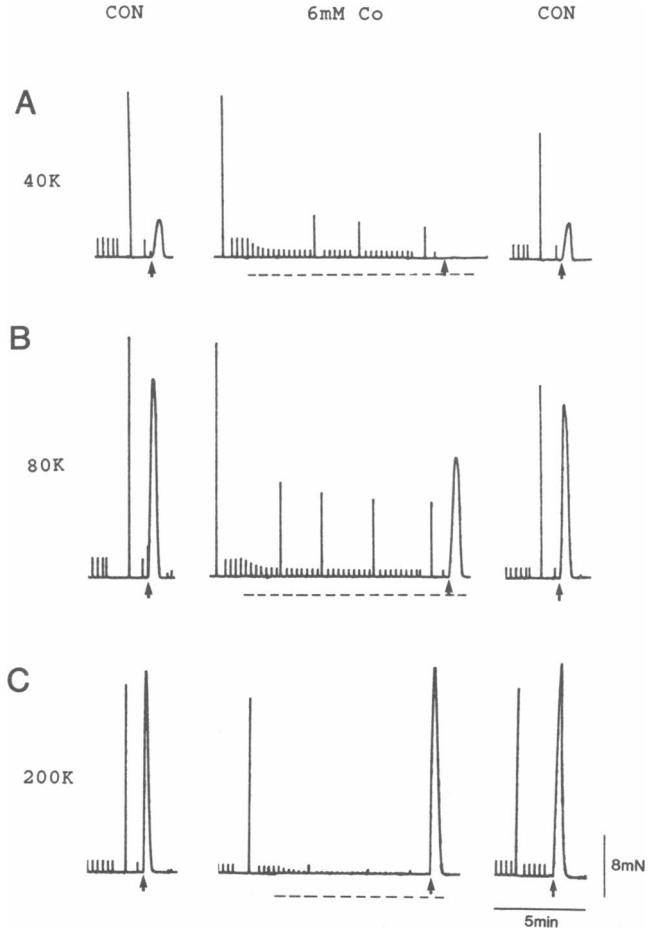

FIGURE 3 The effect of 6 mM cobalt in calcium-free solution on twitch, tetanic, and potassium contracture tension in three different preparations. (*A*) 40 mM potassium (*40K*) contractures evoked by sudden exposure to solution E1 or F1 (Table 1A). (*B*) 80 mM potassium (*80K*) contractures (using an appropriate combination of the 40 mM potassium and 200 mM potassium solutions solutions, i.e., solutions E1 and E2 or F1 and F2, Table 1A). (*C*) 200 mM potassium (*200K*) contractures in solution E2 or F2, (Table 1A). The first column (*CON*) shows records in which the control solution was the calcium-free solution with 6 mM additional magnesium (solution E, Table 1A). The second column (6 mM Co) shows records obtained during exposure to 6 mM cobalt solution (with 6 mM cobalt replacing 6 mM magnesium in the control solution [solution F, Table 1A]) for the times indicated by the broken horizontal lines. The third column (*CON*) shows records obtained after washout of the cobalt solution with the calcium-containing control solution (solution A, Table 1) followed by equilibration in the calcium-free control solution (solution E, Table 1). Each potassium contracture in the control calcium-free solution (*CON*) was preceded by a potassium contracture in the calcium-containing control solution (solution A, Table 1A). The contractures in the calcium-containing solution were very similar to those in the calcium-free control solution and are not shown. In each record, the potassium solution was added at the time indicated by the arrow and was washed out after the peak of the contracture. Cobalt was added for the period indicated by the dashed horizontal line. The horizontal calibration denotes 5 min and the vertical calibration 8 mN.

TABLE 2 Effects of 6 and 20 mM cobalt and magnesium on twitch and tetanic tension expressed relative to twitch or tetanic tension in control solution

Solution	Relative twitch tension	Relative tetanic tension
6Mg (n = 25)	0.95 ± 0.02	0.96 ± 0.01
6Co (n = 25)	0.43 ± 0.03	0.38 ± 0.03
20Mg (n = 26)	0.51 ± 0.03	0.72 ± 0.01
20Co (n = 26)	0.20 ± 0.03	0.30 ± 0.03

The protocol followed in the experiments is described in the text. The results are shown as mean ± 1 SEM. Number of experiments is shown in parentheses.

magnesium, it was concluded that cobalt did not depress contraction by simply screening surface charge.

If divalent ions bound to negative surface charges that affected the field seen by the voltage sensor for contraction, a higher affinity of cobalt than magnesium for such a site might explain the greater depressant effect of cobalt. If so, the voltage dependence of contraction would be shifted to more positive potentials than normal in solutions to which 6 mM cobalt sulphate had been added but not in solutions containing an added 6 mM magnesium sulphate. This possibility was tested by recording potassium contractures (40–200 mM K) in solutions containing 7 mM magnesium sulphate (solution E) or 6 mM cobalt sulphate (solution F) for comparison with potassium contractures in control solution (solution A).

Average potassium contracture tension recorded in 6–10 preparations in control (solution A), 6 mM magnesium (solution E), and 6 mM cobalt (solution F) solutions are plotted against membrane potential in Fig. 4. It is clear that the tension-membrane potential curve was shifted to the right after addition of 6 mM cobalt sulphate (*open squares*) but not 6 mM magnesium sulphate (*open triangles*) to control solution. The lines show the best (least squares) fits to the three sets of data of the Boltzmann-type expression:

$$T = T_{max}/[1 + \exp(V' - V_m)/k], \quad (1)$$

where T_{max} is maximum tension relative to maximum tetanic tension, V_m is membrane potential, V' is the potential at which tension equals 50% of T_{max}, and k is a slope factor (Dulhunty and Gage, 1985). The values for T_{max}, V', and k used to generate the curves in Fig. 4 are listed in Table 4 and show a 7-mV shift in V' in the 6 mM cobalt solution but no shift in V' in the 6 mM magnesium solution.

To exclude the possibility that the divalent ions were changing the depolarization during potassium contrac-

TABLE 3 The resting membrane potential in control solution (solution A, Table 1A), 6 mM Mg solution (solution E, Table 1) and 6 mM Co (solution F, Table 1) and in the control 40K solution (solution C, Table 1); 6Mg/40K solution (solution E1, Table 1) and 6Co/40K solution (solution F1, Table 1)

	Control	6Mg	6Co
	mV	*mV*	*mV*
3.5K	−83.4 ± 0.4 (72)	−83.7 ± 0.5 (55)	−83.1 ± 0.4 (28)
40K	−35.6 ± 0.6 (32)	−35.3 ± 0.4 (30)	−34.8 ± 0.5 (42)
	Control	20Mg	20Co
	mV	*mV*	*mV*
3.5K	−77.1 ± 2.6 (42)	−80.3 ± 0.6 (21)	−78.0 ± 0.9 (28)
40K	−39.4 ± 0.8 (27)	−38.5 ± 0.7 (22)	−38.1 ± 0.7 (25)
200K	−3.3 ± 0.6 (21)	−3.7 ± 0.3 (18)	−3.3 ± 0.3 (27)

The sequence of solution changes was the same as described in the text for Table 2, with an exposure to a 40K solution between each exposure to the control, high magnesium or high cobalt solutions. The results are shown as mean ± 1 SEM with the numbers of fibers sampled in parentheses.

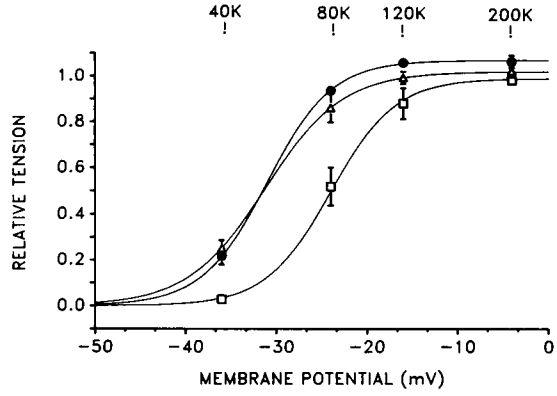

FIGURE 4 The voltage dependence of maximum potassium contracture tension in the control solution containing 7.6 mM calcium (solution A, Table 1; *solid circles*), 6 mM added magnesium solution (solution E, Table 1; *open triangles*) and 6 mM cobalt solution (solution F, Table 1; *open squares*) using average results from experiments similar to those shown in Fig. 3. Potassium contracture tension is expressed relative to tetanic tension (see Methods). Potassium concentrations were converted to membrane potentials (abscissa) using results obtained in other experiments (see text). Potassium concentrations used are shown along the top of the graph. The lines through the three sets of data were obtained by fitting Eq. 1 (see text) to the data, and the constants for each curve are given in Table 4. The symbols show average data from 6 to 10 preparations and the vertical bars show ±1 SEM where this exceeds the dimensions of the symbol.

TABLE 4 Effects of magnesium and cobalt on the parameters T_{max}, V', and k obtained from the best fit of Eq. 1 to average potassium contracture data

	T_{max}	V'	k
Control	1.07	−31.0	3.6
6Mg	1.02	−31.2	4.3
6Co	0.99	−24.0	3.6
Control	1.16	−29.5	3.65
20Mg	1.02	−23.6	4.0
20Co	1.00	−21.1	3.5

Because different preparations were used for the 6Co and 6Mg experiments and the 20Co and 20Mg experiments, two sets of control data are given.

tures, membrane potential was recorded in a large number of fibers exposed to the different potassium concentrations in the presence of the different divalent cations. It was found that neither the addition of 6 mM magnesium sulphate nor 6 mM cobalt sulphate changed the relationship between potassium concentration and membrane potential (Table 3).

In addition to the shift in voltage dependence of tension, there was a depression of maximum tension in the 6 mM cobalt solution (Fig. 4, Table 4). This action was explored further by testing the effects of a higher concentration of cobalt. Twitches, tetanic contractions, and potassium contractures were recorded in solutions containing 20 mM cobalt sulphate (solution H) and, for comparison, in solutions containing 20 mM magnesium

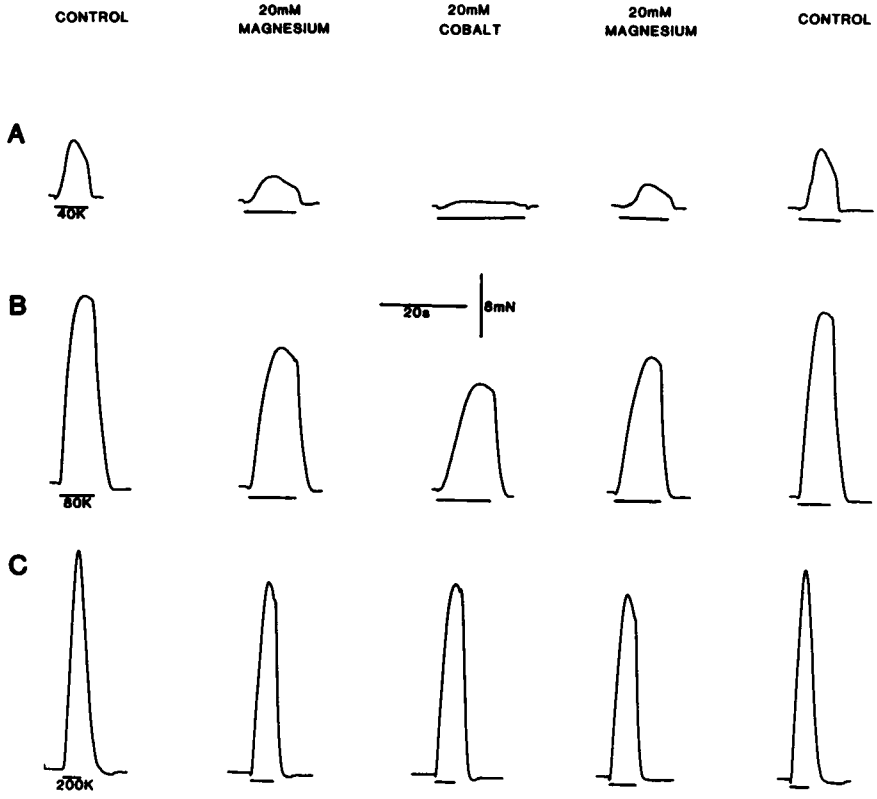

FIGURE 5 The effect of 20 mM magnesium (solution G, Table 1) and 20 mM cobalt (solution H, Table 1) on potassium contractures. All records were obtained from the same preparation. (*A*) 40 mM potassium contractures caused by sudden exposure to solutions C, G1, or H1. (*B*) 80 mM potassium contractures (using appropriate combinations of solutions C and D, G1 and G2, or H1 and H2). (*C*) 200 mM potassium contractures (solutions D, G2, or H2). The duration of exposure to a raised potassium solution is indicated by the horizontal lines under each record. Column 1: control records after equilibration in solution A, Table 1. Column 2: after 5 min equilibration in the 20 mM magnesium solution (solution G, Table 1). Column 3: after return to control solution for 10 min and then exposure to the 20 mM cobalt solution (solution H, Table 1) for 5 min. Column 4: after a 10-min recovery in control solution and 5 min in the 20 mM magnesium solution. Column 5: control records obtained after recovery in control solution from exposure to the 20 mM magnesium solution. Potassium contractures in this series of experiments were induced after a brief exposure to a low sodium solution (solution B, Table 1) to eliminate any contribution of action potential-induced twitch activity to potassium contracture tension. Fibers were exposed to a low sodium control solution for 1 min before the low-sodium plus high-potassium solution. The control contractures were no different from control contractures obtained in sodium-containing solutions (see e.g. Fig. 3). The horizontal calibration denotes 20 s and the vertical calibration 8 mN.

sulphate (solution G). Because tension never fully recovered in preparations exposed to calcium-free solutions containing 20 mM magnesium or 20 mM cobalt sulphate, test solutions contained the normal concentration of calcium as well as magnesium or cobalt. The use of calcium-containing solutions in this second series of experiments avoided the possibility that any effects of cobalt were influenced by removal of calcium which has long been known to have complex effects on excitation-contraction coupling (Luttgau, 1963; Caputo and Giminez, 1967; Caputo, 1972; Luttgau and Spiecker, 1979; Luttgau et al., 1986; Dulhunty and Gage, 1988; Brum et al., 1988).

At these higher concentrations, both magnesium and cobalt now depressed twitch and tetanic tension. In solutions containing 20 mM magnesium sulphate (solution G), twitch tension fell on average to about half the control value, and tetanic tension fell by ~30% (Table 2). 20 mM cobalt sulphate (solution H) had a greater depressant effect than 20 mM magnesium sulphate, causing on average a reduction of 80% in twitch tension and 70% in tetanic tension (Table 2). The depression of tension caused by 20 mM magnesium sulphate and 20 mM cobalt sulphate could not be attributed to effects of the high cation concentration on the resting membrane potential which was not significantly changed by either ion (Table 3).

Potassium contractures were also depressed by 20 mM magnesium sulphate and 20 mM cobalt sulphate, as illustrated in Fig. 5. In this experiment, 20 mM magnesium sulphate caused a 60% reduction in the amplitude of the 40 mM potassium contracture, a 30% reduction in the amplitude of the 80 mM potassium contracture, and a 14% reduction in the amplitude of the 200 mM potassium contracture (columns 2 and 4, Fig. 5). Even more depression of potassium contracture tension was caused by the 20 mM cobalt sulphate solution (column 3) for 40 and 80 mM potassium contractures, but 20 mM magnesium sulphate and 20 mM cobalt sulphate caused much the same amount of depression of 200 mM potassium contractures (Fig. 5 *C*). As the depolarizations caused by these concentrations of potassium were not significantly affected by magnesium or cobalt (Table 3), the depression of the potassium contractures cannot be attributed to less depolarization.

Average potassium contracture tension (relative to tetanic tension) in control solution (*solid circles*), 20 mM magnesium sulphate solution (*open triangles*), and 20 mM cobalt sulphate solution (*open squares*) is shown plotted against membrane potential in Fig. 6. The lines through the symbols are best (least squares) fits of Eq. 1 with values of T_{max}, V', and k as listed in Table 4. The maximum tensions recorded in the 200 mM potassium solution in the presence of 20 mM magnesium sulphate or 20 mM cobalt sulphate were significantly lower than in the controls ($P = 0.001$, Student's t). The average positive shift in V' of 8.4 mV produced by 20 mM cobalt sulphate was not much greater than the 7-mV shift caused by 6 mM cobalt sulphate. There was a positive shift in V' of ~6 mV in the 20 mM magnesium sulphate solution.

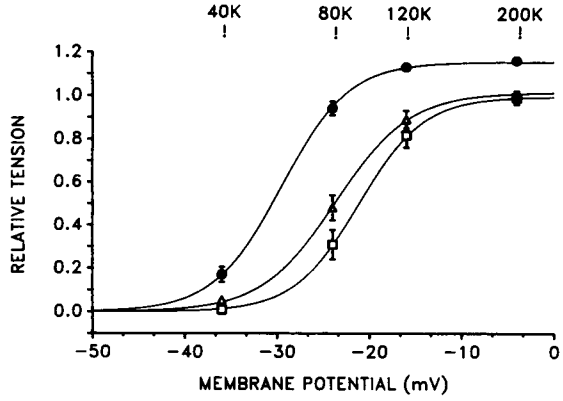

FIGURE 6 Voltage dependence of tension in control solution (solution A, Table 1A; *solid circles*), 20 mM magnesium solution (solution G, Table 1A; *open triangles*) and 20 mM cobalt solution (solution H, Table 1A; *open squares*) using average results from experiments similar to those shown in Fig. 5. The lines through the three sets of points are least squares fits of Eq. 1. Constants for each curve are given in Table 4. Symbols show average data from six to eight preparations and vertical bars show ±1 SEM where this exceeds the dimensions of the symbol.

Not only was there a positive shift in the tension-voltage curve in solutions containing 20 mM magnesium sulphate or cobalt sulphate, but also the rise in tension during a potassium contracture was slowed, as can be seen in Fig. 5. The time to peak of the tension and the 20–80% rise times are shown for the different potassium contractures in Fig. 7, *A* and *B*. The slowed development of tension was more marked with 20 mM cobalt sulphate (*open squares*) than with 20 mM magnesium sulphate (*open triangles*). A slowing of contraction might be expected to accompany a shift in the voltage dependence of tension because the rate of rise of tension is slower for smaller contractures at more negative potentials (Hodgkin and Horowicz, 1960; see also Fig. 7). However the 80 mM potassium contractures in solutions containing 20 mM cobalt sulphate, although greater in amplitude than the 40 mM potassium contractures in control solution (Fig. 5), became much slower (Fig. 7, *A* and *B*). The slower time course cannot therefore be explained simply in terms of the slower time course of smaller potassium contractures.

In one experiment in which exposure to the 200 mM potassium solution was maintained while tension decayed, the decay was also slowed in solutions containing 20 mM magnesium sulphate or cobalt sulphate. The half decay

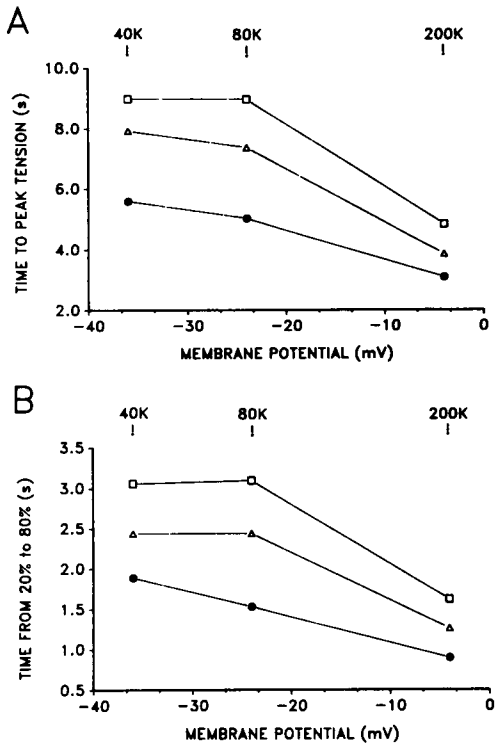

FIGURE 7 The effect of the 20 mM magnesium solution (solution G, Table 1A) and 20 mM cobalt solution (solution H, Table 1A) on the time to peak tension (*A*) and the 20–80% rise time (*B*) of potassium contractures. The times were measured from the contractures shown in Fig. 5. (*Solid circles*) Control data; (*open triangles*) 20 mM magnesium; (*open squares*) 20 mM cobalt. Potassium concentrations are shown above.

time of 12 s in control solution increased to 18 s in the presence of 20 mM magnesium sulphate and to 21.6 s in the solution containing 20 mM cobalt sulphate.

The slowing of the decay of potassium contractures caused by the elevated divalent cation concentrations could have been due to a change in surface potential and a consequent shift to the right in the voltage dependence of the rate of inactivation which is slower at more negative membrane potentials (Hodgkin and Horowicz, 1960). Such an effect would also increase the time to peak tension but should have much less effect on the 20–80% times shown in Fig. 7. Furthermore, a change in the rate of inactivation would not explain the clear delay in the onset of the 40 mM potassium contracture shown in Fig. 5 *A* which was 1 s in control solution, 3 s in 20 mM magnesium sulphate solution, and 5 s in 20 mM cobalt sulphate solution (Fig. 5 *A*). It is clear that 20 mM magnesium sulphate and 20 mM cobalt sulphate alter the kinetics of the activation process in rat soleus fibers and that the effects cannot be explained simply in terms of a change in the rate of inactivation of tension.

The possibility that steady-state inactivation was affected by the magnesium and cobalt sulphate solutions was examined in experiments in which fibers were conditioned by a 3-min exposure to 40, 60, or 80 mM potassium to cause graded inactivation of tension and then tension was tested with a 200 mM potassium solution. Control 200 mM potassium contractures were elicited before and after the inactivated 200 mM potassium contracture and the amplitude of the inactivated contracture was expressed relative to the mean of the two control contractures. In each preparation, control and test contractures were first performed in control solutions (solution A) and after 3 min in the 20 mM cobalt sulphate solution (solution H). The inactivating potassium solutions and the 200 mM potassium solution each contained 20 mM cobalt sulphate and the magnesium sulphate and calcium concentrations were as shown for solutions H1 and H2 in Table 1A. Instead of the shift of the steady-state inactivation membrane potential curve to the right that would be expected if cobalt were reducing the negative charge on the outside surface of the membrane, there was in fact a small shift to more negative membrane potentials. The inactivated potassium contracture tension was lower than normal after exposure to 40, 60, and 80 mM potassium in the 20-mM cobalt sulphate solution (Table 5). There are clearly two separate effects of cobalt on the inactivation process: the shift to the right in the voltage dependence of the *rate* of inactivation may be due to a change in surface charge, whereas the shift to the left in the voltage dependence of the *amount* of inactivation must be due to some other effect of cobalt.

Effects of cadmium

Cadmium shared many of the effects of magnesium and cobalt on excitation-contraction coupling. In the presence of 3 mM cadmium sulphate (solution Q), twitch and tetanic tension were rapidly depressed (Fig. 8 *B*), as were 40 and 80 mM potassium contractures. As seen with magnesium and cobalt, there was little depression of 200

TABLE 5 Steady-state inactivation in 20 mM cobalt

Inactivating potassium concentration	Membrane potential	Relative tension	
		Control	20 mM cobalt
mM	*mV*		
40	−39	0.625 ± 0.016	0.595 ± 0.084
60	−31	0.095 ± 0.019	0.048 ± 0.014
80	−26	0.014 ± 0.005	0.002 ± 0.001

Protocol and solutions used in the experiment are described in the text. The membrane potentials in each inactivating potassium solution (column 2) were measured in separate experiments. The results are given as mean ± SEM.

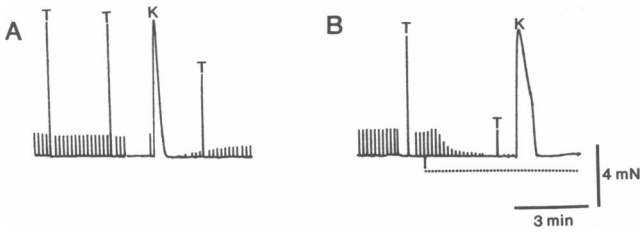

FIGURE 8 Effect of 3 mM cadmium solution on twitch, tetanic (*T*), and 200 mM potassium contracture (*K*) tension. (*A*) Control records obtained in calcium-free solution (solution P, Table 1C). The 200 mM potassium contracture was evoked by sudden exposure to solution R (Table 1C). (*B*) The solution was changed from the control calcium-free solution (solution P, Table 1C) to one in which 3 mM $CdCl_2$ replaced 5 mM of the $MgSO_4$ (solution Q, Table 1C) at the time indicated by the broken line. The 200 mM potassium contracture was evoked by sudden exposure to solution S (Table 1C). The horizontal calibration denotes 3 min and the vertical calibration 4 mN.

mM potassium contractures (Fig. 8, *A* and *B*) in solutions containing 3 mM cadmium sulphate although the time course of contractures was slowed. It can also be seen, by comparing Figs. 1 and 8, that the depressions of twitch and tetanic tension were greater with 3 mM cadmium sulphate than with 6 mM cobalt sulphate.

Depression of twitch tension in solutions containing 3 mM cadmium sulphate was often preceded by a period of twitch potentiation, as can be seen in Figs. 8 and 9. Furthermore, after some time in a solution containing cadmium (solutions U or W), there was normally a spontaneous slow contracture (Fig. 9 *B*). When the cadmium solution was washed out with control solution (solutions T or V), after the development of the slow contracture, there was always a very large contracture with an amplitude equal to or greater than the maximum tetanic tension (Fig. 9, *A* and *B*). Potassium contracture tension was depressed during the cadmium withdrawal contracture (Fig. 9 *B*). It can be seen that twitch and tetanic tension were depressed during the contractures (Fig. 10). These cadmium-withdrawal contractures had a similar amplitude whether or not solutions contained calcium (Table 1C, Figs. 9 and 10) but the decay was accompanied by oscillations in tension only when calcium was present (Fig. 9 *B*).

The contractures were not caused by membrane depolarization because, as can be seen in Fig. 9 *C* and Table 6, neither exposure to cadmium nor washout of cadmium caused a depolarization great enough to explain the increase in tension. A brief depolarization of 6–8 mV was seen on washout of the cadmium in three fibers (Table 6) (tension was blocked with 20 mM butanedione monoxime [Fryer et al., 1988]) but this was insufficient to explain the cadmium-withdrawal contractures.

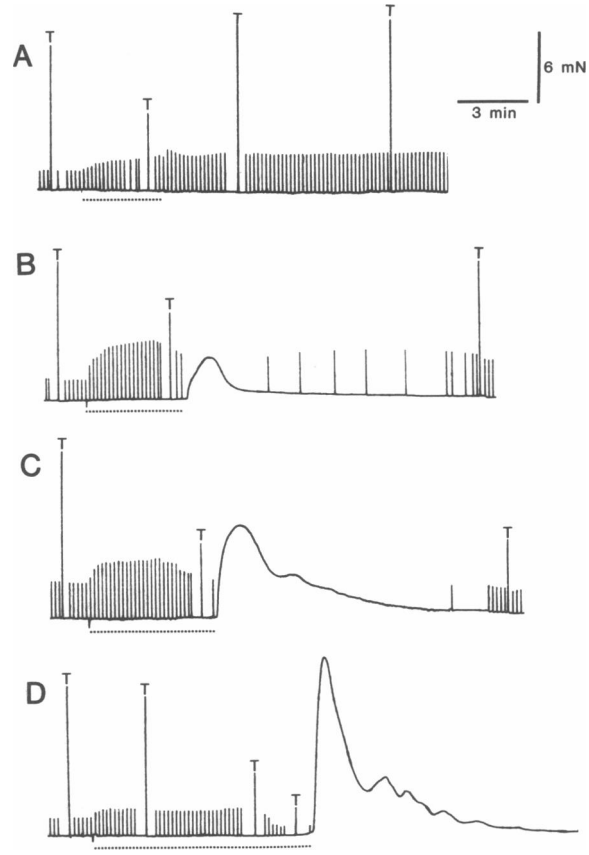

FIGURE 9 Cadmium-withdrawal contractures after exposure to 3 mM cadmium in (*A*) calcium-free solution (solution Q, Table 1C) and (*B*) in normal (2.5 mM calcium) solution (solution U, Table 1C). Broken lines denote the periods of exposure to 3 mM cadmium. The 200 mM potassium contracture (*K*) in *B* was elicited using solution D (Table 1A). *C* shows a continuous record of membrane potential obtained during the sequence of solution changes used in *B*. Exposure to the cadmium-containing solution is indicated by the dashed line. In *A*, the fibers were equilibrated in control, calcium-free solution (solution V, Table 1C) before exposure to the cadmium solution (solution W, Table 1), which was replaced by solution V at the end of the dashed line. In *B* and *C*, the bath solution was changed from control, calcium-containing solution (solution T, Table 1C) to the cadmium-containing solution (solution U, Table 1C) and then back to solution T at the end of the dashed line. The horizontal calibration denotes 3 min and the vertical calibration denotes 4 mN for *A* and *B* and 40 mV for *C*.

Large cadmium-withdrawal contractures with oscillating tension changes were seen only after exposure to cadmium of 8 min or longer (Fig. 10 *D*). No contracture was recorded after exposures of up to 3 min (Fig. 10 *A*) and contractures of increasing amplitude were recorded after 4 min (Fig. 10 *B*) and 5 min in cadmium (Fig. 10 *C*). A possible explanation for this dependence on exposure time is that the contracture is secondary to slow accumulation of cadmium in a restricted, possibly intra-

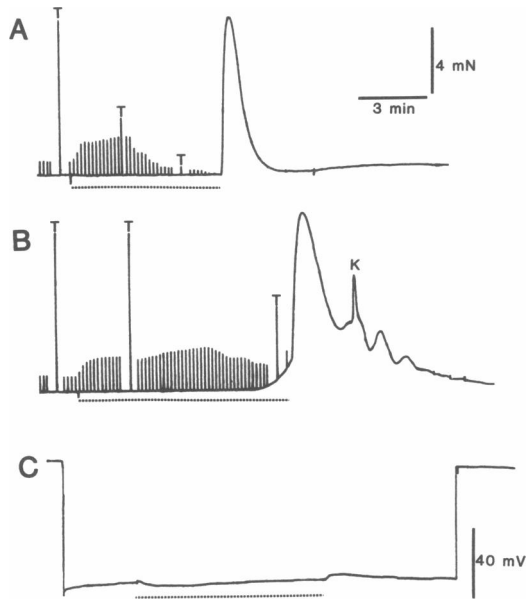

FIGURE 10 The effect of duration of exposure to cadmium on the amplitude of the cadmium-withdrawal contracture. The records in *A*, *B*, and *C* were obtained from one preparation and those in *D* from another preparation. The preparation was allowed to recover for 15 min in control solution (solution T, Table 1C) between *A* and *B* and for 60 min between *B* and *C*. The periods of exposure to cadmium, indicated by the dashed lines, were 3 min in *A*, 4 min in *B*, 5 min in *C*, and 8 min in *D*. For each record, the solution was changed from the control solution (solution T, Table 1C) to the cadmium solution (solution U, Table 1C) at the start of the broken line and then back to solution T at the end of the *dashed* line. The horizontal calibration denotes 3 min and the vertical calibration 6 mN.

cellular, space. Indeed, the slow development of tension during exposure to cadmium (Fig. 9 *B*) suggested that cadmium exerted a second effect at an intracellular site.

DISCUSSION

There have been many reports of the influence of extracellular calcium ion concentration and other divalent cations on contraction (Luttgau, 1963; Caputo and Gimenez, 1967; Luttgau and Speicker, 1979; Caputo, 1972; Luttgau et al., 1986). The effects of the divalent cations on the activation properties are consistent with changes in surface charge as a result of either screening or binding but effects on the inactivation process suggest that the ions may have a specific effect on the voltage sensor for contraction (Luttgau et al., 1986; Brum et al., 1988). Many of the results described in this paper suggest that divalent cations affect both activation and inactivation in a manner that cannot be completely explained by a decrease in surface charge.

The divalent cations tested, magnesium, cobalt, and cadmium, all affected excitation-contraction coupling. Part of the depression of tension and slowing of inactivation may have been due to a shift in the tension-membrane potential curve to more positive potentials (Figs. 4 and 6), because of a decrease in negative fixed charge on the external surface of the membrane. This effect must be due to binding of the ions to, rather than screening of, these sites because there was a clear sequence of effectiveness in blocking contraction, Cd > Co > Mg. Furthermore, the effects were seen with very

TABLE 6 Effects of addition and removal of 3 mM cadmium on the resting membrane potential, continuously recorded in three fibers in which contraction was blocked with 20 mM BDM and recoreded with multiple penetrations in one unblocked preparation during washout of cadmium

Time of exposure to cadmium (*min*)	0	1	2	3	4	5	6	7	8
	mV	*mV*	*mV*	*mV*	*mV*	*mV*	*mV*	*mV*	*mV*
Fiber 1	−78	−80	−80	−78	−78	−78	−78	−78	−78
Fiber 2	−74	−76	−76	−75	−74	−73	−72	−72	−71
Fiber 3	−78	−79	−79	−78	−78	−78	−78	−78	−78
Time after removal of cadmium (*min*)	0	1	2	3	4	5	6	7	8
	mV	*mV*	*mV*	*mV*	*mV*	*mV*	*mV*	*mV*	*mV*
Fiber 1	−78	−74	−74	−75	−76	−78	−79	−80	−81
Fiber 2	−71	−68	−67	−68	−69	−70	−70	−70	−70
Fiber 3	−76	−70	−64	−66	−68	−70	−72	−74	−76
Multiple fibers	−81	−72	−68	−78	−76	−72	−76	−75	−74

Solutions used are given in the legend to Fig. 9.

low free concentrations of the ions (Table 1D). The results give little information about the nature or location of the charged sites but it is interesting that the sequence of effectiveness of the ions is the same as for depression of calcium currents (Almers et al., 1985). It may be that part of the calcium channel is the voltage sensor for excitation-contraction coupling, and the negatively charged sites that influence the voltage sensor are those used by calcium ions in traversing the channels. If cobalt and cadmium have a relatively high affinity for these sites, they would both block calcium currents (Almers et al., 1985) and shift the voltage dependence of tension to more positive potentials. A reduction in negative charged site density is not a sufficient explanation for all of our observations. Neither the depression of 200 mM potassium contractures by magnesium and cobalt (Figs. 4 and 6), nor the potentiation of contraction followed by spontaneous contractures in the presence of cadmium (Figs. 9 and 10), nor the shift in the steady-state inactivation curve to more negative potentials (Table 5) can be explained by a positive shift in the tension-membrane potential relationship. Furthermore, the slowing of the rise time of potassium contractures in 20 mM cobalt or 20 mM magnesium (Figs. 5 and 7) is far greater than expected from the positive shift in the activation curve. Many of these effects can be explained if it is assumed that the calcium channel protein involved in excitation-contraction coupling undergoes a sequence of conformational changes from a precursor, to an active and then to an inactivate state, and that these conformational changes depend on membrane potential and external calcium concentration (Dulhunty and Gage, 1988). The binding of the cations to the calcium-sensitive sites could then influence the amount of protein in the active or inactive forms and the rate at which each transition occurred.

Another possible mode of action of the divalent cations is related to their potential intracellular effects. If entry of calcium ions normally accelerates breakdown of a messenger involved in excitation-contraction coupling, suppression of the calcium current would potentiate tension and persistence of the messenger might also underlie the slower decay of potassium contracture tension in the presence of the foreign divalent ions. Without more evidence, further speculation of this kind is probably not warranted.

It may appear paradoxical that 20 mM cobalt caused a positive shift in the asymmetric charge-voltage curve of +15 to +20 mV in mammalian muscle (Dulhunty and Gage, 1985; Lamb, G. D., and P. W. Gage, unpublished observations) yet shifted the tension-voltage curve by only +8 to +9 mV (Fig. 6). However, the concentration of free cobalt would have been much higher in the charge movement experiments in which bromide was the predominant anion.

The slow onset of cadmium-induced contractures and the cadmium withdrawal contractures may be due to slow access of the ion to a site where cadmium potentiates contraction. It seems probable that such a site would be in the intracellular compartment where cadmium concentration would build up more slowly than in the T-system. The initial potentiation of twitch tension in cadmium which develops more rapidly than other effects on contraction probably reflects an action of the ion in the T-system. Alternatively the potentiation may be due to an intracellular effect of very low concentrations of cadmium that could be reached in a very short time. For example, it has been proposed that cadmium enhances the effectiveness of inositol trisphosphate in producing contractures in skinned skeletal muscle fibres by inhibiting an enzyme that catalyzes hydrolysis of inositol trisphosphate (Storey et al., 1984; Vergara et al., 1985). The large cadmium withdrawal contractures such as those illustrated in Figs. 9 and 10 could then be due to rapid reversal of the depressant effects of cadmium on excitation-contraction coupling in the T-system with unmasking of the full potentiation that may be due to a persistent increase in the concentration within the fiber of a messenger for calcium release from the sarcoplasmic reticulum. Finally, it is possible that cadmium slowly enters fibers and causes direct activation of the contractile proteins (Stephenson and Theileczk, 1986).

The tension-voltage curve obtained from potassium contractures shows saturation of tension at potentials more positive than −10 mV (Figs. 4 and 6). If the overshoot during an action potential in the T-system were +20 mV or more, as on the surface membrane, the tension-voltage curves in Figs. 4 and 6 indicate that a negative shift in membrane potential as large as 20 mV should not depress twitch or tetanic tension. Comparison of the tension-voltage curves in Figs. 4 and 6 and the degree of depression of twitches and tetanic tension caused by these ions (Figs. 1, 3, and 8) indicates that the equivalent voltage across the T-system membrane during an action potential may normally be as low as 0 to −30 mV (Adrian and Peachey, 1973). This would be consistent with a lower density of sodium channels in the T-system than on the surface membrane (Jaimovich et al., 1976). The high chloride permeability in the T-system (Dulhunty, 1979) might further reduce the overshoot of action potentials in normal solutions containing chloride ions.

In conclusion, divalent ions may affect excitation-contraction coupling in several ways: by influencing the membrane field, by binding to the voltage-sensing activator in the wall of the T-system and hence affecting both

activation and inactivation, by blocking calcium channels and thus preventing the normal rise in calcium concentration at the inner surface of the membrane, by entering the fiber and interacting with a second messenger system involved in excitation-contraction coupling or by interacting directly with the contractile proteins.

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