EFFECTS OF EXTRACELLULAR CALCIUM CONCENTRATION AND DIHYDROPYRIDINES ON CONTRACTION IN MAMMALIAN SKELETAL MUSCLE

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SUMMARY

1. Twitches, tetanic contractions and potassium contractures were recorded isometrically from small bundles of rat soleus muscle fibres.

2. Solutions with reduced calcium concentrations (low-calcium solutions), whether buffered with EGTA (85 and 3 μM-Ca^{2+}) or not (15 μM-Ca^{2+}), caused an initial potentiation of contraction followed by depression.

3. The decay of potassium contractures (200 mM-potassium) was more rapid than normal in low-calcium solutions.

4. Recovery from the inactivation produced by a 200 mM-potassium contracture was slowed in low-calcium solutions but full recovery was seen within 10–15 min after return to a solution containing 2·5 mM-Ca^{2+}.

5. Nifedipine (50 μM) in solutions containing 2·5 mM-Ca^{2+} potentiated contraction whereas, in low-calcium solutions, contraction was depressed and the depression was more pronounced the lower the Ca^{2+} concentration.

6. As with low-calcium solutions, potassium contractures decayed more rapidly in solutions containing nifedipine. Nifedipine slowed still further the rate of recovery from inactivation in low-calcium solutions.

7. (−) Bay K 8644 (50 μM) depressed contraction, increased the rate of decay of potassium contractures and slowed recovery from inactivation, like nifedipine. The racemate of Bay K 8644 was less effective.

8. In explanation of these and other observations, it is proposed that there is a dihydropyridine-binding molecule in the walls of the transverse tubular system that normally exists predominantly in a 'precursor' form at the resting membrane potential and is converted by membrane depolarization to an 'activator' form essential for excitation–contraction coupling. Conversion of the precursor to activator involves both conformational change and dissociation of calcium. Prolonged depolarization converts activator to an inactivated form by inducing further conformational change and dissociation of calcium. Recovery from inactivation requires reverse conformational changes and rebinding of calcium. The dihydropyridines affect contraction by reducing the affinity of the molecule for calcium.

INTRODUCTION

It has been amply demonstrated that an inflow of calcium ions (Ca^{2+}) from the extracellular medium is not necessary for contraction in skeletal muscle. In muscles exposed to 'calcium-free' solutions buffered with EGTA, twitches (Armstrong, Bezanilla & Horowicz, 1972) and calcium release from the sarcoplasmic reticulum (Miledi, Parker & Zhu, 1984) can still be elicited. On the other hand, changes in extracellular Ca^{2+} concentration can affect contraction. When the extracellular Ca^{2+} concentration is lowered, there is an increase in the rate of decay (inactivation) of slow contractures produced by long depolarizations, a shift in the steady-state inactivation curve to more negative potentials (Luttgau & Spiecker, 1979; Cota & Stefani, 1981) and a slowed recovery from inactivation (Luttgau, Gottschalk & Berwe, 1987). Indeed, contraction can be blocked when extracellular Ca^{2+} concentration is buffered at very low concentrations with high concentrations of EGTA (Barrett & Barrett, 1978). Such results clearly demonstrate that extracellular Ca^{2+} can at least modulate contraction.

Although calcium current across the T-tubule membrane does not appear to be essential for contraction in skeletal muscle, several types of drug that block calcium channels have been reported to affect contraction. D600 and diltiazem cause a use-dependent block of contraction (Eisenberg, McCarthy & Milton, 1983; Gottschalk & Luttgau, 1985; McCleskey, 1985; Gallant & Goettl, 1985; Luttgau *et al.* 1987) and there are conflicting reports about the effects of the dihydropyridine calcium channel blockers, nitrendipine and nifedipine, on contraction in skeletal muscle. They have been variously reported to depress contraction (Ildefonse, Jacquemond, Rougier, Renaud, Fosset & Lazdunski, 1985; Avila-Sakar, Cota, Gambroa-Aldeco, Garcia, Huerta, Muniz & Stefani, 1986; Rios, Brum & Stefani, 1986), to potentiate contraction (Gallant & Goettl, 1985) or to have no effect (McCleskey, 1985; Lamb, 1986; Luttgau *et al.* 1987; Rakowski, Olszewska & Paxson, 1987). These observations, although somewhat confusing and seemingly contradictory, indicate that extracellular Ca^{2+} and the dihydropyridines can modulate contraction in skeletal muscle under appropriate conditions.

The aim of the experiments described in this paper was to explore whether, and how, extracellular calcium and the dihydropyridines affect contraction in mammalian skeletal muscle. It was found that both can affect contraction and the observations can be explained in a straightforward way if it is assumed that calcium must dissociate from a 'precursor' molecule accessible from the T-tubule lumen for normal contraction to occur and that recovery from inactivation requires binding of calcium ions.

METHODS

Preparation. Soleus muscles were dissected from 250–300 g male Wistar rats (killed with an overdose of halothane), pinned out in a Petri dish lined with Sylgard (Dow Corning) and dissected into small bundles containing from five to ten fibres.

Stimulation and recording. The methods for eliciting and recording isometric twitches, tetanic contraction and potassium contractures have been described in detail elsewhere (Dulhunty & Gage, 1985). Briefly, a bundle of fibres was mounted in a small volume, rapid-flow bath, with a solution change-over time of less than 500 ms. The fibres were electrically stimulated through massive

platinum electrodes which extended along either side of the bath. 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. The duration of trains was set to give a steady plateau of tension and varied between 0·8 and 1·5 s from preparation to preparation. Potassium contractures were produced by rapidly changing from the control solution to a solution containing a raised potassium concentration (Table 1). Tension was continuously recorded via an Akers semiconductor transducer on a chart recorder (Hewlett–Packard 7402 A) and selected twitches, tetanic contractions or potassium contractures were stored on disc in a computer for later analysis. The experiments were done at 25·5 ± 0·5 °C.

TABLE 1. Composition of solutions (mM)

Solution	Na_2SO_4	NaCl	K_2SO_4	KCl	$CaSO_4$
Control	32·25	16	1·75	0	7·6
40 mM-K^+	80	0	12	16	7·6
200 mM-K^+	0	0	92	16	7·6
Ca^{2+} free	32·25	16	1·75	0	0
85 μM-Ca^{2+}	32·25	16	1·75	0	0
3 μM-Ca^{2+}	32·25	16	1·75	0	0

	$MgSO_4$	EGTA	Ca–EGTA	Sucrose	*Ca^{2+}
Control	1	0	0	170	2·5
40 mM-K^+	1	0	0	0	—
200 mM-K^+	1	0	0	0	—
Ca^{2+} free	10	0	0	170	0·015
85 μM — Ca^{2+}	10	0	20	140	0·085
3 μM-Ca^{2+}	10	10	10	100	0·003

2 mM-TES buffer (pH = 7·4) was added to all solutions except the 85 μM-Ca^{2+} and the 3 μM-Ca^{2+} solutions which contained 10 mM-TES (see Methods). For potassium contractures in low-calcium solutions, the Ca^{2+} concentration was lowered in the potassium solutions as in the low-calcium solutions and 10 mM-$MgSO_4$ added.

* Measured Ca^{2+} concentration.

Solutions. All experiments were done in low-chloride solutions to give a rapid change in membrane potential during potassium contractures (Dulhunty & Gage, 1985). The compositions of the different solutions used are given in Table 1. The concentrations of free calcium ions in the solutions were measured directly with a calcium-sensitive electrode (Radiometer Ion 83 Ion Meter). The free calcium concentration in the control solution was 2·5 mM. Calcium in the 85 and 3 μM-Ca^{2+} solutions was added as Ca–EGTA, prepared by dissolving equimolar amounts of EGTA and $CaCO_3$ in distilled water (final pH ≈ 4) at 80 °C for 10 min (Ashley & Moisescu, 1977) and the 7·6 mM-$CaSO_4$ in the normal solution was replaced with 10 mM-$MgSO_4$. The concentration of TES (N-tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid) buffer was increased in the EGTA-containing solutions to counteract the hydrogen ion buffering capacity of EGTA. Solutions containing high potassium concentrations and low calcium concentrations were prepared in the same way as the 40 mM-potassium and 200 mM-potassium solutions containing 2·5 mM-Ca^{2+} (Table 1). Nifedipine (Sigma, U.S.A), Bay K 8644 and (−) Bay K 8644 (kindly supplied by Bayer, F.R.G.) were made up as 10 mM stock solutions in absolute ethanol and stored in light-resistant containers at 4 °C.

RESULTS

Effects of lowered calcium concentration

In general, lowering the extracellular free-calcium concentration produced reproducible changes in the amplitude of twitches, tetanic contractions and potassium contractures, increased the rate of decay of potassium contractures and
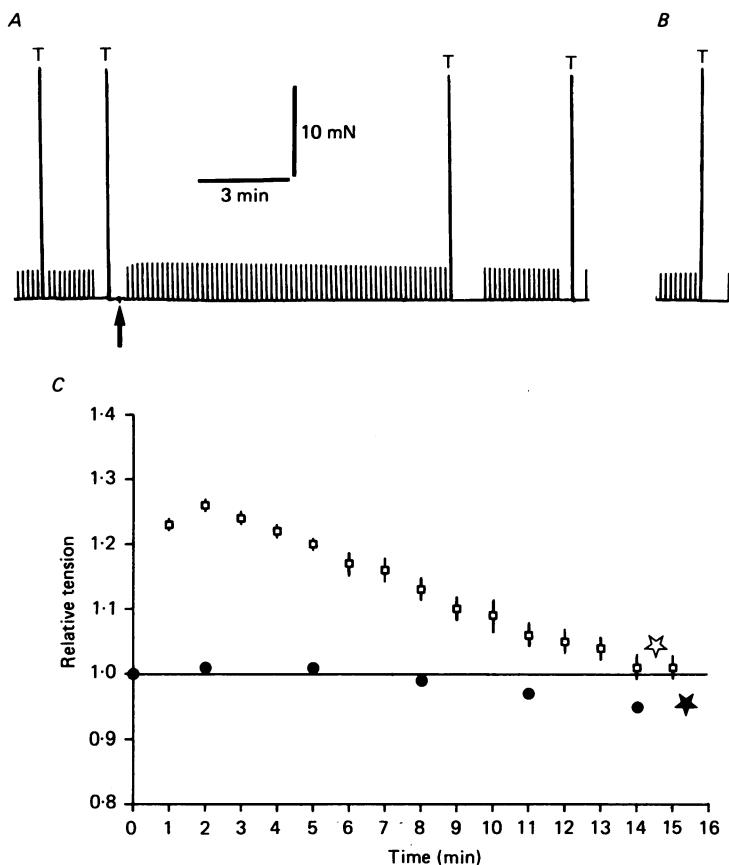
Fig. 1. Effect of calcium-free solution on twitch, tetanic and potassium contracture tension. *A* and *B*, chart records of twitches (small vertical deflections) and tetanic contractions (large vertical deflections, T). The first two tetanic contractions in *A* were in control solution containing 2·5 mM-Ca^{2+}. The arrow marks the change to calcium-free solution. Records in *B* were obtained after 15 min recovery in control solution. Horizontal calibration, 3 min. Vertical calibration, 10 mN. *C*, graph of average twitch (□) and tetanic (●) tension from twenty-seven preparations plotted against time in calcium-free solution. Tension was normalized to the mean tension in control solution before and after exposure to calcium-free solution. ☆: average 40 mM-potassium contracture tension in six preparations after 14–15 min in calcium-free solution. ★: average 200 mM-potassium contracture tension produced by seven preparations after 15–16 min in calcium-free solution. The vertical lines show ± 1 S.E.M. where this is greater than the dimensions of the symbols.

slowed recovery of contractions after potassium contractures. There was no detectable effect on the time course of twitches or tetanic contractions (20–80 % rise time or half-decay time).

Twitch and tetanic tension. In solutions containing no added calcium and no EGTA (Ca^{2+} free), there was an initial potentiation of the amplitude of twitches and, to a much lesser extent, of tetanic tension; the effect subsided within 5–30 min and was often followed by depression. These effects are illustrated in Fig. 1. In Fig. 1*A*, the potentiation of twitches produced by the Ca^{2+}-free solution (introduced at the arrow)
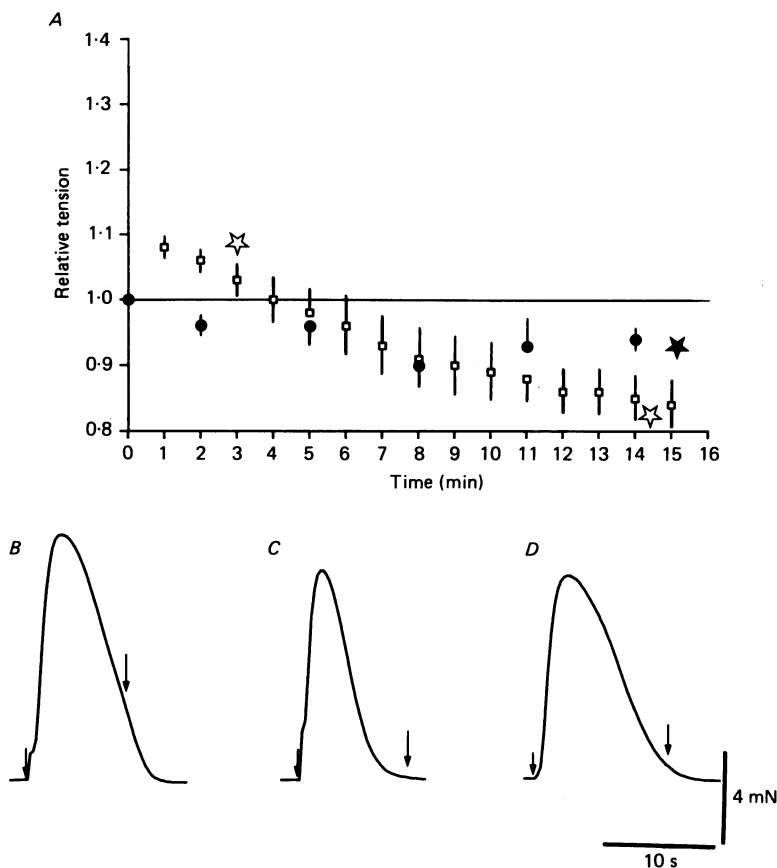
Fig. 2. Effects of 85 μM-Ca^{2+} on tension. *A*, graph of tension (relative to controls before and after exposure to 85 μM-Ca^{2+}) against time in 85 μM-Ca^{2+}. $\square$ and $\bullet$ (defined for Fig. 1) show average tension in seventeen bundles of fibres and the vertical bars indicate ± 1 S.E.M. $\stackrel{\wedge}{\approx}$: 40 mM-potassium contracture tension in one bundle of fibres after 3 and 14·5 min in 85 μM-Ca^{2+}. $\bigstar$: average 200 mM-potassium contracture tension in nine bundles exposed to 85 μM-Ca^{2+} for 15 min. *B, C* and *D*, 200 mM-potassium contractures recorded sequentially in control solution (*B*), after 35 min in 85 μM-Ca^{2+} (*C*), and after 15 min recovery in control solution (*D*). The arrows mark the start and stop of 200 mM-potassium flow. Horizontal calibration, 10 s. Vertical calibration, 4 mN.

was rapid in onset and then slowly disappeared. There was a slight depression of tetanic tension after 11 min in the Ca^{2+}-free solution when the twitch was still potentiated. After wash-out of the Ca^{2+}-free solution with control solution, twitch and tetanic tension returned to their former amplitude (Fig. 1*B*). The average time courses of the effects of a Ca^{2+}-free solution on twitches ($\square$) and tetanic contractions ($\bullet$) recorded in twenty-seven preparations are shown in Fig. 1*B*. The average twitch tension was increased by 25% after 2 min in the Ca^{2+}-free solution but there was comparatively little potentiation of tetanic tension. After 15 min, twitch tension had returned to the control level while tetanic tension was slightly reduced in amplitude (about 5%).

When the extracellular Ca^{2+} concentration was buffered at 85 μM with EGTA

(Table 1), there was less initial potentiation which lasted for a much shorter time, and more eventual depression of the twitch than in Ca^{2+}-free solution. The average time course of these changes measured in seventeen preparations is illustrated in Fig. 2. The twitch was potentiated by less than 10 % for 3–4 min and was depressed by more than 15 % after 15 min. There was no potentiation of tetanic tension which was depressed up to 10 % during the 15 min recording period.

Membrane potential. Muscle fibres become depolarized in solutions containing high concentrations of EGTA (Barrett & Barrett, 1978). It seemed possible that the changes in contraction described above could have been secondary to changes in membrane potential (Sandow & Khan, 1952; Hodgkin & Horowicz, 1960; Caputo & Giminez, 1967). However, the solutions containing EGTA together with both calcium and magnesium ions caused no depolarization: the average membrane potential in thirty-eight fibres in 2·5 mM-Ca^{2+} was $-77·8\pm0·8$ mV (mean ± 1 S.E.M.) and the average potential in twenty-four fibres in 85 μM-Ca^{2+} was $-75·9\pm1·1$ mV. Furthermore, membrane potentials in the solutions containing 50 μM-nifedipine or $(-)$ Bay K 8644 were respectively $-83·5\pm2·5$ (nine fibres) or $-77·64\pm1·2$ mV (fourteen fibres). Therefore the changes in contraction observed with these solutions cannot be attributed to membrane depolarization. The small hyperpolarization in 50 μM-nifedipine may have been due to blockage of a small steady-state calcium current but this was not investigated further.

Potassium contractures. The changes in twitch and tetanic tension induced by reducing Ca^{2+} concentration could have been secondary to changes in action potentials. However, the amplitude of potassium contractures was also affected by alterations in Ca^{2+} concentration. Two concentrations of potassium were tested: 40 mM-potassium which produced submaximal contractures of similar amplitude to twitches in soleus fibres (Dulhunty & Gage, 1985) and 200 mM-potassium which produced maximal contractures with tension similar to tetanic tension. In general, changes in 40 mM-potassium contracture tension mimicked changes occurring in twitches, whereas 200 mM-potassium contracture tension followed changes in tetanic tension. For example, after about 15 min in the Ca^{2+}-free solution, the average contracture produced by a 200 mM-potassium solution (seven preparations) was reduced in amplitude (★ in Fig. 1C) by much the same degree as tetanic tension, whereas a contracture produced by a 40 mM-potassium solution (☆ in Fig. 1C, six preparations) was potentiated like the twitches. In the 85 μM-Ca^{2+} solution (Fig. 2A), 40 mM-potassium contracture tension was at first potentiated (☆ at 3 min, one preparation) and then depressed (☆ at 14·4 min, one preparation) to much the same degree as twitch tension: at about 15 min, 200 mM-potassium contracture tension (★, nine preparations) was depressed less than the twitches but to much the same degree as tetanic tension.

In addition to affecting the amplitude of potassium contractures, the extracellular Ca^{2+} concentration also influenced their time course. In general, the decay of potassium contractures became faster when the Ca^{2+} concentration was lowered. This effect is illustrated in Fig. 2B. The decay of the 200 mM-potassium contracture in a solution containing 85 μM-Ca^{2+} (Fig. 2C) is clearly faster than in control solution before (Fig. 2B) or after (Fig. 2D) exposure to the low-Ca^{2+} solution.

The extracellular Ca^{2+} concentration also influenced the rate of recovery of twitch

and tetanic tension following a potassium contracture, as illustrated in Fig. 3. In control solution (Fig. 3A), twitch and tetanic tension had recovered their normal amplitude within 4–5 min after a 200 mM-potassium contracture whereas, in 85 μM-Ca^{2+} solution (Fig. 3B), recovery of twitch and tetanic tension after a 200 μM-potassium contracture were clearly much slower. The average rates of
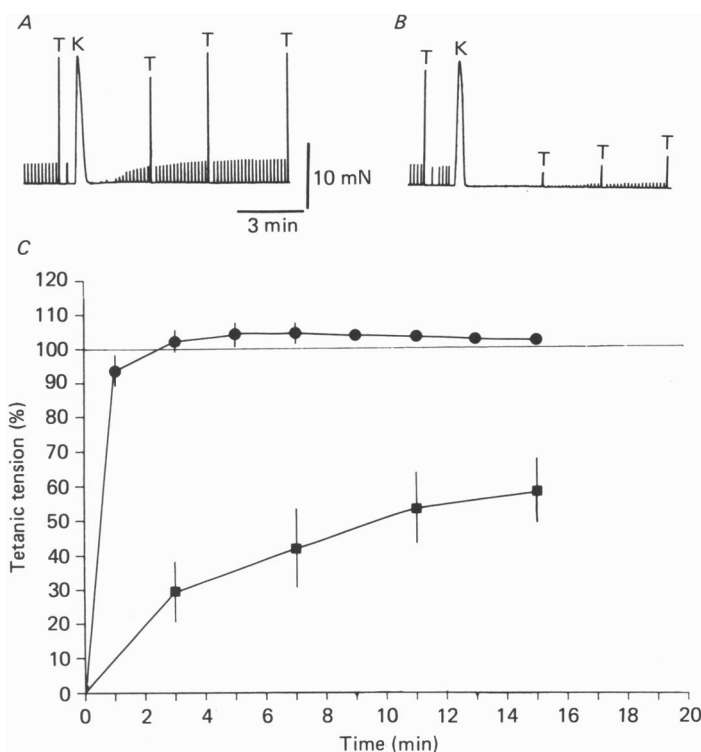

Fig. 3. Recovery of twitch and tetanic tension (T) following a 200 mM-potassium contracture (K) is slower in 85 μM-Ca^{2+} solution (B) than in control solution (A). The record in B was obtained after 15 min in 85 μM-Ca^{2+}. Horizontal calibration, 3 min. Vertical calibration, 10 mN. C, graph of tetanic tension against time after 200 mM-potassium contractures in control solution (●, mean ± 1 S.E.M. from seven preparations) and in 85 μM-Ca^{2+} solution (■, mean ± 1 S.E.M. from four preparations). Tension is expressed as the percentage of the tetanic tension recorded immediately before the potassium contracture.

recovery of tetanic tension following 200 mM-potassium contractures in control (●, seven preparations) and in 85 μM-Ca^{2+} solution (■, four preparations) are shown graphically in Fig. 3C. In control solution, recovery occurred within 3–4 min whereas in the 85 μM-Ca^{2+} solution there was still about 40% depression of tetanic tension 15 min after contractures. The effect of 85 μM-Ca^{2+} on the rate of recovery from 200 mM-potassium contractures varied considerably from preparation to preparation and this is reflected in the height of the error bars in Fig. 3. Full recovery was observed in about 20% of preparations in the 85 μM-Ca^{2+} solution and in 100% of preparations after return to the control solution.
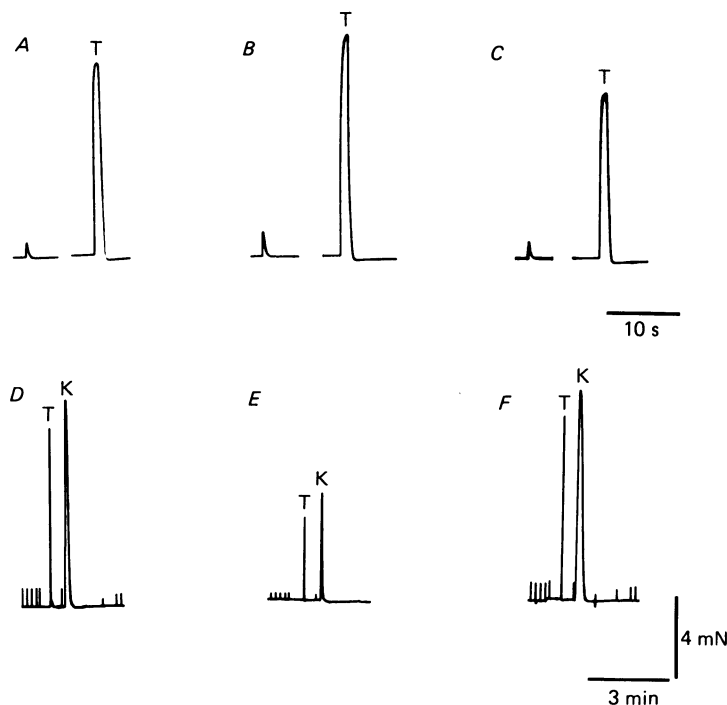
Fig. 4. Effects of 50 μM-nifedipine on twitches, tetanic contractions (T) and potassium contractures (K). A, B and C were obtained in the same preparation. A, control solution. B, after 15 min exposure to nifedipine in control solution. C, after wash-out of the nifedipine with control solution for 60 min. Horizontal calibration, 10 s. Records D, E and F were recorded from another bundle of fibres. D, after 20 min in 85 μM-Ca^{2+}. E, after 15 min in 85 μM-Ca^{2+} plus nifedipine. F, after 10 min wash-out with control solution. Horizontal calibration, 3 min. Vertical calibration for all traces, 4 mN.

Graf & Schatzmann (1984) found that tension remained depressed after a potassium contracture in calcium-free solution, even when preparations were returned to solutions containing the normal Ca^{2+} concentration for 22–25 min. In contrast, there was full recovery from inactivation of twitch, tetanus and potassium contracture tension caused by a 200 mM-potassium contracture in a solution containing 85 or 3 μM-Ca^{2+}, within 10–15 min of return to the normal solution containing 2·5 mM-Ca^{2+}. Examples of fully recovered twitches, tetanic contractions and potassium contractures following 200 mM-potassium contractures in low Ca^{2+} solutions can be seen in Figs 4F and 7H.

Effects of nifedipine

The influence of Ca^{2+} concentration on contraction could have been mediated by changes in depolarization-activated calcium currents across the membrane of the transverse tubules. Because dihydropyridines also block calcium currents, their effects were tested for comparison with the effects of lowered Ca^{2+} concentration. To ensure that calcium channels were blocked effectively (Lamb & Walsh, 1987), a high nifedipine concentration of 50 μM was used to probe the effects of the drug.
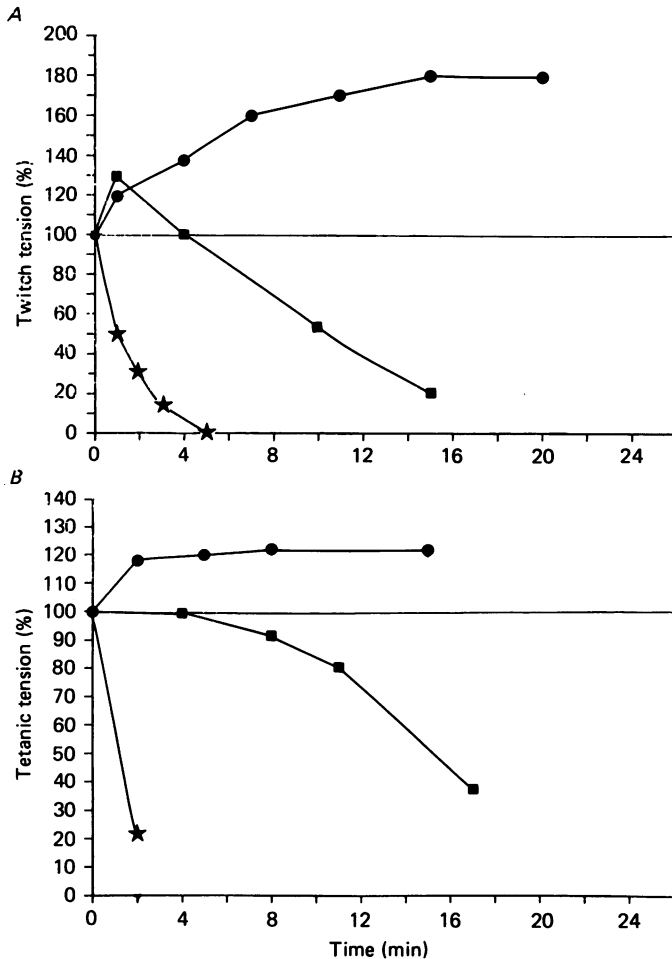
Fig. 5. The influence of 50 μM-nifedipine on twitch (A) and tetanic (B) tension in one bundle of fibres in solutions containing normal (●) and reduced (■ and ★) Ca^{2+} concentrations. Tension is expressed as the percentage of tension in the control or low-Ca^{2+} solution immediately before addition of 50 μM-nifedipine at time = 0. ●, 2·5 mM-Ca^{2+}. ■, 85 μM-Ca^{2+}. ★, 85 μM-Ca^{2+} following a 30 min exposure to 10 μM-nifedipine in 85 μM-Ca^{2+}.

Twitch and tetanic tension. In control solutions (2·5 mM-free calcium), nifedipine affected both twitch and tetanic tension, as illustrated in Fig. 4. There was a clear increase in twitch and tetanic tension after exposure to the solution containing nifedipine for 15 min (Fig. 4B) and these effects could be reversed by washing out the drug (Fig. 4C). It can also be seen that the increase in twitch tension (65 %) was larger than the increase in tetanic tension (25 %). Full development of the potentiation was slower for twitches (Fig. 5A, ●) than for tetanic contractions (Fig. 5B, ●). Similar effects were seen in three preparations.

When the extracellular free-Ca^{2+} concentration was buffered at 85 μM, both twitch and tetanic tension were reversibly depressed by nifedipine, as illustrated in Fig. 4$D–F$: both twitch and tetanic tension were clearly much smaller after 15 min

exposure to nifedipine (Fig. 4E) than in control solution (Fig. 4D) and had recovered to pre-exposure levels 10 min after wash-out of the drug. It was found that the depression of the twitch in these solutions was preceded by a brief period of potentiation, as illustrated in Fig. 5A (■). The progressive depression of twitch and tetanic tension appeared not to be complete after 15–18 min exposure to nifedipine when twitch tension had fallen to 20% and tetanic tension to 36% of control in this
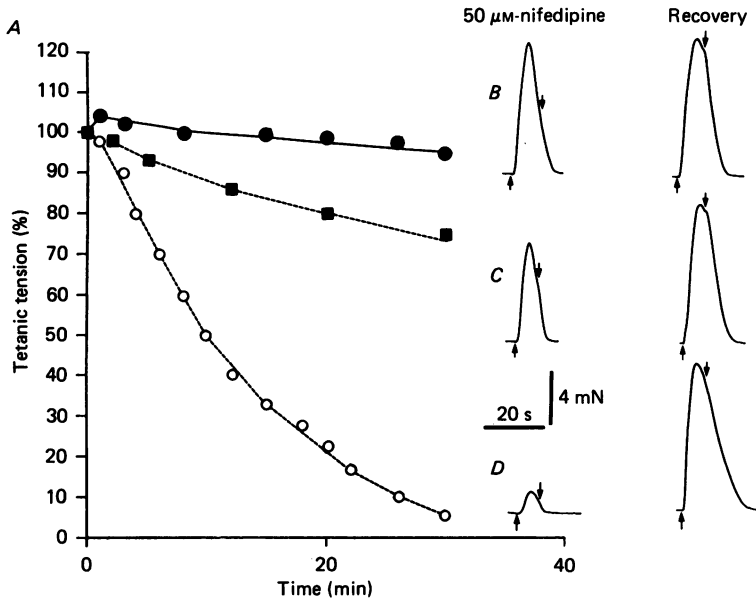

Fig. 6. The influence of Ca^{2+} concentration on the effect of 50 μM-nifedipine on tetanic and potassium contracture tension in one preparation. A, tetanic tension against time after addition of 50 μM-nifedipine to calcium-free (●), 85 μM-Ca^{2+} (■) and 3 μM-Ca^{2+} (O) solutions. Tension was calculated as described for Fig. 5B. B, C and D contain 200 mM-potassium contractures: the first trace after 30 min in nifedipine, the second following 15–20 min wash-out with the appropriate low-calcium solution. Arrows indicate the start and stop of 200 mM-potassium flow. B, calcium-free solution. C, 85 μM-Ca^{2+}. D, 3 μM-Ca^{2+}. Horizontal calibration, 20 s. Vertical calibration, 4 mN.

preparation. Similar effects were recorded in eight preparations. It was assumed that the time course of the effect was influenced by the rate at which nifedipine reached equilibrium at the site where it acted in the membrane. This assumption was supported by the observation that, in the same preparation, the depression of twitch and tetanic tension developed much more rapidly on changing from a control solution containing 85 μM-Ca^{2+} and 10 μM-nifedipine to a test solution containing 85 μM-Ca^{2+} and 50 μM-nifedipine (Fig. 5A and B, ★).

The rates of depression of twitch and tetanic tension by nifedipine were more rapid the lower the extracellular Ca^{2+} concentration. Results obtained in one preparation exposed successively to the Ca^{2+}-free, 85 μM-Ca^{2+} and 3 μM-Ca^{2+} solutions (●, ■ and O, respectively) are illustrated in Fig. 6A. In each case, nifedipine was added after the bundle of fibres had equilibrated in the low-calcium solution for 20 min. In

calcium-free solution, nifedipine caused an initial potentiation, followed by a slowly developing and relatively small depression (to about 93% of control after 30 min). In the solution containing 85 μM-Ca^{2+}, there was no initial potentiation and tetanic tension fell to about 75% of control at 30 min. With a lower buffered Ca^{2+} concentration (3 μM), tetanic tension had fallen to about 5% of control at 30 min.

Potassium contractures. As mentioned above, 200 mM-potassium contractures closely followed changes in tetanic tension in the one preparation. This is illustrated
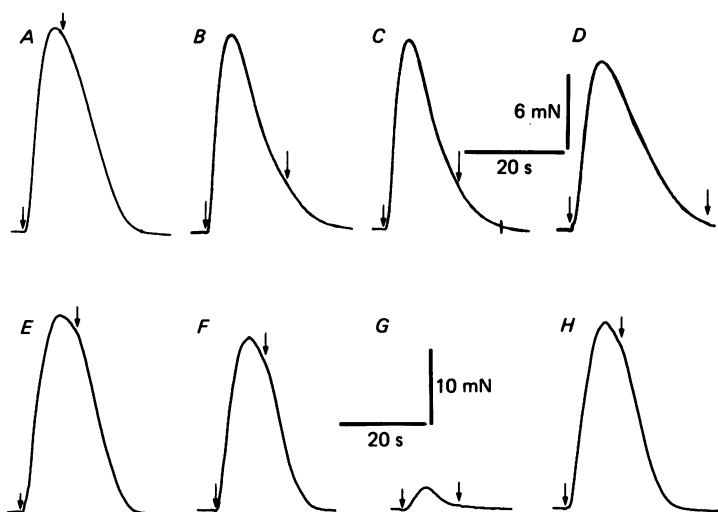

Fig. 7. The effect of nifedipine on 200 mM-potassium contractures in 2·5 mM-Ca^{2+} (*A–D*) and 85 μM-Ca^{2+} (*E–H*). The upper row of records were from one preparation, the lower row from another. Arrows denote the start and stop of 200 mM-potassium flow. *A*, control 2·5 mM-Ca^{2+}; *B* and *C*, control solution plus 50 μM-nifedipine for 14 min (*B*) and 35 min (*C*); and *D*, after 50 min wash-out of nifedipine with control solution. Vertical calibration, 6 mN. *E*, control 2·5 mM-Ca^{2+}; *F*, after 15 min in 85 μM-Ca^{2+}; *G*, after 25 min in 85 μM-Ca^{2+} plus 50 μM-nifedipine. *H*, after 25 min recovery in control solution. Horizontal calibration, 20 s. Vertical calibration, 10 mN.

graphically in the records in Fig. 6*B–D* (obtained from the same preparation as in Fig. 6*A*) of potassium contractures at the end of (and after) periods of exposure to nifedipine in solutions containing different Ca^{2+} concentrations (*B*, Ca^{2+} free; *C*, 85 μM-Ca^{2+}; *D*, 3 μM-Ca^{2+}). These records and those in Fig. 7 illustrate the depression of potassium contractures by nifedipine when the Ca^{2+} concentration is lowered. It can be seen in Fig. 7*A–D* that there was little if any depression of potassium contractures by nifedipine in the control solution (2·5 mM-free calcium) and it was possible to elicit many potassium contractures in the presence of 50 μM-nifedipine without causing paralysis (Fig. 7*C*). There was only slight depression of potassium contractures in Ca^{2+}-free solution (Fig. 6*B*). There was clear depression of the amplitude of potassium contractures in solutions containing nifedipine and 85 μM-Ca^{2+} (Figs 6*C* and 7*E–H*) or 3 μM-Ca^{2+} (Fig. 6*D*) and the effects could be reversed by washing out the drug (Figs 6*D* and 7*H*).

Nifedipine not only depressed the amplitude of potassium contractures but it also

made them decay faster. This effect was seen at all Ca^{2+} concentrations, from 2·5 mM to 3 μM, in eight preparations. The faster decay in the presence of nifedipine can be seen in some of the records in Figs 6 B–D and 7. However, the 200 mM-potassium solution was normally washed out before the contracture had decayed back to the baseline and this produced a faster decay than would be obtained as a result of inactivation if the potassium solution were not washed out. In an experiment in
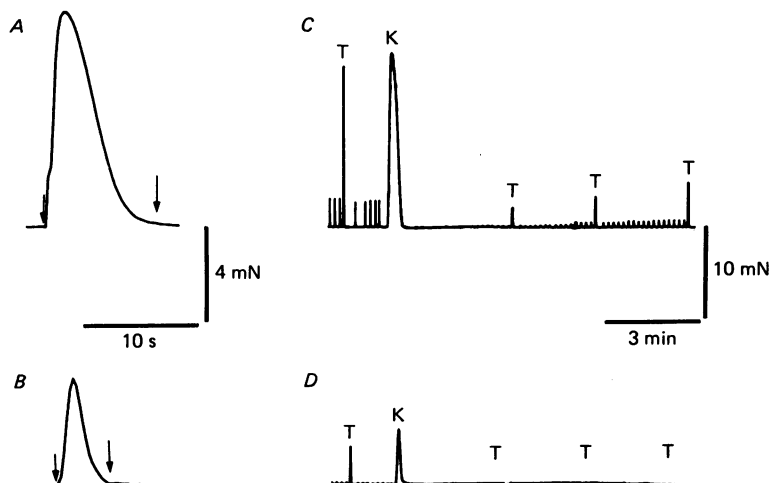

Fig. 8. Effect of 50 μM-nifedipine in 85 μM-Ca^{2+} on the time course of 200 mM-potassium contractures and the subsequent recovery of tension. A and B were taken from one bundle of fibres, 200 mM-potassium was added at the first arrow and was left on longer than usual (until the second arrow). A, 85 μM-Ca^{2+}. B, after 30 min in 85 μM-Ca^{2+} plus 50 μM-nifedipine. Horizontal calibration, 10 s. Vertical calibration, 4 mN. The records in C and D, from a different preparation, show twitches, tetanic contractions (T) and 200 mM-potassium contractures (K). C, recovery in 85 μM-Ca^{2+}. D, recovery from a contracture after 15 min in 85 μM-Ca^{2+} plus 50 μM-nifedipine. Horizontal calibration, 3 min. Vertical calibration, 10 mN.

which the 200 mM-potassium solution was not washed out early, the increased rate of decay produced by the nifedipine is clearly demonstrated (Fig. 8A and B).

The slower than normal rate of recovery from potassium contractures in the low-calcium solutions was slowed even further by addition of nifedipine. Fibres were essentially paralysed after a 200 mM-potassium contracture in a solution containing 85 μM-Ca^{2+} and 50 μM-nifedipine, as illustrated in Fig. 8C–D. Some recovery of twitch and tetanic tension could be obtained if the nifedipine was washed out with the 85 μM-Ca^{2+} solution, but a 20–30 min wash in control solution with 2·5 mM-Ca^{2+} was always required for full recovery. In contrast, addition of nifedipine to the normal solution (2·5 mM-Ca^{2+}) did not significantly affect the rate of recovery from potassium contractures.

Effects of (−) *Bay K 8644*

It could be argued that a calcium current across the surface membrane is essential for contraction but that contraction continues in solutions with a low free-calcium

concentration because the tubular Ca^{2+} concentration is maintained by intrinsic calcium buffers and a calcium pump (Barrett & Barrett, 1978). If nifedipine did not block all of the calcium channels, there might be sufficient calcium entry in the 2·5 mm-Ca^{2+} solution for excitation–contraction coupling: a combination of low calcium plus nifedipine might then totally block calcium entry. The negative isomer of another dihydropyridine, (−) Bay K 8644, enhances rather than depresses
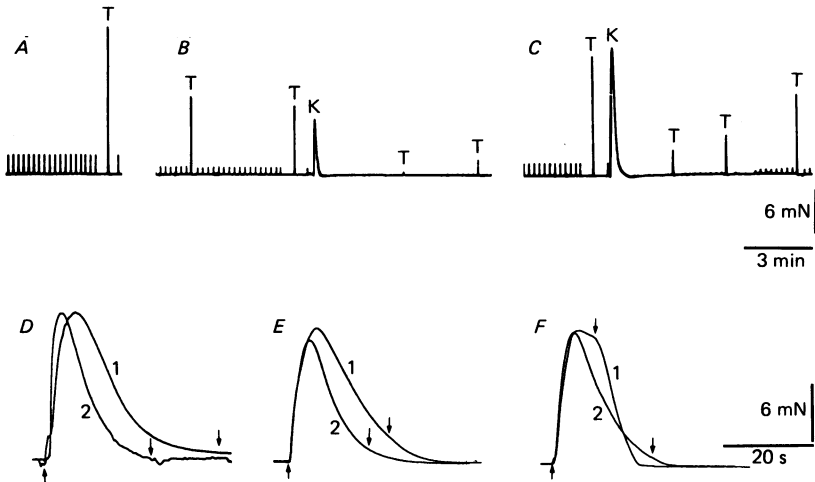

Fig. 9. Effects of Bay K 8644 on contraction. A–C, effects of (−) Bay K 8644 on twitches, tetanic contractions (T), 200 mm-potassium contractures (K) and recovery from contractures. A, after 20 min in 85 μm-Ca^{2+}. B, after 20 min in 85 μm-Ca^{2+} plus 50 μm-(−) Bay K 8644. C, after wash-out of (−) Bay K 8644 for 20 min with 85 μm-Ca^{2+}. Horizontal calibration, 3 min. Vertical calibration, 6 mN. D, E and F contain superimposed records of potassium contractures. Arrows show the start and stop of 200 mm-potassium flow. D, 85 μm-Ca^{2+} (1) and 85 μm-Ca^2 plus 50 μm-(−) Bay K 8644 (2). E, 85 μm-Ca^{2+} (1) and 85 μm-Ca^{2+} plus 50 μm of the racemate of Bay K 8644 (2). F, control 2·5 mm-Ca^{2+} (1) and control plus 50 μm of the racemate of Bay K 8644 (2). Horizontal calibration, 20 s. Vertical calibration, 6 mN.

calcium currents (Franckowiak, Bechem, Schramm & Thomas, 1985; Lamb & Walsh, 1987). The effects of this drug on contraction were investigated to test this idea.

Surprisingly, in solutions containing 85 μm-Ca^{2+}, 50 μm-(−) Bay K 8644 depressed tension (five preparations) as illustrated in Fig. 9A–C. The twitch, tetanic and potassium contracture tension in the presence of the drug fell to 50, 47 and 42 % of the control tension, respectively. The record in Fig. 9C, obtained following wash-out of the drug for 20 min, shows partial recovery of twitch, tetanic and potassium contracture tension. The depression of tension by 50 μm-(−) Bay K 8644 was less than the depression by 50 μm-nifedipine. In the same preparation as that illustrated in Fig. 9A–C, 50 μm-nifedipine caused a reduction in twitch, tetanic and potassium contracture tension to 25, 36 and 26 % of the controls, respectively.

As with nifedipine, the decay of potassium contractures was faster in the presence of (−) Bay K 8644 and this can be seen in the superimposed scaled records of the

potassium contractures in the presence (2) and after wash-out (1) of the drug (Fig. 9D). This is opposite to the reported lengthening of submaximal contractures produced by depolarization in voltage clamped fibres exposed to solutions containing Bay K 8644 (Ildefonse *et al.* 1985). Recovery from a potassium contracture in 85 μM-Ca^{2+} solution was slowed by exposure to ($-$) Bay K 8644, as can be seen by comparing traces in Fig. 9B and C.

The racemate of Bay K 8644, tested in four preparations, caused much less depression of twitch, tetanic and potassium contracture tension than nifedipine or ($-$) Bay K 8644 at the same concentration (50 μM). The records in Fig. 9E–F, obtained in one of the preparations, are unscaled: the racemate reduced potassium contracture tension to 87 % of normal in 85 μM-Ca^{2+} (Fig. 9E) and to 95 % in 2·5 mM-Ca^{2+} (Fig. 9F). However, the racemate and negative isomer of Bay K 8644 had similar effects on the rate of decay of potassium contractures. The racemate caused a clear increase in the rate of decay of the potassium contractures in the presence of both 2·5 mM-Ca^{2+} (Fig. 9F) and 85 μM-Ca^{2+} (Fig. 9E).

DISCUSSION

The results described in this paper provide information about the influence of extracellular calcium ions on contraction in a mammalian skeletal muscle and reveal a striking synergism between the effects of lowered calcium concentration, nifedipine and Bay K 8644, suggesting that they may act at common sites in the excitation–contraction coupling process.

Surprisingly, there was less initial potentiation and greater later depression of twitches in the EGTA-buffered, 85 μM-Ca^{2+} solution than in the unbuffered, Ca^{2+}-free solution containing 15 μM-Ca^{2+}. This could occur if there was an effective reservoir of calcium in the transverse tubular system, conceivably a buffered system or a pool constantly replenished by a calcium pump (Malouf & Meissner, 1979), that maintains calcium concentration above 85 μM in a Ca^{2+}-free (non-buffered) solution.

Some of the observations confirm and extend some apparently conflicting and paradoxical observations described previously, mostly in amphibian skeletal muscle. Potentiation of twitches seen in low-calcium solutions in amphibian skeletal muscle has been attributed to changes in membrane potential or surface charge (Caputo & Giminez, 1967), or to repetitive firing of action potentials (Armstrong, Bezanilla & Horowicz, 1972). The potentiation seen in our experiments cannot be explained in any of these ways. There was no membrane depolarization in low-calcium solutions containing 6 or 10 mM-magnesium. Furthermore, substitution of 6 mM-MgSO$_4$ for 8 mM-CaSO$_4$ caused no shift in the potential for half-maximum tension measured from potassium contracture tension–voltage curves, indicating that there was no change in surface potential (A. F. Dulhunty & P. W. Gage, unpublished observations). Finally, it has been shown that repetitive firing of action potentials increases twitch contraction time (Close & Hoh, 1968) but there was no change in time course of the potentiated twitches in low-calcium solutions. (In an occasional preparation, some signs of repetitive action potential activity were observed in low-calcium solutions containing 10 mM-magnesium. However, this activity was unsynchronized in the five to ten fibres in a bundle and resulted in a long tail on the

twitch without a change in peak amplitude.) In addition, 40 mM-potassium contractures, that do not depend on action potentials, were increased in amplitude in low-calcium solutions. It would seem that lowering calcium concentration has an effect on excitation–contraction coupling not arising from changes in membrane field or from repetitive action potentials.

Depression of contraction in low-calcium solutions has also been reported previously in both amphibian and mammalian muscle (Luttgau, 1963; Edman & Grieve, 1964; Caputo & Giminez, 1967; Armstrong *et al.* 1972; Barrett & Barrett, 1978; Luttgau & Spiecker, 1979; Cota & Stefani, 1981; Caputo, 1981; Bolanos, Caputo & Velaz, 1986; Kostius, Muchnik & Paz, 1986; Luttgau *et al.* 1987). The depression is not due to membrane depolarization because tension remains depressed when depolarization is prevented by adding magnesium ions (Luttgau & Spiecker, 1979; Cota & Stefani, 1981) or clamping the membrane potential at normal levels (Barrett & Barrett, 1978; Caputo, 1981; Bolanos *et al.* 1986; Luttgau *et al.* 1987). Nor is the depression of contraction due to suppression of transmembrane calcium currents as contraction can persist when these currents are inhibited, e.g. by nifedipine (Lamb, 1986) or a variety of calcium channel blockers (Gonzalez-Serratos, Valle-Aguilera, Lathrop & del Carmen Garcia, 1982).

The increased rate of decay (inactivation) of potassium contractures in low-calcium solutions has been observed both with (Luttgau & Spieker, 1979; Cota & Stefani, 1981; Caputo, 1981; Bolanos *et al.* 1986; Luttgau *et al.* 1987) and without calcium buffers (Luttgau, 1963; Caputo & Giminez, 1967) and the slowed recovery of excitation–contraction coupling (restoration) after potassium contractures in low-calcium solutions has been described in amphibian preparations (Caputo, 1981; Luttgau *et al.* 1987).

The effects of the dihydropyridines on contraction have previously been examined only in the presence of normal calcium concentrations. Nifedipine causes potentiation of twitches (Gallant & Goettl, 1985) but little change in tetanic or maximal contracture tension (Luttgau *et al.* 1987) in non-depolarized fibres. In depolarized fibres, nifedipine depresses calcium transients and contraction (Rios & Brum, 1987; Rakowski *et al.* 1987). It was not expected that Bay K 8644 would have similar effects to nifedipine on contraction as it normally has an opposite effect on calcium currents. However, it has been found that Bay K 8644 like nifedipine can depress calcium currents in depolarized calf Purkinje fibres (Sanguinetti, Krafte & Kass, 1986).

The observations described above can be explained in terms of a simple model which is essentially an extension of several previous models (e.g. Hodgkin & Horowicz, 1960). It is proposed that a protein 'precursor' molecule, P, in the membrane of the transverse tubules is converted by membrane depolarization to an activated state, A, essential for excitation–contraction coupling. In order to account for the potentiation of twitches when calcium concentration is lowered, it is proposed that formation of the activated state involves a rapid, depolarization-induced conformational change followed by rapid dissociation of calcium. During prolonged depolarization, A becomes slowly converted to an inactive state, I, and perhaps further inactivated (paralysed) states (see review by Luttgau *et al.* 1987). In order to explain the slowed recovery from inactivation following potassium contractures in

the presence of low calcium concentrations, it is proposed that conversion of A to I involves further dissociation of calcium. These processes can be represented by

$$P \rightleftharpoons Q \underset{Ca^{2+}}{\rightleftharpoons} A \underset{Ca^{2+}}{\rightleftharpoons} I,$$
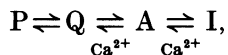
where P to Q represents a conformational change induced by membrane depolarization, reversed by membrane repolarization, and Q to A and A to I are calcium dissociation steps. There is not sufficient information to determine whether reconversion of I to P is through A as shown or via a separate multistep pathway. The effects of the dihydropyridines can be explained by proposing that they reduce the affinity of A and I for calcium, perhaps by binding to allosteric sites. It should be emphasized that this model is surely an oversimplification and that single steps almost certainly represent many steps.

In terms of this reaction scheme, a brief depolarization would increase k_{PQ} (rate constant of P to Q) to generate Q, with subsequent formation of A as calcium dissociates from Q. At low Ca^{2+} concentrations or in the presence of the dihydropyridines, the rate of reconversion of A to Q would be slower than normal so that the average dwell time in A would be increased and this would tend to increase the amount of A (potentiation of twitches) and to increase the probability of transition into an inactivated state. This latter effect could, with time, lead to a decrease in A (depression of twitches) as more of the molecules enter an inactivated state, especially as recovery from the inactivated state would also be slowed in low-calcium solutions or in the presence of the dihydropyridines. During prolonged depolarizations, k_{AI} would become more significant and, as more of the molecules became inactivated, tension would be depressed as seen during potassium contractures. This would occur more rapidly at low Ca^{2+} concentrations or in the presence of dihydropyridines because of depression of k_{AQ} and k_{IA}. Furthermore, in such solutions, the rate of recovery of excitation–contraction coupling after potassium contractures would be depressed because of the decrease in k_{IA}. It is interesting that this scheme is similar in many respects to that recently proposed to explain the effects of low Ca^{2+} concentrations on depolarization-activated potassium channels (Armstrong & Lopez-Barneo, 1987) and may reflect a general mechanism of channel activation.

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