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EFFECT OF CAFFEINE ON INTRAMEMBRANE CHARGE MOVEMENT AND CALCIUM TRANSIENTS IN CUT SKELETAL MUSCLE FIBRES OF THE FROG

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SUMMARY

1. The authors have studied the effect of caffeine in subthreshold concentration (0·5 mmol l^{-1} at 2–4 °C) on the contraction threshold, on intramembrane charge movement and calcium transients in voltage-clamped frog skeletal muscle fibres.

2. The single-gap technique (Kovács & Schneider, 1978) was used for the voltage clamping of terminated segments of cut fibres. Ionic conductances were minimized by using caesium glutamate at the open end pool and tetraethylammonium sulphate and tetrodotoxin at the closed end pool.

3. Myoplasmic calcium transients evoked by depolarizing pulses were recorded by measuring the changes in absorbance of the fibres at 720 nm after the intracellular application of Antipyrylazo III dye.

4. The strength–duration curve for contraction threshold was shifted towards more negative membrane potentials in the presence of caffeine. Shift was more definite at shorter pulse durations than at the rheobase.

5. The total amount of charge moving during the depolarizing pulses at different membrane potentials was not changed by caffeine treatment, whereas the threshold amounts of charge moved during the critical periods of the contraction threshold decreased at different voltages (by about 23 %).

6. In the presence of caffeine, calcium transients accompanying long (100 ms) depolarizing pulses showed increased voltage-dependent peak amplitudes, rising phases and rate coefficients referring to calcium release, but a decreased voltage-dependent re-uptake rate either during or after the pulse.

7. Calcium transients evoked by depolarizing pulses along the strength–duration curve for contraction threshold gave the same peak amplitudes (ranging from 0·9 to 2·8 μmol l^{-1} free myoplasmic calcium on different fibres), but membrane-potential-dependent latency times and rising phases. The rate coefficients for declining phase did not depend on the preceding pulse voltage.

8. On applying caffeine, the calcium transients related to the contraction threshold also had equal but smaller peak amplitudes, shorter latency times and the same magnitude of voltage-independent rate coefficients for the declining phase as in the control solution.

9. The twitch potentiating effect of caffeine can be explained by its facilitating

calcium release from the sarcoplasmic reticulum, while the re-uptake rate is not modified. The apparent inhibition of re-uptake can be related to the enhanced release of calcium due to caffeine effect. Due to the sensitizing effect of caffeine on the sarcoplasmic reticulum membrane, smaller amounts of charge are needed to reach the contraction threshold than without caffeine.

INTRODUCTION

The excitation–contraction coupling of skeletal muscle fibres consists of a sequence of steps leading from depolarization to contraction. The depolarization of T-tubules results in the increase of myoplasmic calcium concentration which initiates the activity of myofilaments. The movement of charged particles is considered as a voltage-sensitive step of the activation of contraction (Schneider & Chandler, 1973). The use of metallochromic indicator dyes has made the recording of myoplasmic calcium changes possible with a time resolution convenient to study the relation of charge movement to calcium release (Kovács, Rios & Schneider, 1979). Experimental results have provided evidence that the movement of charges is capable of controlling calcium release (Schneider, Rios & Kovács, 1981; Schneider, 1981).

Twitch potentiators can be useful tools for studying the connexions between charge movement and calcium release. One of the widely used drugs is caffeine, which can lead to the contracture of skeletal muscle fibres without changing the membrane potential (Axelsson & Thesleff, 1958). At subthreshold concentrations it can potentiate twitches without changing the shape of the action potentials (Sandow, Taylor, Isaacson & Seguin, 1964), it can shift the activation curve determined by potassium contractures (Lüttgau & Oetliker, 1968) or can bring about 'sarcomeric oscillations' (Kumbaraci & Nastuk, 1982).

In our experiments we studied the effects of caffeine in subthreshold concentration on the contraction threshold, charge movement and calcium transients in voltage-clamped cut skeletal muscle fibres. It is supposed that the essential effect of caffeine is sensitizing the sarcoplasmic reticulum (s.r.) membrane to the movement of charged particles, since calcium release accompanying depolarizing pulses was enhanced without changing the charge movement or the re-uptake rate of calcium from the myoplasm. Some of the results have been presented previously (Kovács & Szűcs, 1981).

METHODS

Preparations and solutions

The experiments were carried out on single fibres dissected from the semitendinosus muscle of frogs (*Rana esculenta*) in solution C1. (The composition of the solutions is given in Table 1.) The fibres were cut in relaxing solution (solution A1) at 4–5 mm from the tendon and mounted in a two-pool chamber where a single vaseline gap separated the pool containing the open end (Pool A) from the pool containing the closed end (Pool C). The length of intact terminated segments (l_C) was in the range 355–543 μm, the diameter of the fibres (d) was 77–112 μm with sarcomere lengths (s) of 2·4–3·0 μm. After completing separation, the solutions were changed for solutions A2 (Pool A) and C2 (Pool C) to block all ionic conductances. This procedure decreased the potential decrement along the terminated segment and made the measurement of charge displacement currents possible. Further details of the dissection and mounting procedures have been described by Kovács & Schneider (1978).

The chamber was cooled by Peltier devices (VILKUT, Hungary) to 2–4 °C, temperature was monitored with a thermistor positioned near the fibres in Pool C.

Voltage-clamping of the fibres

A compensation circuit (Kovács & Schneider, 1978) was used to follow the membrane potential (V'_m) and the membrane current (V'_I). Compensation was set to have a V'_m signal superimposable on the transient recorded by micro-electrodes (V_m). The membrane potential of the intact segment was set to −100 mV. The depolarizing and hyperpolarizing pulses were induced by a digital pulse

TABLE 1. Composition of experimental solutions

	External solutions*						
	Na	K	TEA	Cs	Ca	Cl	SO_4
C1	115	2·5	—	—	1·8	121·1	—
C2	—	—	150	10	8	—	88
	Internal solutions						
	K	Cs	Mg	Glutamate	Cl	EGTA	ATP
A1	120	—	2	120	4	0·1	—
A2	—	120	2	120	4	1	0·5

All concentrations are in mmol l^{-1}. Solution C1 contained 2 mmol l^{-1}, the others 5 mmol l^{-1} Tris–maleate buffer (pH 7·0).

* Solution C2 contained $3·1 \times 10^{-7}$ mol l^{-1} TTX. Abbreviations: ATP, adenosine 5′-triphosphate; TEA, tetraethylammonium sulphate; TTX, tetrodotoxin; EGTA, ethyleneglycol-bis(β-amino-ethylether)*N*,*N*′-tetraacetic acid.

generator (HTSZ, Hungary) and pulse conditioner. To avoid oscillation, the time constant of pulses was set 0·1–0·05 ms.

Caffeine was applied by changing the solution of the closed end pool (solution C2) for the caffeine-containing one. While changing the solutions the membrane potential was maintained by a steady voltage (E_P) equal to the output voltage of the clamping amplifier.

Optical set-up

The absorbance of the fibres was measured in a way similar to that described by Kovács & Schneider (1977). To decrease the noise caused by the vibration of the environment, the whole optical set-up was placed on a heavy plate (wooden and iron plates glued together, weighing 200 kg) standing on tennis balls. The light of a tungsten–halogen bulb (12 V, 100 W Tungsram) powered by two lorry batteries was collected by condensing lenses into a horizontal beam, then projected through an adjustable slit and interference filters (Hungarian Optical Works and Ditric Optics). The monochromatic light beam was directed upward by a front-silvered mirror and focused by a microscope objective (× 10) to have a sharp-edged rectangular cross section at the level of the fibre. The illuminated part of the terminated segment was about 150 μm long and half as wide as the diameter of the fibre.

The transmitted light was collected by a water immersion objective with long working distance (× 40, 0·75 N.A., Zeiss 561702) and projected on a photo-diode (PV 100, EG and G) located in the phototube of a compound microscope. The photo-diode was connected to a low noise amplifier (Analog Devices 52 J) also placed in the phototube. The resting light intensity (I) was measured by a d.c. coupled digital volt-meter, the changes in light intensity (ΔI) due to the depolarizing pulses were monitored using a high-gain a.c. coupled amplifier with 1 kHz upper and 0·1 Hz lower frequency cut-off (MIKI, Hungary).

To test the reliability of our optical system the absorbance spectra of calcium-free and calcium-containing Antipyrylazo III solutions were determined. The measurements were carried out in a glass tube of 1 mm internal diameter at room temperature. Then 0·1 mmol l^{-1} dye was dissolved either in the relaxing solution (solution A2) or in the same solution containing 2 mmol l^{-1} $CaCl_2$. The relative absorbance spectra referred to the A_{550} were the same as the identical spectra determined with a spectrophotometer.

Digital processing of the data

Both the current (V_I') and the optical (ΔI) transients were digitized and averaged by an Averaging Converter (EMG). Each signal was sampled 202 times by the 8 bit A/D converter of this device. The averaged data were stored on punched paper tapes and were analysed off-line by a microcomputer (HT 680X, HTSZ) having an 8 bit microprocessor (Motorola 6800). The programmes for data analysis were made in Basic. The digital data were converted into analogue form by a 10 bit D/A converter and recorded by a plotter.

Strength–duration curves for contraction threshold

Contraction threshold was determined by depolarizing voltage-clamped pulses with increasing amplitude at constant duration. The just-visible movement at the tendon end of the fibres was observed through a compound microscope ($\times 400$) equipped with a water-immersion objective. The observer applied the pulse without knowing its amplitude. The 100 ms duration was sufficient to determine the rheobase, so longer depolarization was not applied.

After the mounting procedure, the fibres were repolarized by the voltage-clamp circuit. The rheobase was determined 15 and 30 min following repolarization and the strength–duration curve was recorded only if the rheobase was stable. The rheobase was determined at the beginning and at the end of each sequence of measurements. The strength–duration curve was analysed only if the difference between the two rheobase data did not exceed 1 mV. In most cases the same values were obtained.

For the quantitative description of strength–duration curves in a certain potential range the eqn. (1) originally suggested by Adrian, Chandler & Hodgkin (1969) was used:

$$(V+C)t_C = B_T, \tag{1}$$

where V is the membrane potential during the pulse, t_C is the pulse duration, while C and B_T are the fitted variables.

In our experiments this function was found to be valid for membrane potential values more positive than -30 mV, or sometimes more than -40 mV, that is, for shorter pulse durations (5–10 ms). To determine the threshold amount of charge or to evoke calcium signals at the contraction threshold sometimes we used the calculated critical duration to reach the contraction threshold at different membrane potentials. With this procedure the uncertainties in the individual determinations of contraction threshold were considerably reduced.

Measurement of myoplasmic calcium transients

The changes in intracellular calcium concentration in response to pulse depolarization have been monitored by Antipyrylazo III in a similar way as described previously (Kovács *et al.* 1979). Calcium transients were recorded by measuring changes in absorbance of the fibres at 720 nm (ΔA). The ΔA values were approximated by $-0{\cdot}43\,\Delta I/I$. The dye applied in 1 mmol l^{-1} concentration at the open end pool (solution A2) entered the myoplasmic space of the fibre, causing a shift in the absorbance spectrum of the terminated segment. The most significant increase was observed at 550 nm. By increasing intracellular dye concentration the size of calcium transients was also increased, thus, to get comparable data, the calcium transients were normalized to the identical resting absorbance value at 550 nm ($\Delta A/A_{550}$).

The increase of intracellular dye concentration was much slower than that found by Kovács *et al.* (1979) using a double-gap chamber. The longer diffusion time can be explained by the longer path length for the dye in the single-gap system where, in most cases, 2–3 h of waiting were necessary to reach a dye concentration sufficient to render calcium transients detectable. To maintain the excitation–contraction coupling intact, the fibres were repolarized soon after mounting and cooling. In the waiting period the change in contraction threshold did not exceed 2 mV.

The changes in myoplasmic calcium concentration (ΔCa) were determined using the procedure suggested by Rios & Schneider (1981) and Kovács, Rios & Schneider (1983). They have found that the calcium dye reaction is of second order in dye (CaD_2) so to calculate ΔCa both the ΔA values and the total dye concentration (D_T) have to be taken into account. By their calibrating procedure D_T was determined using a molar extinction coefficient (ϵ_{550}) of $2{\cdot}55 \times 10^4$ M^{-1} cm^{-1}. The dissociation constant (K) of $1{\cdot}32 \times 10^{-8}$ M^2 was substituted by the apparent dissociation constant ($K' = 1{\cdot}33 \times K$) owing to the presence of free magnesium (~ 1 mmol l^{-1}) in the myoplasmic space. Because of the

uncertainties in estimating the absolute ΔCa, the $\Delta A/A_{550}$ records are presented in the Figures. The calculated D_T and ΔCa values are indicated in the legends of the Figures and in the Tables.

Measurement of intramembrane charge movement

To obtain charge displacement current records (I_Q) in the single-gap system, the authors used a procedure similar to the one described by Horowicz & Schneider (1981*a*). Depolarizing and hyperpolarizing pulses of 100 ms were applied and the current transients (V_I') were recorded. Generally an average of eight signals was necessary to reach an acceptable signal-to-noise ratio. To avoid very strong contractions the pulse durations were decreased to 60 ms at large depolarizations, in particular in the presence of caffeine. Each depolarizing pulse was followed by a hyperpolarizing one with half amplitude, allowing the mean linear capacitative transients to be constructed.

In order to isolate I_Q records, each V_I' transient was analysed separately. First of all the ionic current components were subtracted then, using transients related to the hyperpolarizing pulses, the mean linear capacitative current record was calculated. The linear capacitative components were subtracted from the on and off parts of the records belonging to the depolarizing pulses, using the first forty points of the off part of the mean linear capacitative transient (Horowicz & Schneider, 1981*a*).

The amounts of charge displaced during depolarization (Q_{on}) and moving back due to repolarization (Q_{off}) were determined at each membrane potential by integrating the on and off parts of each I_Q record. Both the I_Q and Q values were expressed relative to the linear capacitance of the fibres, calculated from the off part of the mean linear capacitative transients. Both the mean linear capacitative transient and the linear fibre capacitance were determined for each sequence of measurements separately, before and after caffeine treatment.

Analysis of the data

The peak amplitude of the optical signals was calculated from the five consecutive points with the highest average value. The time-to-peak value was determined as the time from pulse onset to the middle of the five points representing the peak amplitude. The rate of rise was calculated by a least-squares fit to the apparently linear rising phase of the signals. Latency time was taken as the time from the start of the pulse to the point which is the first of two consecutive points deviating from the base line by more than twice its standard deviation. The rate coefficients for calcium movement between intracellular compartments were calculated from calcium transients by a non-linear least-squares fit described by Scarborough (1966).

The steady-state distribution of Q_{on} values as a function of membrane potential was characterized by the two-state Boltzmann model using a non-linear least-squares curve fitting (Scarborough, 1966).

The significance of differences was determined by Student's paired t test.

RESULTS

Strength–duration curves for contraction threshold

In agreement with the published data (Sakai, 1965; Lüttgau & Oetliker, 1968), 1 mmol l^{-1} caffeine was found to cause spontaneous contracture at 2–4 °C. Therefore, the drug was applied in a concentration of 0·5 mmol l^{-1} in every case. A potentiating effect developed within 1 or 2 min after drug application so the measurements were started in the 5th minute of caffeine treatment. On repeating the determination of the strength–duration curves for contraction threshold in the presence of caffeine, no change in drug effect was found. After washing out the caffeine, complete recovery could occur.

The strength–duration relation for just-detectable contraction was shifted towards more negative membrane potentials due to caffeine treatment (Fig. 1). This shift was more definite at shorter pulse durations than at longer ones. To describe this behaviour, the changes in both the rheobase and the membrane potentials at brief

pulses were analysed separately. The rheobase determined by 100-ms-long pulses on ten fibres decreased significantly ($P < 0.001$) due to caffeine treatment (Table 2).

The data obtained in the more positive potential range of the strength–duration curve (that is at brief pulses) were fitted by eqn. (1) and the slope of the curve (B_T) which can be taken as a mechanically effective area was determined. Adrian *et al.*

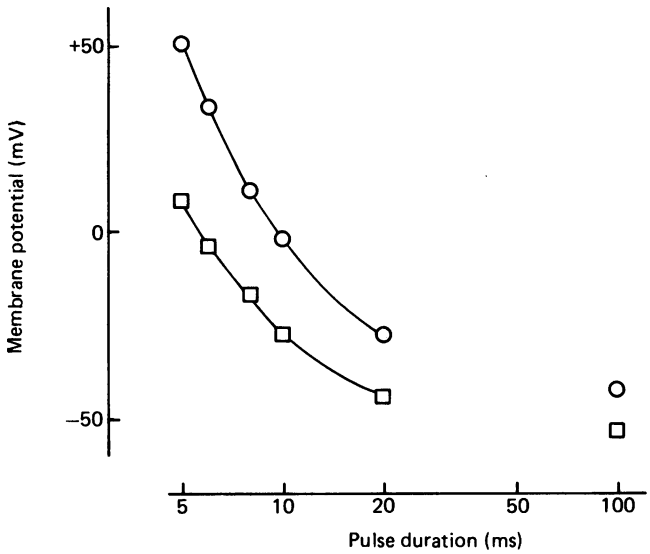

Fig. 1. Effect of caffeine (0·5 mmol l^{-1}) on the strength–duration curve for the contraction threshold. Membrane potential values during the pulses necessary to get just-detectable contraction in an individual fibre are plotted *vs.* pulse duration. The continuous lines represent the best fit to eqn. (1); $B_T = 523{\cdot}86$ and $350{\cdot}21$ mV ms; $C = -54{\cdot}15$ and $-61{\cdot}98$ mV in the control solution (○) and in the presence of caffeine (□), respectively. Fibre 00117, $d = 90$ μm, $s = 2{\cdot}45$ μm.

TABLE 2. Effect of caffeine on strength–duration curve for contraction threshold

	Control	Caffeine	Control − caffeine
Rheobase (mV)	$-48{\cdot}6 \pm 5{\cdot}0$	$-55{\cdot}4 \pm 6{\cdot}4$	$6{\cdot}8 \pm 3{\cdot}5$
B_T (mV ms)	$408{\cdot}8 \pm 105{\cdot}5$	$315{\cdot}3 \pm 88{\cdot}3$	$93{\cdot}5 \pm 43{\cdot}7$

Values represent the means (±S.D.) of data obtained on ten different fibres. The changes (control – caffeine) were significant ($P < 0{\cdot}001$).

(1969) have found that in case of membrane potentials more positive than −10 mV, the area of a portion of the depolarizing pulses (B_T) was the same even in different fibres (about 120 mV ms at 4 °C). In our experiments the value of B_T varied from fibre to fibre in accordance with the finding of Costantin (1974) and we could extend the fitting procedure over the range of more negative membrane potentials (up to −30, −40 mV). Due to the caffeine effect, B_T decreased on the above mentioned ten fibres ($P < 0{\cdot}001$, Table 2) although a parallel shift was found on a few fibres.

Caffeine effect on charge movement

To gain information on the mechanism which enables caffeine to modify the excitation–contraction coupling of muscle fibres, the charge movement process was

investigated. The cut-fibre preparation was very convenient for this purpose because it allowed depolarizing pulses to reach and go beyond the contraction threshold (Horowicz & Schneider, 1981*a*). Nevertheless, the strong movement, in particular in the presence of caffeine, caused some limitation, therefore, the pulses could not exceed the membrane potential of −20 mV. Determination of the threshold amount of charge necessary to reach the contraction threshold was also restricted to this membrane potential range.

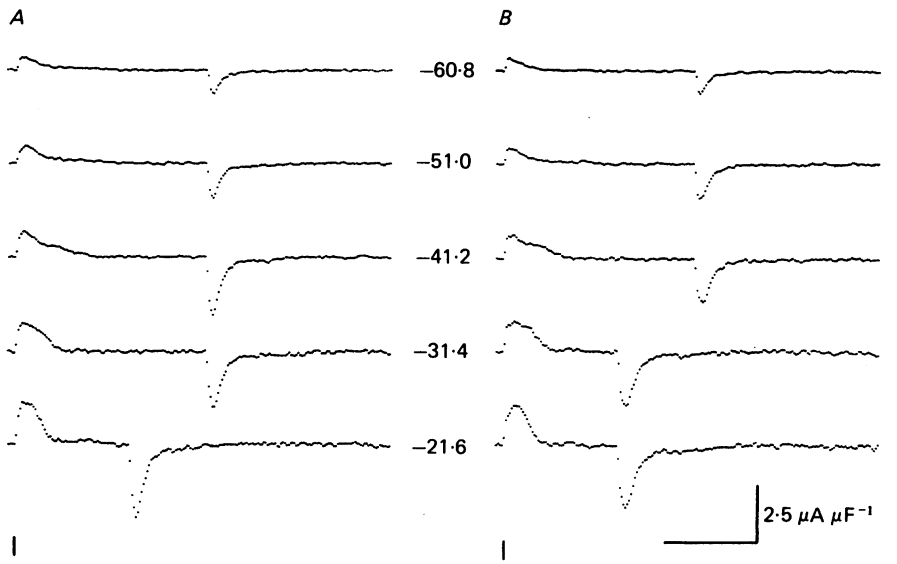

Fig. 2. Charge-displacement currents recorded in control solution (*A*) and in the presence of caffeine (0·5 mmol l^{-1}) (*B*). The onset of depolarizing pulses is indicated by vertical bars. Membrane potentials are shown between the corresponding traces. Time calibration represents 50 ms. Fibre 90410.

Charge-displacement currents recorded in the same fibre are shown in Fig. 2. Although the 8 bit resolution of the A/D converter did not result in very nice records, the time course and the voltage dependence of charge movement seem to be the same within the noise limit before (Fig. 2*A*) and after (Fig. 2*B*) caffeine treatment. Seven similar experiments were carried out applying depolarizing pulses to the same membrane potentials. The mean (±S.E. of the mean) of Q_{on} values obtained in these seven fibres are plotted *vs.* membrane potential in Fig. 3*A*. In two fibres, no V_I' transients were recorded at −30·7 mV and −20·8 mV, either in the control solution or in the caffeine.

To analyse the caffeine effect on the voltage dependence of charge movement, the equation for the two-state Boltzmann model was used which is generally accepted to describe the steady-state distribution of charges (Q) as a function of membrane potential (V) (Schneider & Chandler, 1973; Chandler, Rakowski & Schneider, 1976; Adrian & Almers, 1976):

$$Q = Q_{max}/[1+\exp-(V-\overline{V})/k], \quad (2)$$

where Q_{max} means the maximum charge movement, $\overline{V}$ defines the voltage at which

50% of the charges move, $1/k$ gives the steepness of the Q *vs.* V relationship. The continuous line in Fig. 3*A* is the best least-squares fit of eqn. (2) to the mean values of Q_{on} in the control solution. The parameter values were 33·3 nC μF^{-1} for Q_{max}, −42·3 mV for $\overline{V}$ and 12·7 mV for k. In the presence of caffeine the best fit (interrupted line) was found with the parameter values: Q_{max} = 33·1 nC μF^{-1}, $\overline{V}$ = −44·3 mV, and k = 12·3 mV. It is obvious that caffeine does not modify the intramembrane charge movement, at least in this potential range and at the 'subthreshold' concentration of 0·5 mmol l^{-1}.

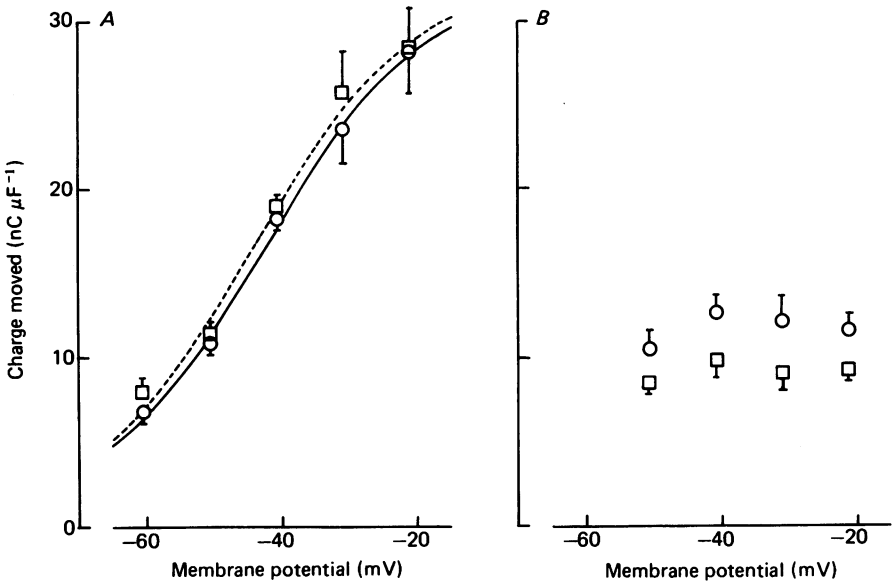

Fig. 3. Effect of caffeine (0·5 mmol l^{-1}) on charge movement during depolarizing pulses of 100 or 60 ms (*A*) and during the critical durations necessary to reach the contraction threshold (*B*). Circles and squares give the mean values of Q_{on} (*A*) and Q_{th} (*B*) obtained from seven fibres in control solution and in the presence of caffeine, respectively. Error bars give ±S.E. of mean. In two fibres no measurements were carried out at the two most positive membrane potentials. Four fibres had rheobase values more positive than −50·5 mV, thus, Q_{th} determinations for this membrane potential were not possible. The curves with continuous and interrupted lines are the best fit of eqn. (2) to the mean values of Q_{on} in control solution and in the presence of caffeine, respectively. Parameter values were: Q_{max} = 33·3 nC μF^{-1}; $\overline{V}$ = −42·3 mV; k = 12·7 mV (in control) and Q_{max} = 33·1 nC μF^{-1}; $\overline{V}$ = −44·3 mV; k = 12·3 mV (in caffeine).

In order to obtain data about the effect of caffeine on the relation between charge movement and calcium release, the threshold amount of charge (Q_{th}) necessary to reach the contraction threshold was calculated. Horowicz & Schneider (1981*b*) reported that the same amount of charge (Q_{th}) was displaced during the time necessary to get just-detectable contractions at different membrane potentials. They found a mean value of 11·5 nC μF^{-1} using a holding potential of −100 mV. In our experiments the strength–duration relation for contraction threshold and the critical durations at different membrane potentials were determined (see Methods) and the Q_{th} displaced during these critical durations were calculated from the time integral of Q_{on} records. The critical durations were determined both before and after the

sequence of recording V_I' transients, and the mean of the two determinations was used to calculate the Q_{th} value.

The mean ($\pm$S.D.) of twenty pairs of determinations at the same membrane potentials before and after caffeine treatment was $11{\cdot}9 \pm 2{\cdot}3$ nC μF^{-1} and $9{\cdot}2 \pm 1{\cdot}8$ nC μF^{-1}, respectively, on the above mentioned seven fibres. The decrease observed (22 %, $P < 0{\cdot}001$) indicates that a smaller amount of charge is enough to reach the contraction threshold in the presence of caffeine, than in the control solution. Since caffeine presumably does not exert any direct effect on the contractile

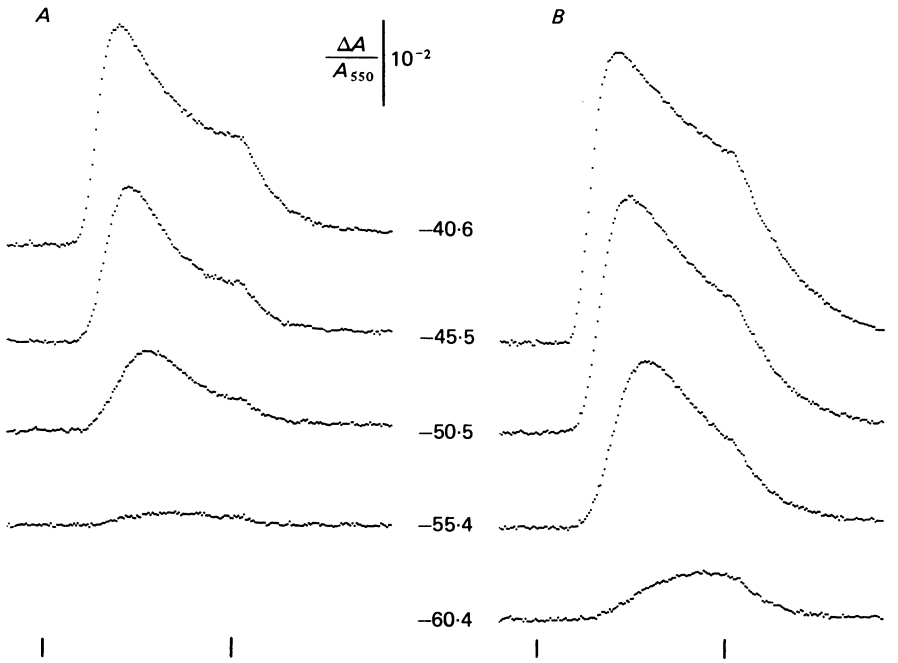

Fig. 4. Calcium transients evoked by long (100 ms) depolarizing pulses (indicated by vertical bars). Traces were recorded *A*, in control solution and *B*, in the presence of caffeine (0·5 mmol l^{-1}). Membrane potential is shown between the traces. $D_T = 0{\cdot}42$ mmol l^{-1}. Vertical calibration corresponds to ΔCa of 2·00 μmol l^{-1}. Fibre 91115, $d = 91$ μm, $s = 2{\cdot}6$ μm. Eight sweeps averaged.

proteins (Korey, 1950; Hasselbach, 1953; Moss, 1979), decrease in the threshold amount of charge might signify an altered transfer function between the charge movement process and the s.r. function, in other words, the increased sensitivity of the calcium release mechanism to the charged particles. In Fig. 3*B* the means of the Q_{th} values at different membrane potentials are plotted separately as a function of membrane potential. The diagram shows that Q_{th} does not depend on pulse voltage either in the control solution or in the presence of caffeine and the drug-induced decrease is roughly the same at different membrane potentials.

Effect of caffeine on calcium transients evoked by long pulses

Previous investigations showed a caffeine-induced calcium release from the fragmented s.r. vesicles (Weber & Herz, 1968; Ogawa, 1970), later the enhancement of

a calcium-induced calcium-release mechanism was suggested in the presence of caffeine in a skinned fibre preparation (Endo, Tanaka & Ogawa, 1970; Endo, 1975). In our experiments calcium transients accompanying depolarizing pulses were recorded to obtain information on caffeine effect on s.r. function in 'intact' cut fibres. After intracellular application of a metallochromic indicator dye (Antipyrylazo III), the change in fibre absorbance (ΔA) at 720 nm was taken as an approximately linear

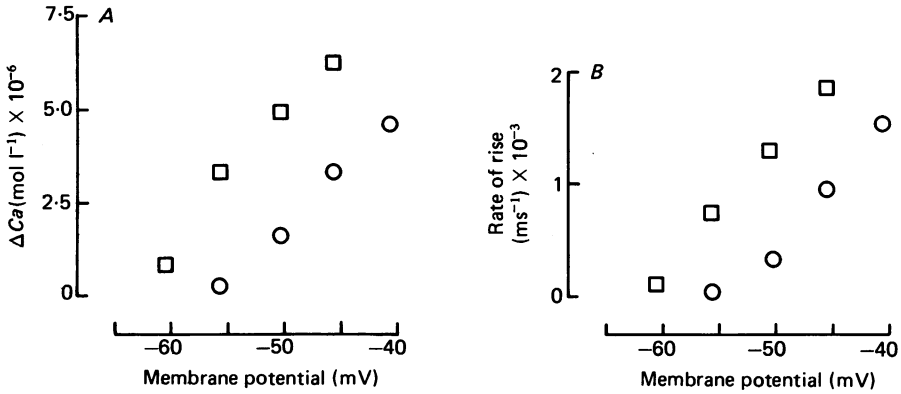

Fig. 5. Effect of caffeine (0·5 mmol l^{-1}) on the peak amplitude (A) and the rate of rise (B) of calcium transients. Same experiment as in Fig. 4. Data obtained in control solution (circles) and in the presence of caffeine (squares) are plotted *vs.* membrane potential during the pulses.

measure of the change in myoplasmic calcium concentration (Scarpa, Brinley & Dubyak, 1978; Rios & Schneider, 1981). As the fibres were not stretched suitably to avoid movement artifacts (sarcomere length varied from 2·4–2·9 μm) the depolarizing pulses could exceed the contraction threshold only by 1 or 2 mV. Therefore, to measure calcium transients by long pulses, fibres were selected that had their contraction threshold at more positive membrane potentials than other fibres in general.

In Fig. 4 calcium transients evoked by 100-ms-long pulses are shown before (Fig. 4A) and after (Fig. 4B) the application of 0·5 mmol l^{-1} caffeine on the same fibre. In the presence of caffeine, the detectable calcium transient appeared at smaller depolarization than in the control solution. At a given membrane potential, caffeine increased the peak amplitude and the rate of rise of the signals. In Fig. 5 where these data are plotted as a function of membrane potential, a clear shift towards negative potentials can be observed.

The calcium transients evoked by long pulses allow the calculation of rate coefficients referring to calcium release and re-uptake at different membrane potentials. Kovács *et al.* (1979) found that during depolarization, the calcium transients (part ΔA_{on}) after an initial transition period could be approximated by the sum of two exponential functions of time plus a constant (D_{on}):

$$A_{on} = B_1 \exp(-\beta_1 t) + B_2 \exp(-\beta_2 t) + D_{on}. \quad (3)$$

This behaviour was consistent with calcium redistribution among three intracellular

compartments with time-independent rate coefficients. Following the pulse after an initial phase, the transients (part ΔA_{off}) can be described by a single exponential time course:

$$A_{off} = C\exp(-\gamma t) + D_{off}. \quad (4)$$

The rate coefficients belonging to both calcium release (β_1) and re-uptake (β_2, γ) were dependent on pulse voltage.

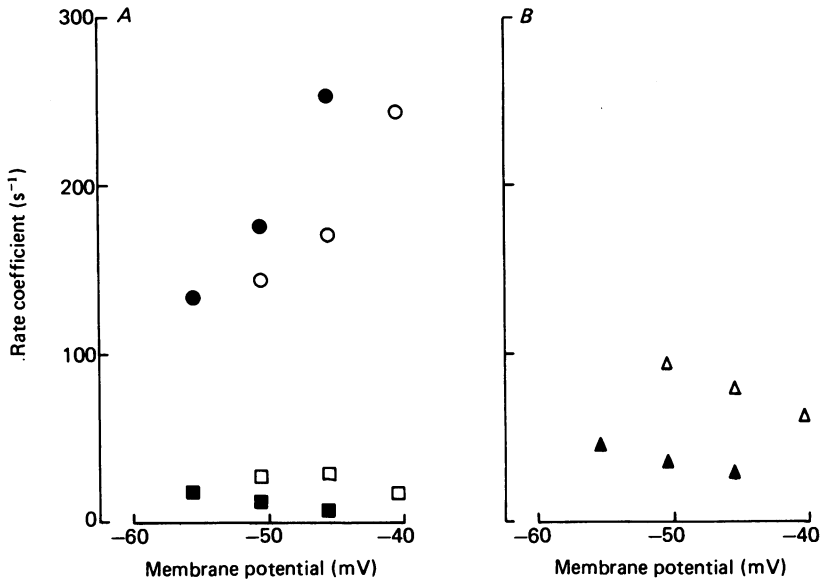

Fig. 6. Effect of caffeine (0·5 mmol l^{-1}) on the voltage-dependent rate coefficients giving the best fit to eqns. (3) and (4). The same experiment as in Fig. 4. Open symbols indicate values obtained in the control solution, while the filled ones were measured in the presence of caffeine. The rate coefficient values, β_1 (circles), β_2 (squares) in *A* and γ (triangles) in *B* are plotted *vs.* membrane potential during the pulse.

The rate coefficient values determined by fitting eqns. (3) and (4) to traces shown in Fig. 4 are plotted as a function of voltage in Fig. 6. After caffeine treatment, the release rate coefficients (β_1) were increased, whereas the re-uptake coefficients related to periods during (β_2) and after the pulse (γ) were decreased.

These results show an obvious potentiating effect of caffeine on the calcium transients evoked by depolarizing pulses. Analysing the rate coefficients related to calcium movements, a caffeine-induced augmentation of calcium release and a slowing down of re-uptake rate can be concluded.

Properties of calcium transients at the contraction threshold

To gain new information on the kinetics of mechanical activation we decided to study the properties of calcium transients at the contraction threshold in control circumstances. This work is capable of filling some of the gaps in our knowledge about the excitation–contraction coupling of muscle fibres and the results will make it

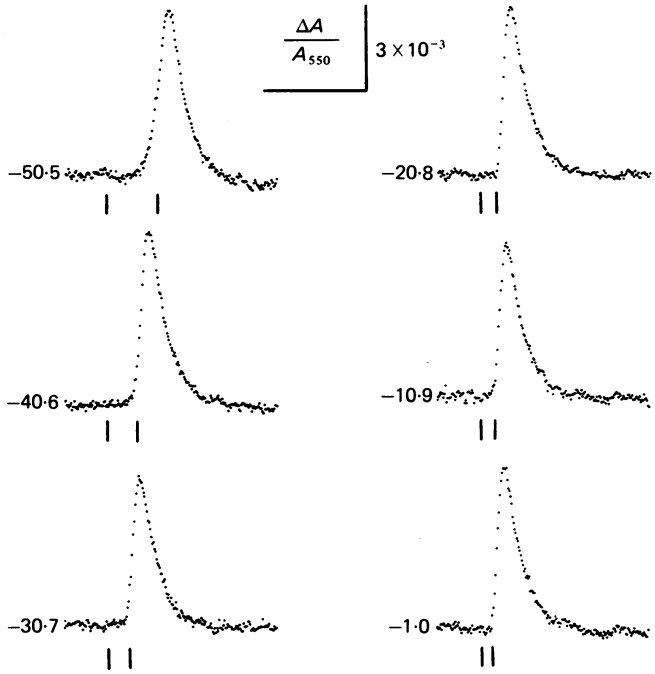

Fig. 7. Calcium transients along the strength–duration curve for contraction threshold. Pulse duration and membrane potential values are indicated next to the individual traces. Time calibration represents 50 ms. D_T = 0·31 mmol l^{-1}. Vertical calibration corresponds to ΔCa of 0·77 μmol l^{-1}. Fibre 91116, d = 105 μm, s = 2·5 μm. Eight sweeps averaged.

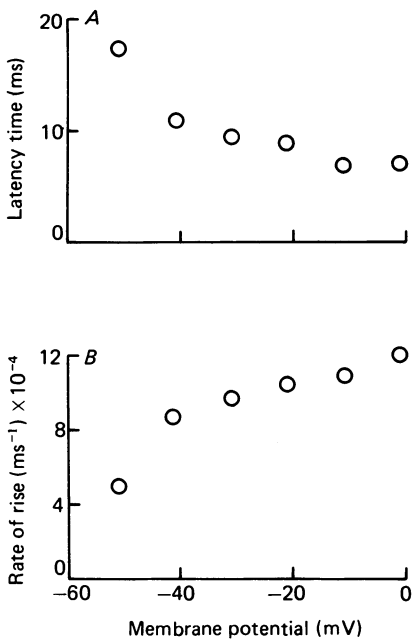

Fig. 8. Voltage dependence of latency time (A) and rate of rise (B) of calcium transients at contraction threshold. The same experiment as in Fig. 7.

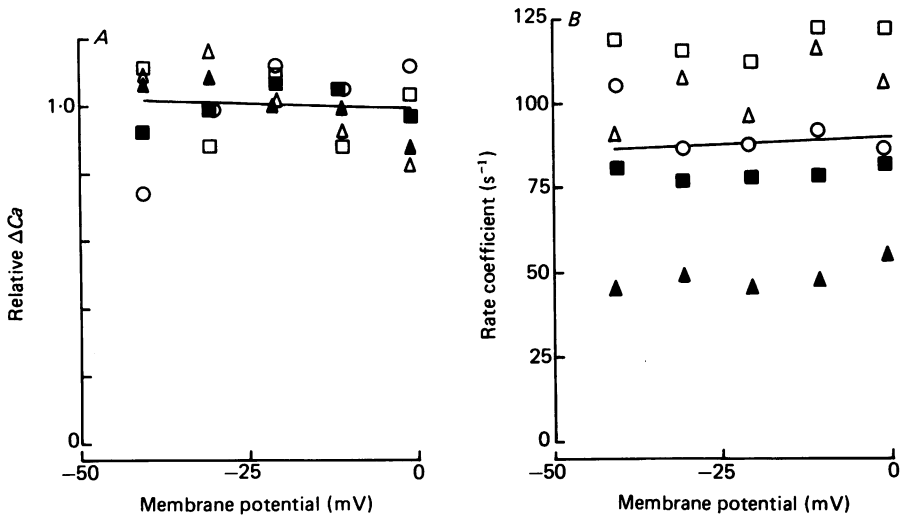

Fig. 9. Relative ΔCa (A) and γ rate coefficient (B) values of calcium transients at contraction threshold *vs.* membrane potential during the pulse. To get relative ΔCa, the ΔCa values were normalized to the mean value in the same fibre. Data were obtained from five different experiments. Fibres 91115 (open circles), 91116 (open squares), 91222 (open triangles), 00131 (filled squares), and 00207 (filled triangles); $d = 84$–$112\ \mu$m. The s, rheobase, D_T and mean ΔCa values for the fibres are listed in Table 3. The continuous lines were fitted by linear regression, the intercepts are 0·98 (A) and 89·78 (B); the slopes are $-1{\cdot}00 \times 10^{-3}$ (A) and $7{\cdot}13 \times 10^{-2}$ (B).

TABLE 3. Changes in myoplasmic calcium concentration at contraction threshold

Fibre	Sarcomere length (μm)	Rheobase (mV)	D_T (mmol l^{-1})	ΔCa (μmol l^{-1})
91115	2·59	−41·6	0·27	2·78
91116	2·52	−54·6	0·31	1·38
91222	2·87	−51·5	0·21	1·93
00117	2·45	−42·6	0·31	1·27
00131	2·52	−57·4	0·26	2·80
00207	2·94	−48·5	0·46	2·07
00924	2·80	−47·1	0·59	1·64
00926	2·64	−49·0	0·68	0·94
01124	2·43	−51·0	0·35	1·46
Mean ± S.D.	2·64 ± 0·19	−49·5 ± 5·40	0·38 ± 0·16	1·81 ± 0·65

possible to investigate the effect of caffeine from new aspects. Therefore, experiments were carried out to record calcium transients along the strength–duration curve for just-detectable movement. The results of such an experiment are shown in Fig. 7. The myoplasmic signals were recorded using depolarizing pulses with appropriate durations, at different membrane potentials for contraction threshold, as indicated for each trace. The kinetic properties and the peak amplitude of the signals were analysed and summarized in Figs. 8 and 9. For the rising phase we found a decreasing latency and an increasing rate of rise towards more positive membrane potentials (Fig. 8). The minimum latency that was found in this and other experiments was 6 ms.

To show the fibre-to-fibre variations, the peak amplitudes obtained in this experiment, together with those of four other ones are plotted *vs.* membrane potential in Fig. 9*A*. The ΔCa values corresponding to $\Delta A/A_{550}$ were determined and normalized to the mean value in the same fibre. The continuous line was calculated by linear regression to the mean values at different membrane potentials. The slope is very small, showing that the peak values of these calcium signals display no voltage dependence. This behaviour is not surprising because they were recorded on the same level of activation, that is, at just-detectable contractions. The mean values of ΔCa, belonging to the contraction threshold in these five fibres and in four other ones, are listed in Table 3 together with the corresponding dye concentration (D_T), rheobase and sarcomere length (s) values. At this sarcomere length (2·43–2·94 μm) the myoplasmic free calcium concentration ranged between 0·94 and 2·80 μmol l^{-1}.

The rate coefficients (γ) calculated from the declining phase of transients obtained in the above-mentioned five fibres are presented in Fig. 9*B*. The continuous line constructed by linear regression to the mean values at different membrane potentials indicates that there is no voltage dependence similar to the peak values.

Our results can be summarized in the following way: the calcium transients at the contraction threshold have voltage-dependent rising phases at different membrane potentials, but the peak amplitudes and the rates of decline are the same. We can conclude that the dependence of the γ rate coefficients on the preceding pulse voltage, obtained by using long pulses (Kovács *et al.* 1979), can be induced by the different calcium levels during the pulses, that is the myoplasmic calcium concentration rather than the membrane potential that controls the re-uptake rate. Further investigation would be necessary to find out the detailed mechanism regulating the declining phase of calcium transients.

Effect of caffeine on calcium transients at the contraction threshold

Because the calcium transients at the contraction threshold have about the same magnitude at different pulse voltages, the purpose of these experiments was to study the effect of caffeine on the voltage-dependent parameters and amplitude values of calcium transients, without the disturbing effect of signal potentiation induced by caffeine. In the presence of caffeine the strength–duration curve for contraction threshold was shifted to the more negative membrane potential range (Fig. 1), therefore, to obtain comparable data, calcium transients were recorded at identical voltages before and after caffeine treatment.

The records obtained from the same fibre are given in Fig. 10. The transients were recorded at the same membrane potentials in the control solution (Fig. 10*A*) and in the presence of caffeine (Fig. 10*B*), but the pulse durations were shorter in the latter case, as indicated in the Figure. The peak amplitudes, the rate coefficients for the declining phases and the latency times were calculated.

The peak amplitude of the signals is about the same at all membrane potentials and decreases in the caffeine-containing solution. The mean values of ΔCa peak amplitudes obtained before and after the caffeine treatment on this and two other fibres are listed in Table 4. The extent of decrease showed a fibre-to-fibre variability, probably in connexion with the caffeine sensitivity of the fibres. As the contractile proteins are probably not modified by caffeine, we have to suppose that there is a

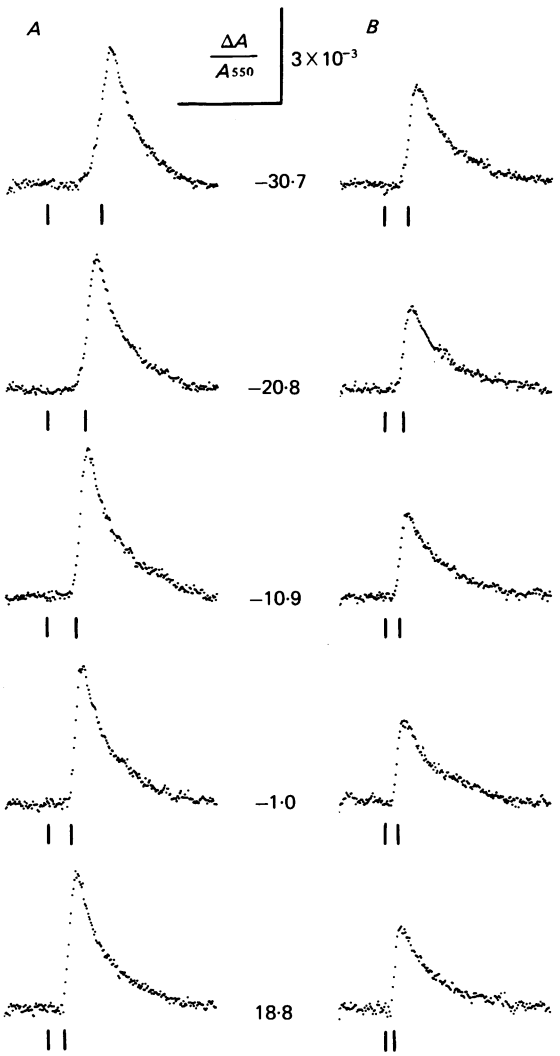

Fig. 10. Calcium transients evoked by depolarizing pulses just necessary to reach the contraction threshold in *A*, the control solution and *B*, in the presence of caffeine (0·5 mmol l^{-1}). Pulse durations are indicated by vertical bars. Membrane potential values are shown between the corresponding traces. Time calibration represents 50 ms. Vertical calibration corresponds to ΔCa values of 0·92 μmol l^{-1} for *A* (D_T = 0·31 mmol l^{-1}) and 0·66 μmol l^{-1} for *B* (D_T = 0·44 mmol l^{-1}). Same fibre as in Fig. 1. Eight sweeps averaged.

small increase in the resting calcium level, which saturates the calcium-binding sites in the myoplasm, therefore, a smaller increase in myoplasmic calcium is enough to reach the contraction threshold. The strongest evidence in support of this view, is that the use of twice the concentration of caffeine (1 mmol l^{-1}) resulted in spontaneous contracture.

On analysing the declining phase of transients, no change was found in the voltage-independent γ rate coefficients of declining phases due to caffeine treatment, as is shown in Table 4, where the mean values of γ rate coefficients from the

above-mentioned three fibres are presented. These results were surprising because the identical values of calcium transients evoked by long pulses with different amplitudes decreased in the presence of caffeine (Fig. 6). We have to suppose that caffeine treatment alone does not influence the re-uptake rate, whereas an elevated myoplasmic calcium level during the pulse exerts a decreasing effect on it.

Latency time was decreased after caffeine treatment at different membrane potentials. In the experiment presented in Fig. 10 the following latency time values were found in the control solution and in the presence of caffeine, respectively: at −30·7 mV, 16·5 and 10·0 ms; at −10·9 mV, 12·0 and 6·5 ms; at 18·8 mV, 8·5 and 5·5 ms. However, we do not have enough convincing data to decide whether the minimum latency is or is not decreased by caffeine treatment.

TABLE 4. Effect of caffeine (0·5 mmol l^{-1}) on the peak amplitudes and declining rate coefficients of the myoplasmic calcium transients at contraction threshold

		ΔCa (μmol l^{-1})		γ (s^{-1})	
Fibre	n*	Control	Caffeine	Control	Caffeine
91116	5	1·38±0·15	0·76±0·03	118·2±4·2	113·4±5·3
00117	5	1·28±0·06	0·63±0·03	66·2±8·5	62·0±6·6
00131	4	2·82±0·20	0·52±0·06	79·2±1·4	88·2±6·4

* No. of determinations on the same fibre at different membrane potentials.

DISCUSSION

Mechanism of caffeine action

The main objective of this work was to study the effects of caffeine on the different steps of excitation–contraction coupling of skeletal muscle fibres. It was found that in sub-threshold concentrations (0·5 mmol l^{-1} at 2–4 °C) caffeine potentiated twitches by increasing calcium release from the s.r., but neither the intramembrane charge movement nor calcium re-uptake was influenced.

The strength–duration curve for contraction threshold was shifted towards more negative membrane potentials under caffeine effect. This shift was more pronounced at short pulses than at longer ones. The change in the rheobase (6–7 mV) was consistent with a shift of 8–10 mV in the contraction threshold, as observed by Caputo, Gottschalk & Lüttgau (1981) on short muscle fibres using voltage-clamped depolarizing pulses and despite of the different conditions it is similar to the data (Lüttgau & Oetliker, 1968) obtained by means of potassium-induced contractures (approx. 15 mV).

The membrane potential values for the short pulses were fitted by eqn. (1). The B_T, considered as the mechanically effective area of depolarizing pulses, was decreased by 23 % due to caffeine treatment, while the amount of charge movement necessary to reach the contraction threshold became smaller to the same extent (22 %). This fact suggests the possibility that the mechanically effective areas belonging to different membrane potentials are constant because the same amount of charged particles has to move to reach the contraction threshold (Horowicz & Schneider, 1981*b*). In the presence of caffeine the sensitivity of the s.r. membrane to charged particles increases, therefore there is a decrease both in the amount of charge necessary to reach the contraction threshold and in the value of B_T.

In the experiments reported here, caffeine does not modify either the voltage dependence of the charge-movement process or the time course of the charge-displacement current at the given membrane potential. This observation, too, suggests that the caffeine effect can be exerted on processes following charge movement. The results are consistent with the previous observations of W. Almers (personal communication) who did not notice any change in the movement of charged particles accompanying depolarizing pulses and are in agreement with the general opinion that the caffeine effect takes place without any modification of the electrical properties of excitable membranes (Axelsson & Thesleff, 1958; Sandow *et al.* 1964; Lüttgau & Oetliker, 1968).

Caffeine enhances the calcium transients elicited by depolarizing pulses without any change in the charge-movement process. Our observation proves unambiguously that this effect can be localized either on the s.r. membrane or on the junction between the T-tubule and the terminal cisterne. On using 100 ms-long depolarizing pulses, the just-detectable calcium transients appear at a more negative membrane potential in the presence of caffeine than in the control solution. In case of larger depolarizing pulses, calcium transients with higher peak amplitudes develop at a faster rate of rise at a given membrane potential due to the caffeine effect (Figs. 4 and 5). The rate coefficient related to calcium release (β_1) is definitively increased in the investigated membrane potential range (Fig. 6). Our results defining the enhancement of calcium release as the essential action of caffeine are in agreement with previous reports on a similar increase of calcium release in fragmented s.r. preparations (Weber & Herz, 1968; Ogawa, 1970) or in skinned fibres (Endo *et al.* 1970; Endo, 1975).

The caffeine effect on the decay of calcium transients seems more difficult to explain. In our experiments, the γ rate coefficient of the declining phase of calcium transients elicited by long depolarizing pulses was decreased in the presence of caffeine (Fig. 6), while in case of calcium transients belonging to the contraction threshold, no change was found (Table 4). There are biochemical investigations indicating a decreasing effect of caffeine on the calcium uptake of fragmented s.r. (Weber 1968; Weber & Herz, 1968). However, to obtain a definitive inhibitory effect, the presence of caffeine (8–10 mmol l^{-1}) was necessary. It is possible, therefore, that caffeine applied in a lower concentration to intact fibres does not influence the calcium uptake by the s.r. On the basis of our data obtained for calcium transients at the contraction threshold, we suggest that caffeine does not modify the re-uptake rate of calcium from the myoplasm. The apparent decrease of the γ rate coefficient of calcium transients evoked by long pulses at a given membrane potential resulted presumably from the caffeine-induced higher calcium level during the pulse (see below).

Our results give further evidence to the idea that caffeine in subcontracture concentration, exerts its twitch potentiating effect by enhancing calcium release without acting directly on the rate of the calcium re-uptake process. The connexion between charge movement and calcium release changes due to the caffeine effect, the membrane of the s.r. grows more sensitive to the charge moving into activating position.

Properties of calcium transients at the contraction threshold

One of the aims of this work was to obtain data about the kinetics of mechanical activation. It is advantageous for this purpose to study the contraction threshold under voltage-clamp conditions where the electrical activity of the membrane is controlled. The strength–duration relation for contraction threshold determined by depolarizing pulses means the same level of contractile activation because microscopically just-observable movement was evoked in every case. During thepulses, the same amount of charges were moved (Horowicz & Schneider, 1981*b*), although the kinetics of charge movement was different at different membrane potentials. As expected, in our experiments we found calcium transients of the same amplitude along the strength–duration curve (Fig. 9*A*). Their latency time and rate of rise were voltage-dependent (Fig. 8) in spite of the fact that in several cases the surface membrane had been re-polarized before the appearance of the transients.

One important finding is that the declining phase of calcium transients can be fitted by a single exponential function. However, the time constant does not depend on the preceding pulse voltage (Fig. 9*B*) as reported previously for long (100 ms) pulses with different amplitudes (Kovács *et al.* 1979). We can conclude, therefore, that the slower rate of calcium re-uptake observed with larger depolarizing pulses is connected with the myoplasmic calcium concentration rather than the membrane potential during the pulses. On applying larger and larger pulses with shorter and shorter durations so that the amplitude of calcium transients will be the same (as, for example, at the strength–duration curve for contraction threshold) the rate of calcium re-uptake and, consequently, the rate of the declining phase will remain the same. A similar conclusion, that it is not the pulse voltage that is the essential factor regulating the re-uptake rate was suggested by E. Rios & M. F. Schneider (personal communication), who found increasing rate coefficients for declining phases of calcium transients evoked by depolarizing pulses with the same amplitude but increasing duration. As an interpretation, they suppose a saturable re-uptake pool in the s.r.

There are at least three basic processes which could explain the rapid following phase of the calcium transient. First, uptake by calcium-binding sites either on the myofilaments or upon parvalbumins; secondly, uptake by the s.r. and thirdly, a decline in a s.r. calcium ion release parameter. The available data concerning the calcium uptake of fragmented s.r. do not explain either the fast decrease in myoplasmic calcium concentration or the rate of relaxation in intact fibres (Ebashi & Endo, 1968). Thus, it is possible that different intracellular binding sites, rather than the calcium dependent ATPase of the s.r. play an important role in decreasing the calcium level.

The above-mentioned properties of calcium transients belonging to the contraction threshold are very similar to the ones found previously in case of other optical signals. Kovács & Schneider (1977) reported that the amplitudes of transparency signals elicited by depolarizing pulses necessary to reach the contraction threshold, were identical at different membrane potentials, but that their latency time and rate of rise depended on voltage. Kovács, Schümperli & Szűcs (1983) measured birefringence signals along the strength–duration curve for the contraction threshold. The transients had equal amplitudes and declined with the same rate constant. The transparency

and birefringence signals of muscle fibres are very similar to the calcium transients, suggesting that these changes are closely connected to intracellular calcium concentration.

Considering the kinetic properties related to the contraction threshold, we can suppose that while the time course of development of myoplasmic calcium transients is controlled mostly by the voltage-dependent intramembrane charge movement, the rate of the declining phase is regulated, amongst other things, by the intracellular calcium level evolving during the time course of the transients.

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