The effects of caffeine on intracellular calcium, force and the rate of relaxation of mouse skeletal muscle

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1. Intracellular calcium concentration ($[Ca^{2+}]_i$) and force were measured from isolated single fibres of mouse skeletal muscle. The effects of 5 mM caffeine on muscle fibres at rest and during short tetani were examined.

2. Caffeine increased tetanic tension and slowed the rate of relaxation. $[Ca^{2+}]_i$ was increased in the presence of caffeine both in the resting muscle and during tetani. The time course of decline of $[Ca^{2+}]_i$ after a tetanus is complex with a large, early, rapid phase followed by a smaller and slower phase. Caffeine accelerated the early phase but slowed the later phase.

3. The sensitivity of the myofibrillar proteins to Ca^{2+} measured in the intact fibre was increased in the presence of caffeine, confirming earlier findings on skinned muscle fibres.

4. Analysis of the late phase of the decline of $[Ca^{2+}]_i$ after a tetanus provides information about the properties of the sarcoplasmic reticulum (SR) Ca^{2+} pump. Caffeine slowed the pump to 60–70% of the control value at a given $[Ca^{2+}]_i$ but had no effect on the Ca^{2+} leak from the SR.

5. Analysis of relaxation made use of the Ca^{2+}-derived force in which the $[Ca^{2+}]_i$ during relaxation was converted to the Ca^{2+}-derived force by means of the steady-state relation between $[Ca^{2+}]_i$ and force. The Ca^{2+}-derived force fell more slowly in the presence of caffeine but the lag between Ca^{2+}-derived force and measured force was unaffected. Thus, the slowed relaxation was caused by changes in Ca^{2+} handling and not by slowed cross-bridge kinetics.

6. A model of the Ca^{2+} movements and force production of muscle was used to examine independently the effects of increased Ca^{2+} sensitivity, slowing of the SR Ca^{2+} pump and increased SR Ca^{2+} permeability. The effects of caffeine on $[Ca^{2+}]_i$, tetanic force and relaxation could be explained by a combination of these three effects.

Caffeine is widely used in muscle research as an agent which increases Ca^{2+} release from the sarcoplasmic reticulum (SR) and thereby increases myoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Weber & Herz (1968) first clearly demonstrated that caffeine caused Ca^{2+} release from isolated SR vesicles and this action was subsequently confirmed in skinned muscle with intact SR (e.g. Nakajima & Endo, 1973). Recently, the SR release channel has been isolated and incorporated in lipid membranes and caffeine has been shown to increase the frequency of opening of Ca^{2+} channels without affecting the elementary conductance (Rousseau, LaDine, Liu & Meissner, 1988). As a consequence of this action, moderate concentrations of caffeine increase twitch and tetanic force; at high concentrations it can produce a prolonged contracture in the absence of stimulation. However, caffeine also slows the time course in a twitch (Konishi & Kurihara, 1987; Fryer & Neering, 1989) and slows relaxation after a tetanus (Pagala, 1980) and these actions are less well explained. There are two main possibilities. (1) Caffeine might slow cross-bridge kinetics and the observation that caffeine increases the Ca^{2+} sensitivity of the isolated myofibrils (Wendt & Stephenson, 1983) without affecting the binding of Ca^{2+} to isolated troponin-C (Palmer & Kentish, 1994) is consistent with this possibility (e.g. Brenner, 1993). (2) Alternatively, caffeine might slow the release and/or uptake of Ca^{2+}. There is already considerable evidence for this possibility as the time course of decline of $[Ca^{2+}]_i$ has been shown to slow in the presence of caffeine (Konishi & Kurihara, 1987; Fryer & Neering, 1989; Simon, Klein & Schneider, 1989). However, this observation has been variously interpreted as an effect on the rate of closure of SR Ca^{2+} channels (Simon *et al.* 1989) or an inhibition of the SR Ca^{2+} pump (Weber & Herz, 1968; Fuchs, 1969; Fryer & Neering, 1989).

We have re-investigated the effects of caffeine on $[Ca^{2+}]_i$ and force from the fast twitch fibres of the mouse with a particular emphasis on the mechanism of the slowing of relaxation. Single mouse fibres were used and $[Ca^{2+}]_i$ was

measured with indo-1 using methods developed in recent studies (Westerblad & Allen, 1993, 1994*a*, *b*). As in earlier studies, caffeine produced a large increase in tetanic $[Ca^{2+}]_i$, a small increase in tetanic force, and a moderate slowing of the rate of relaxation. We confirm that the increased Ca^{2+} sensitivity described by Wendt & Stephenson (1983) in skinned fibres is present in the intact fibres. The combination of increased $[Ca^{2+}]_i$ coupled to increased Ca^{2+} sensitivity would be expected to produce some slowing of relaxation since, in the absence of other changes, it will take longer for the $[Ca^{2+}]_i$ to fall to the level required to achieve a given force. Analysis of the late decline of $[Ca^{2+}]_i$ after a tetanus showed that caffeine reduced the activity of the SR Ca^{2+} pump and this will also contribute to the slowing of relaxation. Using a method which measures the delay associated with Ca^{2+} dissociation from troponin and cross-bridge detachment, we found no evidence that caffeine slowed these processes. We have used a computer model of Ca^{2+} movements and force production (Cannell & Allen, 1984; Westerblad & Allen, 1994*a*) to explore the contributions of these changes to the measured slowing of relaxation. The model shows that the observed changes in tetanic $[Ca^{2+}]_i$, myofibrillar Ca^{2+} sensitivity and SR pump rate are sufficient to explain the observed slowing of relaxation.

METHODS

The methods have been described in detail previously (Westerblad & Allen, 1993, 1994*a*, *b*). Briefly, mice were killed by rapid cervical dislocation and single fibres of the flexor brevis muscle were dissected. The fibres were mounted in a muscle chamber which allowed them to be stimulated and their force recorded. Fibres were superfused at 22 °C with a physiological salt solution of the following composition (mM): NaCl, 121; KCl, 5·0; $CaCl_2$, 1·8; $MgCl_2$, 0·5; NaH_2PO_4, 0·4; $NaHCO_3$, 24; and glucose, 5·5. The solution was gassed with 95% O_2 and 5% CO_2 which gave a pH of 7·3. Fetal calf serum (approximately 0·2%) was added to the solution to help maintain the viability of the fibres (Lännergren & Westerblad, 1987).

Standard tetani were 100 Hz, 0·35 s duration. Caffeine was applied to the fibre 30 s before stimulation and immediately washed out; this process was repeated for each tetanus in caffeine.

Measurement of $[Ca^{2+}]_i$ with indo-1

$[Ca^{2+}]_i$ was measured with indo-1 which was pressure-injected into the fibres (for details see Westerblad & Allen, 1993). The fibre was illuminated with light at 360 nm and fluorescent light measured simultaneously at 400 and 505 nm. The individual fluorescence signals were routinely filtered with a 100 Hz low-pass filter. In some control experiments twitches were recorded with 300 Hz filtering but this did not significantly affect the rate constant of the decline of fluorescence or of the $[Ca^{2+}]_i$ signal (see below). Conversion of the indo-1 ratio signal to $[Ca^{2+}]_i$ made use of the *in vivo* calibration method developed in this laboratory (Westerblad & Allen, 1993). Peak tetanic force was measured before and after injection as a test of whether the indo-1 caused increased Ca^{2+} buffering, but there was no significant change. The intracellular concentration of indo-1 was estimated by measuring the fluorescence caused by a known concentration of indo-1 dissolved in 150 mM KCl, 5 mM Pipes (pH 7·3), 5 mM EGTA and 5% fetal calf serum in a glass capillary tube whose internal dimensions were similar to those of a single fibre. The fetal calf serum was included as soluble proteins are known to simulate some of the change in the properties of fluorescent indicators observed in the intracellular environment (Westerblad & Allen, 1993; Kurebayashi, Harkins & Baylor, 1993). The mean intracellular indo-1 concentration in our fibres was 25 μM (range 15–41 μM).

The Ca^{2+} dissociation rate constant of indo-1 *in vitro* is 130 s^{-1} at 20 °C (Jackson, Timmerman, Bagshaw & Ashley, 1987) but such rates are frequently slower in the intracellular environment (e.g. Kurebayashi *et al.* 1993). In an earlier publication (Westerblad & Allen, 1994*b*) we reported that the fast rate constant of decline of $[Ca^{2+}]_i$ after a twitch was 98 s^{-1} and that this value represented a lower limit for the *in vivo* Ca^{2+} dissociation constant of indo-1. The latter statement was incorrect; the dissociation rate constant for indo-1 should be estimated from a single fluorescent wavelength or from the Ca^{2+} occupancy of indo-1 and when this analysis was performed on the indo-1 signal from a twitch the value of 34 ± 4 s^{-1} ($n = 4$) was obtained; after a tetanus the rate constant was 10 ± 1 s^{-1}. However, the observation that the fast phase of $[Ca^{2+}]_i$ decline after a twitch has a rate constant of 98 s^{-1} remains correct and at rates which are lower than this indo-1 is presumably giving at least a qualitative indication of the changes in the rate of decline of $[Ca^{2+}]_i$. The fast component of decline of $[Ca^{2+}]_i$ after a tetanus in the present experiments has a rate constant of 64 ± 7 s^{-1} ($n = 9$) so that indo-1 should be capable of detecting acceleration or slowing of this rate.

Caffeine is known to quench indo-1 fluorescence at both measurement wavelengths but the ratio is unaffected (O'Neill, Donoso & Eisner, 1990).

Ca^{2+} sensitivity and Ca^{2+}-derived force

In order to determine the Ca^{2+} sensitivity, tetani at 20–100 Hz were produced and the $[Ca^{2+}]_i$ and force were averaged over the final 100 ms (or an integral number of stimuli at low stimulus rates). Each such point was then plotted on a force–$[Ca^{2+}]_i$ plot (e.g. Fig. 2*B*). Full details have been given in Westerblad & Allen (1993). The data points were then fitted to a Hill equation:

$$P = P_{max}[Ca^{2+}]_i^N/(Ca_{50}^N + [Ca^{2+}]_i^N), \quad (1)$$

where P is the relative force, P_{max} is the force at saturating $[Ca^{2+}]_i$, Ca_{50} is the $[Ca^{2+}]_i$ giving 50% of the P_{max}, and N is a constant which describes the steepness of the relation. P_{max} for both control and caffeine was obtained by producing a 100 Hz tetanus in 5 mM caffeine. The continuous relation from this expression was then used to convert the $[Ca^{2+}]_i$ signal during a tetanus to the Ca^{2+}-derived force.

Curve fitting for pump function analysis

Analysis of the SR pump function followed the method first described by Klein, Kovacs, Simon & Schneider (1991) and was subsequently applied to mouse muscle (Westerblad & Allen, 1993, 1994*a*, *b*). The method assumes that the long tail of elevated $[Ca^{2+}]_i$ after a tetanus is a period when SR Ca^{2+} uptake is in a pseudosteady state with the $[Ca^{2+}]_i$ and that Ca^{2+} uptake is proportional to $d[Ca^{2+}]_i/dt$. Thus, a plot of $d[Ca^{2+}]_i/dt$ *versus* $[Ca^{2+}]_i$ represents a SR pump function curve. Differentiation of a noisy $[Ca^{2+}]_i$ signal yields a very noisy $d[Ca^{2+}]_i/dt$ signal and this problem has been overcome by fitting the $[Ca^{2+}]_i$ tail signal with a double exponential function (see Fig. 3*A*) and using the $d[Ca^{2+}]_i/dt$ and $[Ca^{2+}]_i$ from the fitted signal rather than from the original data. In our previous studies we followed Klein *et al.* and fitted the

relation between $d[Ca^{2+}]_i/dt$ and $[Ca^{2+}]_i$ with an equation with three adjustable parameters representing the rate of pumping (A), the power function (N) and the SR Ca^{2+} leak (L):

$$d[Ca^{2+}]_i/dt = A[Ca^{2+}]_i N - L. \quad (2)$$

While this empirical expression fits our limited data well, it does not directly lead to parameters which can be fitted into a more realistic model of SR pump function required for our computer model of muscle (for discussion of choice of the SR model see below). To overcome this problem we used the following expression:

$$\text{SR pump uptake} = B\,d[Ca^{2+}]_i/dt$$
$$= V_{max}([Ca^{2+}]_i/K_m)^N/(1+([Ca^{2+}]_i/K_m)^N) - L, \quad (3)$$

where B is the calcium buffering of the myoplasm, V_{max} is the maximum pump rate of the SR, K_m is a dissociation constant between Ca^{2+} and sites on the SR pump, N is a power function which was set to 4 (see below), and L is a (constant) leak of Ca^{2+} from the SR. Buffering of $[Ca^{2+}]_i$ close to the resting level is dominated by the Ca–Mg sites (Gillis, 1985) on parvalbumin (and troponin). Thus, to a good approximation,

$$B = [CaPa]/[Ca^{2+}]_i = K_{Ca}[Pa], \quad (4)$$

where K_{Ca} is the apparent binding constant of Ca–Mg binding sites for Ca^{2+}, [Pa] is the concentration of free Ca–Mg sites and [CaPa] is the concentration of Ca–Mg sites with Ca^{2+} bound to them. Taking the total Ca–Mg binding sites to be 1 mM, $[Mg^{2+}]_i$ to be 0·8 mM (Westerblad & Allen, 1992), K_{Ca} to be $4{\cdot}5 \times 10^8$ M^{-1} and K_{Mg} to be $3{\cdot}5 \times 10^4$ M^{-1} (references in Westerblad & Allen, 1994*a*), we calculate B to be ~10 000. This is the steady-state buffering; the instantaneous buffering will be somewhat less since the equilibration of Pa and Ca^{2+} take several seconds. We have taken B to be 10 000 which, with appropriate choice of other model variables leads to a good fit between the experimental tails of $[Ca^{2+}]_i$ and the model output (see Fig. 6*C*). The value of V_{max} is also uncertain; published values from isolated skeletal SR have a maximum of about 1 mM s^{-1} and are usually much lower (reviewed by Gillis, 1985) whereas estimates from model fitting give values in the range 3–5 mM s^{-1} (Garcia & Schneider, 1993). The minimum value which allows a reasonable fit of eqn (3) to the data points is 2–4 mM s^{-1} and we have used the value of 4 mM s^{-1}. With these assumed values of B and V_{max}, K_m was obtained by a least squares minimization routine when eqn (3) was fitted to the $d[Ca^{2+}]_i/dt$ *vs.* $[Ca^{2+}]_i$ data (e.g. Fig. 3*B*).

Model

In order to investigate the significance of possible mechanisms of action of caffeine we made use of a model of Ca^{2+} movements and force development. The model is a development of that initially described by Cannell & Allen (1984) and the current version is described in some detail in Westerblad & Allen (1994*a*). The following modifications were incorporated to improve the fit of the model to the data (Fig. 6*A* and *C*). (1) The Ca^{2+} stored in the SR was increased by raising the initial $[Ca^{2+}]$ in the SR (from 2·5 to 4 mM) and by raising the concentration of calcium binding sites due to calsequestrin in the SR (from 30 to 40 mM). The total calcium in the model can be obtained by the sum of each form of calcium in the model (free or bound) multiplied by the fractional volume of the compartment in which it is distributed. In the present version of the model the total calcium is 2·2 mM (cf. Westerblad & Allen, 1994*a*) which compares with recent measurements of 1–2 mM (Fryer & Stephenson, 1993). (2) The time constant (τ_2) for closure of the SR Ca^{2+} channels was increased (from 10 to 20 ms). (3) The myofibrillar Ca^{2+} sensitivity of the model was established by running a modified version of the model, which gave a steady $[Ca^{2+}]_i$ and force at a range of intermediate levels. These $[Ca^{2+}]_i$ and force values were fitted to eqn (1) and subsequently the off rate constant for Ca^{2+} binding to troponin was modified to achieve the Ca_{50} measured experimentally. Under control conditions the off rate constant obtained in this way was 185 s^{-1}, which gave a Ca_{50} of 452 nM. (4) The mathematical form of the SR function was modified from that given in Westerblad & Allen (1994*a*). The experimental data show that the relation between SR uptake rate and $[Ca^{2+}]_i$ has a fourth power function at very low $[Ca^{2+}]_i$ (Klein *et al.* 1991; Westerblad & Allen, 1993, 1994*a*, *b*). While an approximate 4th power relationship has been repeatedly demonstrated this does not reveal the molecular mechanisms involved. In order that the pump function is adequately described over the full range of $[Ca^{2+}]_i$ we need to know the maximum Ca^{2+} uptake rate of the SR (V_{max}) and something about the steepness of the function in the intermediate and high range of $[Ca^{2+}]_i$. This part of the relationship can be characterized by the ratio of $[Ca^{2+}]_i$ which increases the pump rate from 10 to 90% maximum (S_{90}/S_{10}) (e.g. Segel, 1975). For the model used in our earlier study, S_{90}/S_{10} was 18. However, this model proved to have several disadvantages for the present purpose; it required a very large V_{max} (> 8 mM s^{-1}) to give even a moderate fit to the $d[Ca^{2+}]_i/dt$ *vs.* $[Ca^{2+}]_i$ data shown in Fig. 3*B*, and when the muscle model was run with a V_{max} of 8 mM s^{-1} the fit of the model $[Ca^{2+}]_i$ during a control tetanus to the experimental data was poorer than that shown in Fig. 6*A*. For these reasons we chose a Hill-type model (eqn (3)) of SR function which has a S_{90}/S_{10} of 3 (Segel, 1975) and allowed a satisfactory fit to the experimental data with a V_{max} of 4 mM s^{-1}. This model of SR function is not intended to be mechanistically realistic; it was choosen only because it fits the experimental data adequately and allows the computer model of muscle to give reasonable simulations of the data. In addition, we performed all the simulations shown in Figs 5 and 6 with the version of the model given in Westerblad & Allen (1994*a*) and with V_{max} set to 8 mM s^{-1}; with this model the fit between data and model are less good but the main conclusions are qualitatively similar.

With the parameters set to the above values, the time course of the model $[Ca^{2+}]_i$ fitted the experimental data well; the final amplitude fit was obtained by small changes in the permeability of the SR release channels.

Statistics

Results are presented as means ± S.E.M. Unless otherwise stated, all numerical results quoted in the text were statistically significant at $P < 0{\cdot}05$ using Student's paired t test.

RESULTS

Effects of caffeine on force, rate of relaxation and $[Ca^{2+}]_i$

Figure 1 shows a representative record of the $[Ca^{2+}]_i$ and force from a standard tetanus under control conditions and after a 30 s exposure to 5 mM caffeine. The effects of caffeine were completely reversible. A smaller series of experiments was performed with 10 mM caffeine with similar but larger effects which we will not report. The force record illustrates the well-recognized increase in peak force and in nine experiments the increase amounted to 8 ± 2%. Relaxation shows the usual two phases; an early phase in

which the decline of force is close to linear which lasts about 40 ms and a late phase which is close to exponential. It is known that the muscle remains isometric during the linear phase but that internal sarcomere rearrangements occur during the exponential phase (Huxley & Simmons, 1973). The early phase is clearly slower in caffeine, the transition to the late phase occurs somewhat later and the exponential decline of the late phase is slightly slower. In nine experiments the rate of decline over the linear phase was 1930 ± 150 kPa s^{-1} (control) and fell by 440 ± 60 kPa s^{-1} in the presence of caffeine, i.e. the rate of relaxation was reduced by 23% in caffeine.

The $[Ca^{2+}]_i$ in the presence of 5 mM caffeine showed the following changes. The resting $[Ca^{2+}]_i$ was raised (not visible in Fig. 1) and in nine experiments the resting $[Ca^{2+}]_i$ was 31 ± 4 nM which increased by 6 ± 1 nM in the presence of caffeine. The $[Ca^{2+}]_i$ during the tetanus showed a large increase, with the tetanic $[Ca^{2+}]_i$ at the end of a tetanus being 990 ± 80 nM (control), and increased by 1160 ± 120 nM in the presence of caffeine. The tetanic $[Ca^{2+}]_i$ in the presence of caffeine is quite variable. One component of this variation arises from the stimulus frequency and this component is particularly clear at low stimulus frequencies, e.g. Fig. 2A. A second component arises because the indo-1 ratio approaches R_{max} (the indo-1 ratio at saturating $[Ca^{2+}]_i$) and close to R_{max}, random components of noise lead to an increasingly large component of noise in the calculated $[Ca^{2+}]_i$.

The rate of decline of $[Ca^{2+}]_i$ after a tetanus is complex. In mouse muscles there is an early, rapid phase in which $[Ca^{2+}]_i$ is reduced to about 10% of the tetanic level within ~100 ms; the $[Ca^{2+}]_i$ then slowly declines back to the resting level over many seconds (see Figs 1, 3A and 4). In contrast to the early mechanical relaxation, the maximum rate of decline of $[Ca^{2+}]_i$ (measured from 15 to 35 ms after the last stimulus) was faster in caffeine and this observation was confirmed in nine preparations in which the maximum rate of decline of $[Ca^{2+}]_i$ was 29 ± 3 μM s^{-1} (control) and 79 ± 10 μM s^{-1} (caffeine). The significance of this observation is hard to assess because the $[Ca^{2+}]_i$ level is so much higher in caffeine, so we also fitted an exponential to the rapid phase of $[Ca^{2+}]_i$ decline (by fitting two exponentials to data from 15 ms to 4 s after the last stimulus). The rate constant of the fast exponential was 64 ± 7 s^{-1} (control) and 86 ± 12 s^{-1} (caffeine), showing that the rate constant of the fast phase was increased in the presence of caffeine. The late phase of decline of $[Ca^{2+}]_i$ was larger and slower in the presence of caffeine (see Fig. 3A) and will be discussed in a later section.

We also measured the time from the one stimulus to the following peak of $[Ca^{2+}]_i$ in tetani at 20–40 Hz in four fibres. The time to peak $[Ca^{2+}]_i$ did not change in tetani at the different frequencies and there was no significant difference between control ($9{\cdot}9 \pm 0{\cdot}3$ ms) and caffeine ($11{\cdot}4 \pm 0{\cdot}4$ ms). This result is quite different to Simon *et al.* (1989) who observed a > 5-fold increase in the time from

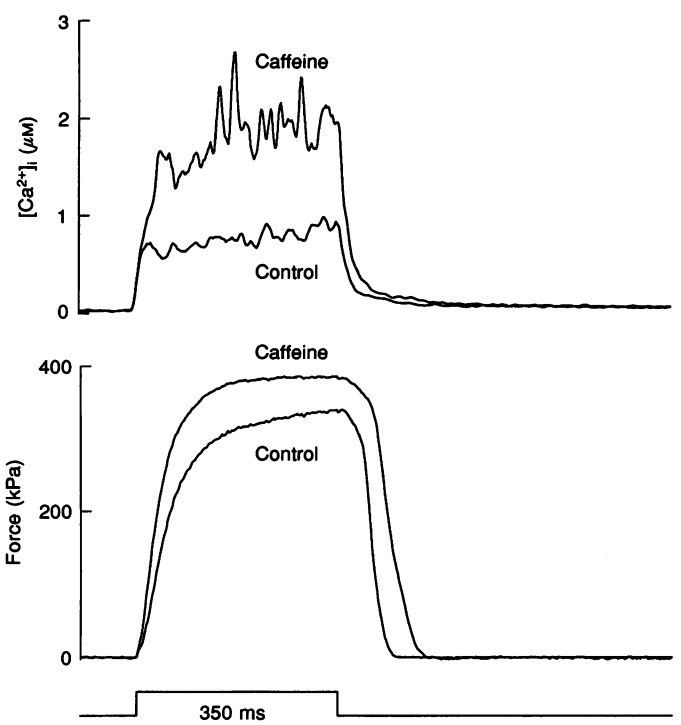

Figure 1. Effect of caffeine on $[Ca^{2+}]_i$ and force in a tetanus

Stimulus timing shown on lowest trace; 100 Hz, 350 ms tetanus. Caffeine (5 mM) was applied for 30 s before the tetanus. The increased noise in the $[Ca^{2+}]_i$ signal in the caffeine record during the tetanus is an artifact caused by the indo-1 fluorescence ratio approaching saturation.

the end of a depolarizing pulse to the start of the fall in $[Ca^{2+}]_i$ in the presence of caffeine.

Sensitivity of the myofibrillar proteins to $[Ca^{2+}]_i$

In vivo $[Ca^{2+}]_i$–force curves were produced using our standard protocol (e.g. Westerblad & Allen, 1993) which is illustrated in Fig. 2*A*. Tetani at a range of stimulus frequencies (between 20 and 100 Hz) were elicited which produced tetani giving force spanning the range between 10% and maximum. The same procedure was then repeated in 5 mM caffeine. Note that in Fig. 2*A* a 50 Hz tetanus under control conditions produced about the same force as a 20 Hz tetanus in the presence of caffeine but the mean $[Ca^{2+}]_i$ was substantially lower in caffeine. The relation between steady-state $[Ca^{2+}]_i$ and force was estimated in each tetanus by averaging the $[Ca^{2+}]_i$ and force over the final 100 ms of a tetanus. The points obtained were plotted as normalized force as shown in Fig. 2*B*. Clearly there is a shift in the Ca_{50} in this example. The smooth curve is a least squares fit of eqn (1). In four

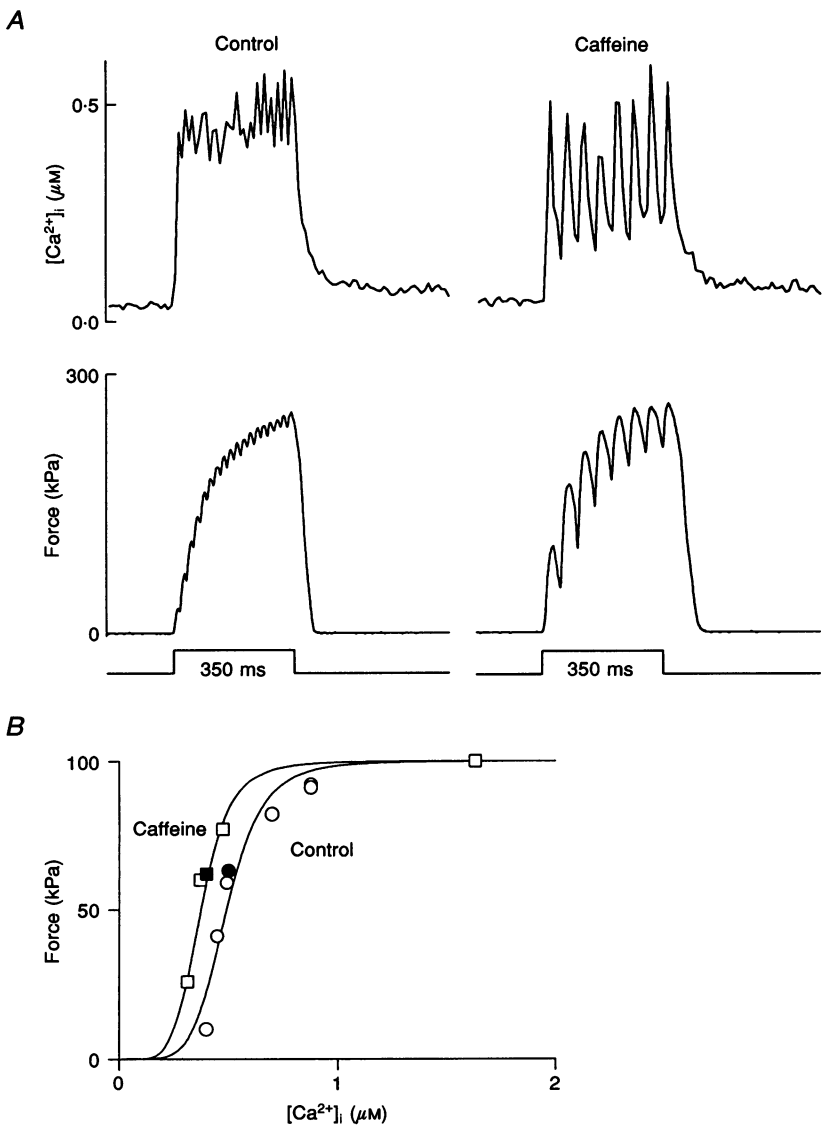

Figure 2. Effect of caffeine on the relation between $[Ca^{2+}]_i$ and force

A, sample records used to measure the relation between $[Ca^{2+}]_i$ and force at intermediate levels of force. The records marked Control were in the absence of caffeine and at a stimulation frequency of 50 Hz, which gave an unfused tetanus that reached about 60% of the maximum force from this fibre. The records marked Caffeine were in the presence of 5 mM caffeine and at a stimulus frequency of 20 Hz, which again gave about 60% of the maximum force. Note that the mean $[Ca^{2+}]_i$ in the final 100 ms of the tetanus was substantially lower than that in the control record. *B*, plot of the mean $[Ca^{2+}]_i$ and force in the final 100 ms of a series of tetani at different stimulus frequencies in the absence (○) and presence (□) of 5 mM caffeine. The filled symbols are measurements from the two examples shown in *A*. Continuous lines are Hill curves (eqn (1)) fitted to the experimental points.

experiments Ca_{50} was 450 ± 30 nM (control) and 330 ± 30 nM (caffeine) whereas there was no significant difference in the values of N (4·9 ± 0·5, control; 5·1 ± 0·8, caffeine). Thus, 5 mM caffeine increased Ca^{2+} sensitivity by a factor of 1·35 (0·13 log units) but did not affect the steepness of the relation.

SR pump function analysis

The methods and assumptions used have been described previously in detail and are not repeated here. One assumption of this method is that caffeine does not change the Ca^{2+} buffering; the changes in Ca^{2+} binding to troponin which we assume to occur are probably too small and too early to affect the present analysis. Figure 3 illustrates the two main stages in the procedure. The noisy lines in Fig. 3*A* show the decline of $[Ca^{2+}]_i$ averaged from nine experiments with the control record below and 5 mM caffeine above. Note the slow time base and the fact that the ordinate is restricted to 200 nM; as a consequence the panel shows mainly the slow phase of the decline of $[Ca^{2+}]_i$. A double exponential fit was made to each of these, starting at 120 ms after the last stimulus (control) and 200 ms after the last stimulus (caffeine) so as to completely exclude the fast phase of $[Ca^{2+}]_i$ decline. The results of these fits are

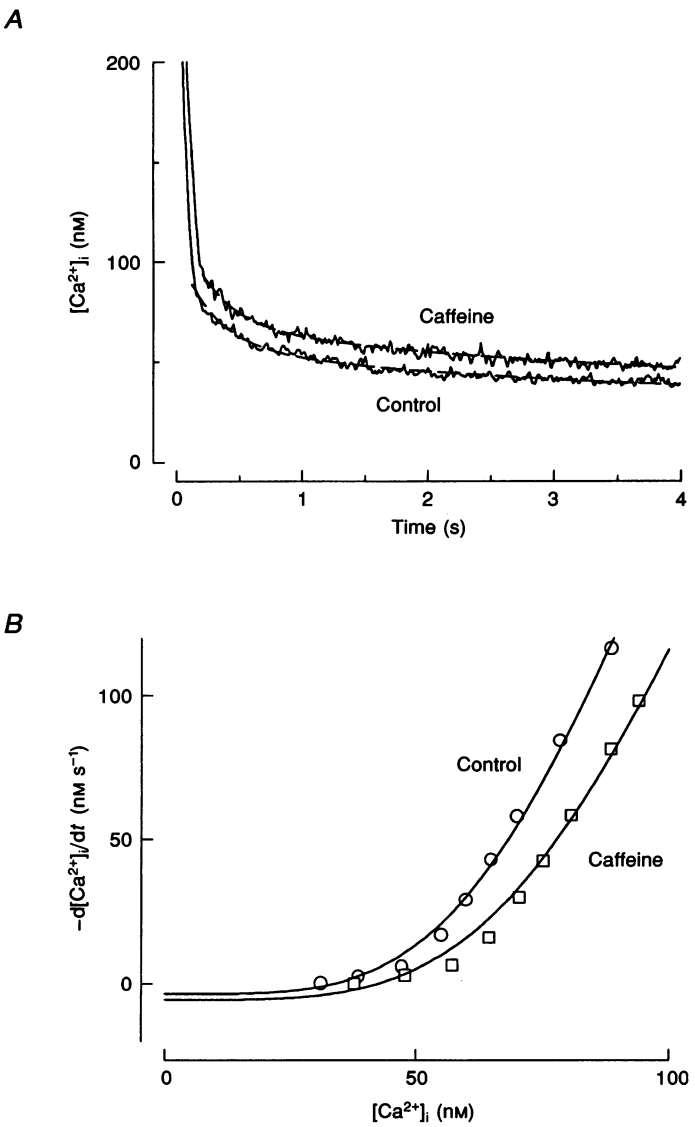

Figure 3. SR pump analysis in the presence and absence of caffeine

A, $[Ca^{2+}]_i$ signals following a 350 ms tetanus; zero time represents the last stimulus. Note the initial (truncated) rapid phase followed by a slow phase which is still above resting levels after 4 s. Data from 9 experiments averaged (continuous traces); upper trace in the presence of 5 mM caffeine. Dashed lines show double exponential fits to the slow phase. *B*, plots of $d[Ca^{2+}]_i/dt$ *vs.* $[Ca^{2+}]_i$ taken from the double exponential fits shown in *A* (control, ○; 5 mM caffeine, □). Continuous curves are theoretical pump function curves (eqn (3)) fitted to the experimental curves. Fits of the alternative pump function curves (not shown, eqn (2)) were almost indistinguishable from the curve shown.

shown as the dashed lines on Fig. 3*A*. Then $d[Ca^{2+}]_i/dt$ was measured at selected $[Ca^{2+}]_i$ on the exponential fits and the results plotted in Fig. 3*B*. Equation (2) was first fitted to this data and gave fits very similar to the lines shown with N values of 3·2 (control) and 3·9 (caffeine) which were not significantly different. Subsequently, the data points were fitted to eqn (3) and the K_m values found to be 109 ± 0·7 nM (control) and 122 ± 1·1 nM (caffeine). The smooth curves in Fig. 3*B* show the fit of eqn (3) to the data using the above values of K_m. A simple way to characterize the difference in pump rate in the two conditions is to compare the pump rate at a given $[Ca^{2+}]_i$. By this criterion the pump rate was reduced to 60–70% of the control value in the presence of caffeine. The differences in the leak (L) were small and not significant.

$[Ca^{2+}]_i$-derived force

Caffeine accelerates the early rate of decline of $[Ca^{2+}]_i$ after a tetanus but it is difficult to interpret how this will affect the rate of relaxation because tetanic $[Ca^{2+}]_i$ is much higher in caffeine. An additional difficulty in interpreting possible effects of $[Ca^{2+}]_i$ on force is that caffeine clearly changes the relationship between $[Ca^{2+}]_i$ and force (Fig. 2). Both these problems can be circumvented by converting the $[Ca^{2+}]_i$ signal obtained during a tetanus to the force which would

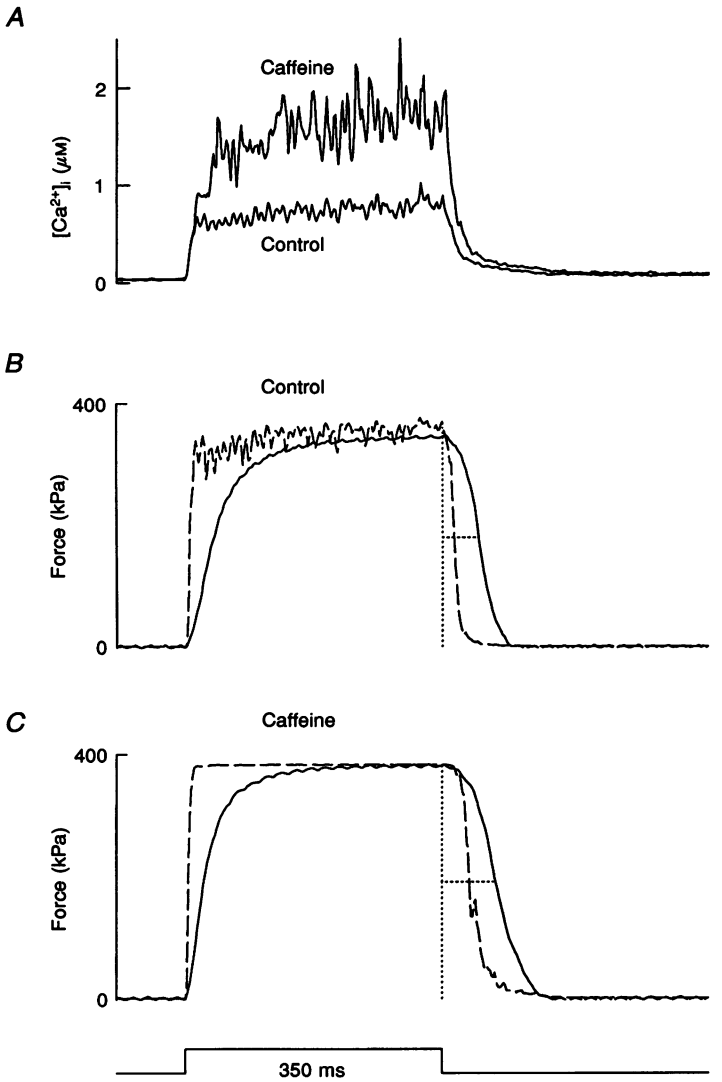

Figure 4. Comparison of force with Ca^{2+}-derived force for tetani in the absence and presence of caffeine

A, averaged $[Ca^{2+}]_i$ signal from 4 experiments in the absence (lower trace) and presence (upper trace) of 5 mM caffeine. *B*, continuous line shows the force under control conditions (averaged from the same 4 experiments as *A*). Dashed line shows the Ca^{2+}-derived force derived from the $[Ca^{2+}]_i$ signal in *A* after transformation by the steady-state relation between $[Ca^{2+}]_i$ and force (as shown in Fig. 2*B*). Vertical dotted line shows the timing of the last stimulus and horizontal dotted line shows 50% force. *C*, same format as *B* but all data in the presence of 5 mM caffeine. Bottom trace shows the timing of stimulation.

have occurred if the steady-state relation between $[Ca^{2+}]_i$ and force had occurred instantly (e.g. Westerblad & Allen 1993, 1994*a*). To do this we have determined the relation between $[Ca^{2+}]_i$ and force in each of four experiments (as depicted in Fig. 2*B*), fitted the points to eqn (1), and then used this relation to convert $[Ca^{2+}]_i$ to Ca^{2+}-derived force throughout a tetanus. Thus, Fig. 4*A* shows the mean $[Ca^{2+}]_i$ signals from four experiments in the presence and absence of caffeine. In Fig. 4*B* the noisy curve (dashed line), which rises faster and falls faster, is the Ca^{2+}-derived force and the smoother curve which lags behind is the true force. The lag between the two curves results from the time taken for Ca^{2+} bound to troponin to equilibrate with the $[Ca^{2+}]_i$ (which is a function of the on and off rate constants of Ca^{2+} binding to troponin) and for cross-bridge attachment and detachment to occur. Figure 4*C* shows the same analysis in the presence of caffeine. Comparison of Fig. 4*B* and *C* shows that the lag of the Ca^{2+}-derived force (measured from the last stimulus until force had declined to 50% of maximum) is greater in caffeine than in control, despite the fact that $[Ca^{2+}]_i$ falls faster in caffeine than control as noted above. In four experiments the lag of the Ca^{2+}-derived force was 15 ± 2 ms (control) and 38 ± 4 ms (caffeine). However the lag between the Ca^{2+}-derived force and the true force does not show any apparent changes (34 ± 3 ms control; 36 ± 5 ms caffeine). This analysis therefore suggests that the slowing of relaxation in caffeine arises from the increased tetanic $[Ca^{2+}]_i$ coupled to the increased myofibrillar Ca^{2+} sensitivity. Slower detachment of cross-bridges does not seem to be a factor. While this conclusion seems clear, the fact that indo-1 kinetics are limited so that the true rate of decline of $[Ca^{2+}]_i$ is faster than the measured rate of decline needs to be borne in mind and this would be expected to shorten the lag of the Ca^{2+}-derived force in both the control and caffeine experiments.

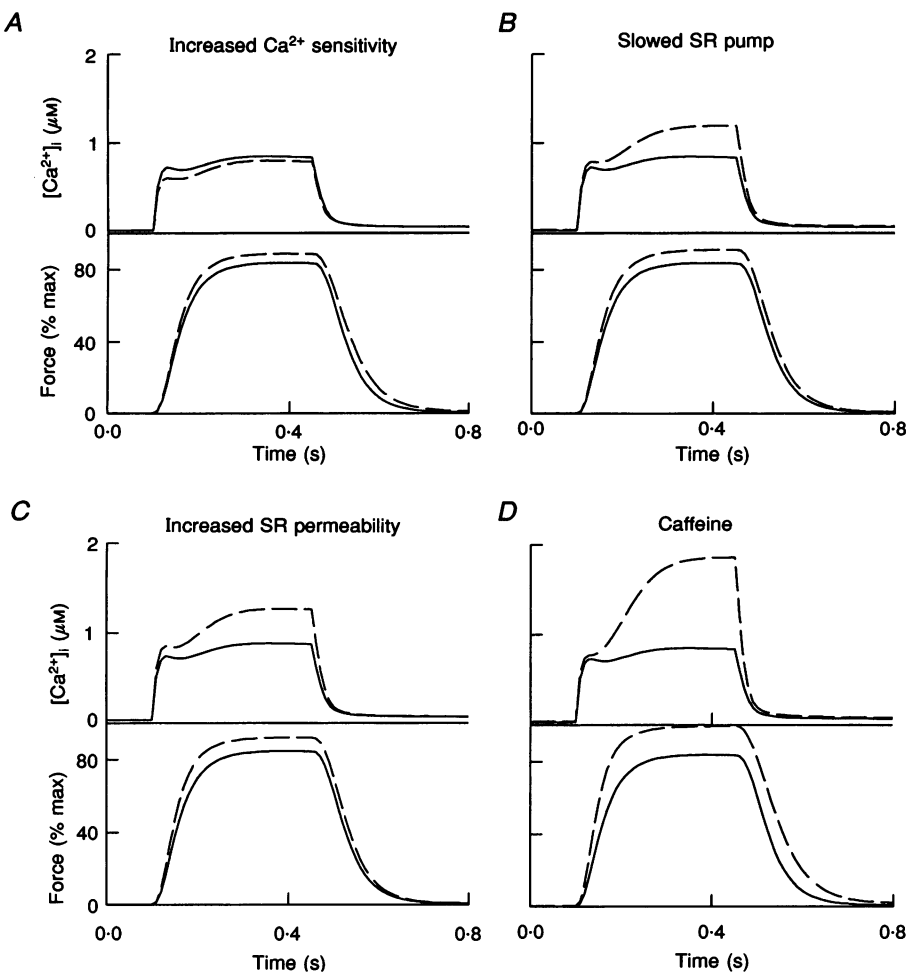

Figure 5. Plots of the output of the computer model

Each panel shows $[Ca^{2+}]_i$ (upper panel) and force (lower panel); continuous lines are the same in each panel and show the control version of the model (see Fig. 6*A* for comparison with experimental data). Dashed lines are the output of the model with the changes indicated by the panel label. In *D*, the version of the model labelled Caffeine has each of the changes shown in the preceding 3 panels incorporated (see Fig. 6*B* for comparison with experimental data).

Modelling

The aim of the modelling was to independently determine the effects on $[Ca^{2+}]_i$, force and rate of relaxation of (1) increased myofibrillar Ca^{2+}-sensitivity, (2) slowing of the SR Ca^{2+} pump, and (3) increased SR Ca^{2+} permeability. Since (1) and (2) have been measured directly, the magnitude of the permeability change was estimated by varying SR permeability in a model with (1) and (2) included until the optimal fit was obtained between the model and the experimental data in the presence of caffeine. As a starting point the variables in the model were choosen to obtain the optimal fit between the model and the control data (see Fig. 6*A* and *C*). There are two obvious discrepancies between the model and the data. Firstly, during the final phase of mechanical relaxation the measured force declines more rapidly than the model force. However, this is because the model represents an isometric muscle with uniform sarcomere spacing whereas it is known that during the final phase of relaxation there is sarcomere rearrangement (Huxley & Simmons, 1973) and this causes the more rapid phase of force decline. Secondly, the peak force is smaller in the model than in the data. This arises because the relation between $[Ca^{2+}]_i$ and force is less steep at relatively high $[Ca^{2+}]_i$ in the model compared with the data (discussed in Westerblad & Allen, 1994*a*).

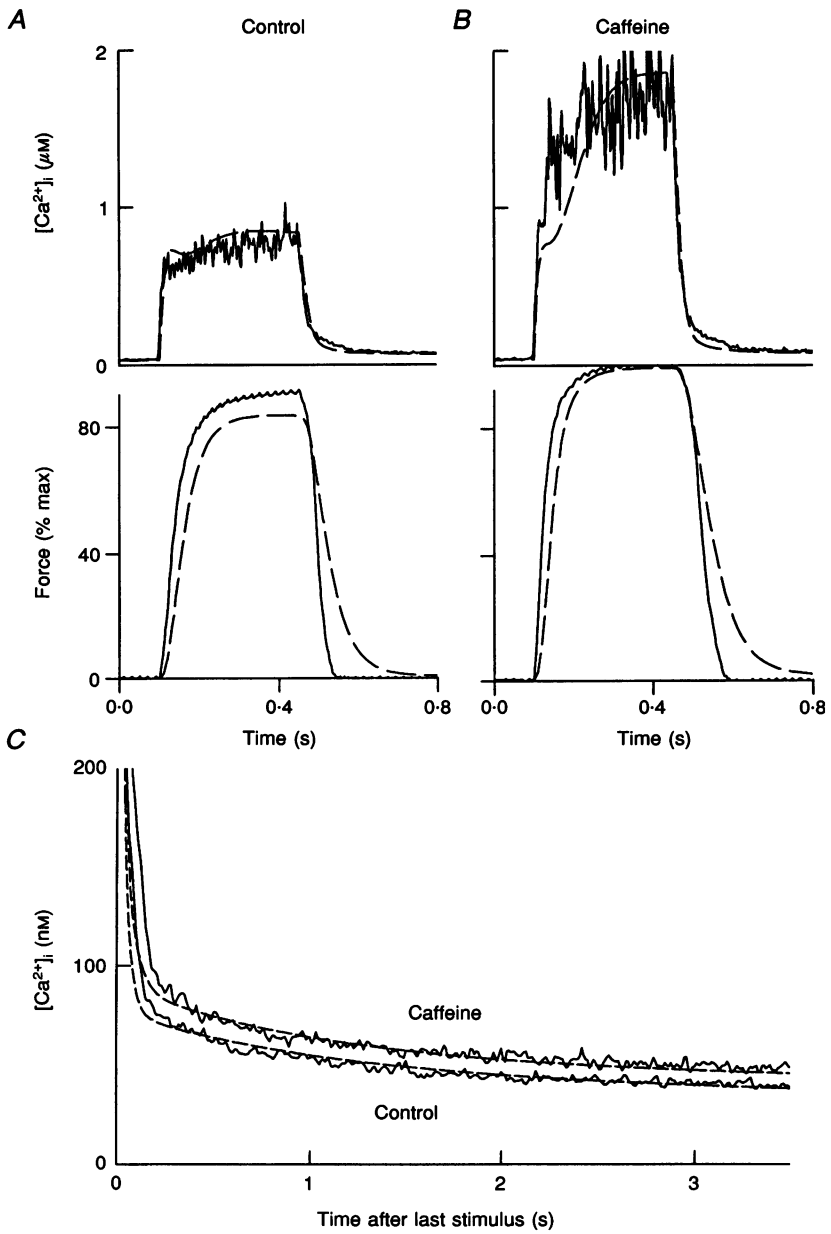

Figure 6. Comparison of experimental data (continuous lines) with model data (dashed lines)
A, control tetani. *B*, tetani in the presence of 5 mM caffeine. *C*, slow time-base plot of slow phase of $[Ca^{2+}]_i$ decline after a tetanus.

Figure 5*A* shows model outputs representing the control (continuous line) and a version with the Ca_{50} decreased from 452 to 347 nM by decreasing the rate constant of dissociation of Ca^{2+} from troponin from 185 to 140 s^{-1}. This simulates the increased Ca^{2+} sensitivity observed in the presence of caffeine. The model shows that this would produce a small increase in tetanic force, a small decrease in tetanic $[Ca^{2+}]_i$, and a small reduction of the relaxation rate (11% slower than control measured between 10 and 40 ms after the last stimulus). The slowing of relaxation arises because Ca^{2+} leaves the troponin more slowly; as a further consequence the initial $[Ca^{2+}]_i$ fall is more rapid since the SR can lower the free $[Ca^{2+}]_i$ more effectively when Ca^{2+} remains bound to troponin for longer. The faster fall in $[Ca^{2+}]_i$ is reflected in the rate constant of the rapid fall in $[Ca^{2+}]_i$ increasing from 45 s^{-1} (control) to 50 s^{-1} (increased sensitivity). Note that this is similar to the increase in rate constant noted in the experimental data and is the probable cause of this effect of caffeine.

Figure 5*B* shows the effect of slowing the SR Ca^{2+} pump to the extent measured in Fig. 3*B* by increasing K_m from 109 to 122 nM. Figure 6*C* shows that this change leads to an increase in the size of the tails of $[Ca^{2+}]_i$, which accurately mirrors the experimental results. Slowing the pump leads to a moderate increase in tetanic $[Ca^{2+}]_i$ and a small increase in tetanic force and to a small reduction of the rate of relaxation (7%, measured from 10–40 ms after the last stimulus). In the model used for Fig. 5 the SR Ca^{2+} pump is running at its maximum rate throughout a tetanus so that reduction of the binding constant of Ca^{2+} for the pump has no effect on the maximum rate; instead the increased tetanic $[Ca^{2+}]_i$ arises because the increased resting $[Ca^{2+}]_i$ causes the Ca^{2+} buffers in the myoplasm to be fuller and an unchanged release of Ca^{2+} consequently causes an increased tetanic $[Ca^{2+}]_i$. However the effect of caffeine on the pump can also be simulated by reducing V_{max} (from 4 to 2·8 mM s^{-1}) and keeping K_m constant, with an equally good fit to the data in Fig. 3*B*. When the model is run with pump inhibition produced in this way (not shown), the increase in tetanic $[Ca^{2+}]_i$ (4500 nM) is much greater than the experimental data and rises linearly throughout the tetanus. Both features are quite unlike the experimental data so it seems unlikely that caffeine reduces V_{max} of the SR pump in a situation where V_{max} is already saturated.

Figure 5*C* shows the effects of increasing SR permeability alone. In the version of the muscle model shown in Fig. 5, because the SR Ca^{2+} pump is saturated during the tetanus, only a very small increase in SR permeability (11%) is needed to produce the observed increase in tetanic $[Ca^{2+}]_i$ when the other known effects of caffeine had been included in the model. Because a model in which the SR Ca^{2+} pump is saturated in a tetanus may be unrealistic, we also ran a version of the model in which the SR function was as described in Westerblad & Allen (1994*a*). In this version the SR pump was not saturated during the tetanus and a larger increase in SR permeability (27%) was required to produce an appropriate $[Ca^{2+}]_i$ increase. The permeability change also had a small effect on the rate of relaxation (9% reduction), presumably simply because the tetanic $[Ca^{2+}]_i$ was higher.

Finally, Fig. 5*D* shows the model with increased Ca^{2+}-sensitivity, decreased pump function and increased SR Ca^{2+} permeability to simulate all the effects of caffeine. The overall fit of this model can be judged from Fig. 6*B*. The only significant discrepancy (apart from the final phase of relaxation as noted above) is that the tetanic $[Ca^{2+}]_i$ in the model rises somewhat more slowly than the experimental data. Interestingly, the alternative version of the model (SR Ca^{2+} pump not saturated, see above) simulates this aspect of the experimental data somewhat better. The slowing of relaxation in this combined model shown in Fig. 5*D* (24%) fits the observed effects of caffeine well. Note that the model has no changes in cross-bridge function associated with caffeine but is nevertheless capable of explaining the slowing of relaxation in the linear phase.

DISCUSSION

Our results confirm earlier reports that caffeine increases tetanic force, increases $[Ca^{2+}]_i$ during the tetanus and slows the rate of relaxation (Pagala, 1980; Konishi & Kurihara, 1987; Fryer & Neering, 1989). In this discussion we consider the mechanisms of increased tetanic force, increased $[Ca^{2+}]_i$, and slowed relaxation.

Mechanisms of increased force in the presence of caffeine

Two main possibilities for the increased force in the presence of caffeine are increased Ca^{2+}-sensitivity and increased tetanic $[Ca^{2+}]_i$. The increased sensitivity was first described by Wendt & Stephenson (1983) in skinned extensor digitorum longus fibres from rat. They showed that 5 mM caffeine produced a 0·06 log unit shift in Ca_{50} which compares with our observed 0·13 shift. Wendt & Stephenson (1983) found no change in the maximum Ca^{2+}-activated force with 5 mM caffeine. Thus, our results confirm that an equivalent or larger increase in sensitivity occurs in intact muscle. The size of the resulting increase in force will depend on how close the tetanic $[Ca^{2+}]_i$ is to saturation; during a 100 Hz tetanus $[Ca^{2+}]_i$ was around 1 μM so that the force increase would be less than 5–10%. The large increase in tetanic $[Ca^{2+}]_i$ will also tend to increase force but again, the increase will depend on the initial $[Ca^{2+}]_i$ level. The combination of increased sensitivity and increased tetanic $[Ca^{2+}]_i$ means that application of 5 mM caffeine to a 100 Hz tetanus is a reliable method of producing the maximum Ca^{2+}-activated force.

Mechanism of increased $[Ca^{2+}]_i$

A number of possible mechanisms for the increased $[Ca^{2+}]_i$ exist: (1) inhibition of the SR Ca^{2+} pump leading to increased myoplasmic $[Ca^{2+}]_i$ (Weber & Herz, 1968; Fuchs, 1969; Fryer & Neering, 1989). (2) increased frequency of SR Ca^{2+} channel opening leading to increased Ca^{2+} release (Weber & Herz, 1968; Rousseau *et al.* 1988) and (3) slower closing of SR channels after the end of an action potential (Simon *et al.* 1989).

Possibility (1) is slowing of the SR Ca^{2+} pump. The data in Fig. 3*B* show that net uptake of Ca^{2+} by the SR is reduced in the presence of caffeine. This could arise either because the rate of leakage of Ca^{2+} from the SR was increased or because the rate of pump was reduced. Since patch clamp studies show unequivocally that caffeine can increase SR Ca^{2+} permeability (Rousseau *et al.* 1988), it is important to consider whether this effect alone can explain the reduced net uptake rate. While it is not completely possible to exclude this possibility without unidirectional flux data, several features of our data make it seem unlikely. Firstly, there was no significant increase in the leak as determined by SR pump function analysis. Secondly, if the difference between the two curves in Fig. 3 does reflect an increased leak through SR Ca^{2+} channels, it would have to be a leak which increased in proportion to the 4th power of $[Ca^{2+}]_i$. Instead, it seems simpler to assume that the pump rate has declined. It is known that SR pump inhibition can elevate tetanic $[Ca^{2+}]_i$ and force in this preparation (Westerblad & Allen, 1994*a*), so the question becomes a quantitative one; how much of the elevated tetanic $[Ca^{2+}]_i$ can be explained by the observed slowing? Figure 5*B* shows an attempt to answer this question using our model and the result is that the slowing of the pump can explain about half of the elevation of tetanic $[Ca^{2+}]_i$.

Possibilities (2) and (3) will both lead to a raised tetanic $[Ca^{2+}]_i$ and the only obvious distinction in the present experiments would be a slowing of the early fall in $[Ca^{2+}]_i$ following a tetanus with possibility (3). Our results, however, show both that the time to peak $[Ca^{2+}]_i$ after a single stimulus was unchanged and that the first phase of the fall of $[Ca^{2+}]_i$ was accelerated in caffeine. These observations make it unlikely that the time constant of closure of the SR Ca^{2+} release channel was slowed. This conclusion is reinforced by the observations from the model (not shown) that slowing of the closure caused an obvious slowing in the time course of the early $[Ca^{2+}]_i$ decline. This conclusion contrasts with that of Simon *et al.* (1989) whose experiments were on frog muscle activated by 20–100 ms voltage clamp pulses. They found that the $[Ca^{2+}]_i$ could continue to rise for 10–60 ms at the end of a brief voltage-clamp pulse and then fall very slowly, implying a drastic prolongation in the time constant of channel closure. It seems that the actions of caffeine are different in mouse and frog and this conclusion is supported by the very different sensitivities of mouse and frog muscle to other effects of caffeine. For instance, Kanaya, Takauji & Nagai (1983) found that 8 mM caffeine was sufficient to produce a maximal contracture in frog fibres whereas Lännergren & Westerblad (1991) found that mouse fibres were resistant to contracture even at 25 mM caffeine. The magnitude of the SR permeability increase associated with each action potential required to explain the residual increase in tetanic $[Ca^{2+}]_i$ can be determined from the model as shown in Fig. 5*C* and *D*. As noted in the Results, permeability increases of 11–27% were required depending on the model of the SR pump used.

The present analysis suggests that the elevated $[Ca^{2+}]_i$ is caused both by slowed pump function and increased SR permeability. While we cannot unequivocally distinguish between these two effects during the tetanus, our modelling suggests that the two mechanisms both make contributions to the observed increase in $[Ca^{2+}]_i$.

Mechanisms of slowing of relaxation in the presence of caffeine

Several possible causes of the slowing of relaxation exist. (1) The reduced rate of closure of the SR Ca^{2+} channels (Simon *et al.* 1989). (2) Increased Ca^{2+} sensitivity (Wendt & Stephenson, 1983) so that the $[Ca^{2+}]_i$ has to fall further to achieve a given reduction in force. Alternatively, another consequence of increased sensitivity is that Ca^{2+} might dissociate more slowly from the troponin-binding sites, leading to an increased lag preceding cross-bridge dissociation. However the lag between the Ca^{2+}-derived force and measured force was unchanged in the presence of caffeine so if Ca^{2+} does dissociate more slowly from troponin, the effect is too small to be detectable by this method. (3) Slowing of the SR Ca^{2+} pump (Weber & Herz, 1968; Fuchs, 1969; Fryer & Neering, 1989). (4) Cross-bridge cycling may be slowed by caffeine leading to reduced rates of cross-bridge detachment. Possibility (1) seems to have been eliminated in the present preparation (see above). Increased Ca^{2+} sensitivity and slowing of the SR Ca^{2+} pump both independently cause a small slowing of relaxation. The magnitude of their effects has been estimated using the computer model and amounted to 11 and 7% respectively. The elevated tetanic $[Ca^{2+}]_i$ associated with the increased SR permeability also makes a comparable contribution (9% reduction) and the overall slowing in the computer model which includes all these effects was 24%, which is very close to the observed slowing (23%). This conclusion from the computer modelling fits well with the experimental analysis which showed that all the increased slowing occurred in the Ca^{2+}-derived force while there was no significant change in the cross-bridge component.

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