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The effect of length on the relationship between tension and intracellular [Ca^{2+}] in intact frog skeletal muscle fibres

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1. The relationship between tension and intracellular calcium concentration ([Ca^{2+}]$_i$) in intact frog skeletal muscle fibres was determined at two fibre lengths, corresponding to mean sarcomere lengths (SL) of 2·2 and 2·9 μm. Tension and [Ca^{2+}]$_i$ were recorded during the slow decline of tension following stimulation in the presence of cyclopiazonic acid (CPA), a sarcoplasmic reticulum Ca^{2+}-uptake pump inhibitor. [Ca^{2+}]$_i$ was estimated by injecting the K^+ salt form of the fluorescent dye fura-2 into the fibres. Experimental temperature was 3·0 °C.
2. At a SL of 2·2 μm, where thick and thin filaments fully overlap, the [Ca^{2+}]$_i$ corresponding to 50% tension generation ([Ca^{2+}]$_{50}$) was 1·09 ± 0·02 μM (mean ± S.E.M., $n = 61$ contractions). At a SL of 2·9 μm, where overlap is approximately 50%, the [Ca^{2+}]$_{50}$ was significantly lower, 0·69 ± 0·02 μM ($n = 22$ contractions). This is in agreement with previous results from skinned fibres.
3. The relationship between tension and [Ca^{2+}]$_i$ was very steep, as reported previously from experiments at a SL of 2·2 μm in which the membrane permeant acetoxymethyl ester form of fura-2 was used. The fall in tension from 90 to 10% occurred in 0·12 ± 0·01 pCa units (mean ± S.E.M., $n = 61$) for a SL of 2·2 μm and 0·17 ± 0·01 pCa units ($n = 22$) for a SL of 2·9 μm, corresponding to Hill coefficients of 15·4 and 10·9, respectively.
4. We conclude that the increase in sensitivity of tension to [Ca^{2+}] that occurs in skinned skeletal muscle fibres upon stretch also occurs in intact fibres, that the steepness of the relation between tension and [Ca^{2+}]$_i$ in intact fibres reported previously cannot be attributed to the use of the acetoxymethyl ester form of fura-2 to report [Ca^{2+}]$_i$, and that the steepness decreases as myofilament overlap decreases.

Tension production by skinned twitch skeletal muscle fibres exhibits an increase in sensitivity to [Ca^{2+}] as fibre length is increased over the descending limb of the relationship between length and tetanic tension (hereafter referred to simply as 'the descending limb of the length–tension relation'). This effect was first demonstrated over a limited range of the tension–[Ca^{2+}] relation in frog fibres by Endo (1972, 1973), and has since been confirmed for a complete range of [Ca^{2+}] in frog skinned fibres (Moisescu & Thieleczek, 1979; Stephenson & Williams, 1983) and mammalian skinned fibres (Stephenson & Williams, 1982). Stephenson & Wendt (1984) predicted that, as a consequence of this increased sensitivity with increasing length, the relationship between tension and fibre length in partially activated fibres would not be strictly dictated by the degree of overlap of thick and thin filaments as is the case in fully activated fibres for lengths beyond those resulting in full overlap (Gordon, Huxley & Julian, 1966). Instead, the optimum of the length–tension relation was predicted to shift to longer lengths during partial activation. Such a shift has been shown experimentally in skinned fibres from both amphibian (Endo, 1972, 1973) and mammalian (Moss, Swinford & Greaser, 1983) skeletal muscle.

Rack & Westbury (1969) and Close (1972) have reported that the optimal length for tension generation during twitch contractions in whole muscle falls on the descending limb of the length–tension relation. Because whole muscle might not be fully activated during twitch contractions, this shift in the length–tension relation could be a manifestation of the increase in [Ca^{2+}] sensitivity observed with increasing fibre length in skinned fibres. Balnave & Allen (1996) reported a similar shift in the optimum of the length–tension relation towards longer lengths during partial activation of intact single skeletal muscle fibres from mice. In that study, partial

activation was achieved by varying stimulus frequency during short tetanic contractions. In addition, Balnave & Allen (1996) used the Ca^{2+}-sensitive fluorescent dye indo-1 to determine the relationship between tension and intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and found no increase in the sensitivity of tension to $[Ca^{2+}]_i$ when fibre length was increased over the descending limb of the length–tension relation. Thus, the shift that occurs in the tension–$[Ca^{2+}]$ relation in skinned skeletal muscle fibres with increasing length has not been shown to exist in an intact preparation.

Morgan, Claflin & Julian (1977) reported a very steep tension–$[Ca^{2+}]_i$ relation in intact skeletal muscle fibres, and proposed that it is due to strongly co-operative binding of calcium ions and myosin molecules to thin filaments. This hypothesis predicts that decreased overlap will result in reduced co-operativity and, consequently, a less steep curve.

The purpose of the study reported here was to measure steady-state tension–$[Ca^{2+}]_i$ curves at different sarcomere lengths and examine them for both a shift in the $[Ca^{2+}]_i$ at 50% tension and changes in steepness. Measurements were obtained from fibres injected with the K^+ salt form of the Ca^{2+}-sensitive fluorescent dye fura-2 and treated with cyclopiazonic acid (CPA), a sarcoplasmic reticulum Ca^{2+}-uptake pump inhibitor that slows the decline of $[Ca^{2+}]_i$ following stimulation such that the relationship between tension and $[Ca^{2+}]_i$ is effectively steady state (Morgan *et al.* 1997). The resulting tension–$[Ca^{2+}]_i$ relations demonstrated a significant, reversible shift towards lower $[Ca^{2+}]_i$ with increased length over the descending limb of the length–tension relation, consistent with the results from skinned twitch skeletal muscle fibres. The results also showed a small average decline in steepness of the tension–$[Ca^{2+}]_i$ relation at long length, consistent with the prediction of reduced co-operativity as myofilament overlap is reduced.

METHODS

Dissection, mounting and apparatus

The experiments were performed on intact single twitch fibres isolated from the tibialis anterior muscle of the frog (*Rana temporaria*). Frogs were stored at 4 °C and killed by decapitation followed by double-pithing immediately upon removal from cold storage. Dissections were performed under dark field illumination at room temperature in a Ringer solution with the following composition (mM): NaCl, 115; KCl, 2·5; $CaCl_2$, 1·8; Na_2HPO_4, 2·15; NaH_2PO_4, 0·85; pH 7·2. After dye injection (see below), fibres were mounted horizontally in a chamber filled with the same solution and maintained at a temperature of 3·0 °C. One end of each fibre was attached to a fixed anchor and the other to a force transducer (Cambridge Technology, Inc.; model 400A). The floor of the chamber was made of polished quartz to allow unattenuated transmission of ultraviolet light for excitation of fluorescent dyes. The chamber was mounted onto the stage of an inverted microscope (Nikon Diaphot 300) fitted with an illumination and photometer system (Photon Technology International, South Brunswick, NJ, USA; model Deltascan 4000). Cyclopiazonic acid (CPA) was introduced by incubating the fibre in Ringer solution containing 1 μM CPA (Sigma) for 2 h. Huchet & Léoty (1993) have reported that CPA has no effect on the tension–$[Ca^{2+}]$ relation of skinned mammalian skeletal muscle fibres at concentrations of 10 μM and lower. All records are from fibres maintained at a mean sarcomere length (SL) of 2·2 μm or 2·9 μm. At SL = 2·2 μm, overlap of thick and thin filaments in these fibres is nearly optimal for tension generation; at SL = 2·9 μm, filament overlap is reduced to approximately 50% of optimal (Morgan, Claflin & Julian, 1991). Details of the dissection, mounting, solutions and apparatus have been described previously (Claflin, Morgan & Julian, 1990).

Monitoring $[Ca^{2+}]_i$

The fluorescent dye fura-2 (Molecular Probes) was used to estimate $[Ca^{2+}]_i$. For this study, fura-2 was introduced into the fibres by iontophoretic injection of the membrane impermeant K^+ salt form of the dye. This is in contrast to our previous studies in which dyes were introduced by soaking in the membrane permeant acetoxymethyl ester (AM) form. By using the membrane impermeant form of fura-2, all potential artifacts associated with loading of membrane-enclosed intracellular compartments (Morgan *et al.* 1997) are avoided. Injection was accomplished using filament-filled micropipettes made with a Flaming-Brown puller (Sutter Instruments, model P-87). Micropipette resistance was approximately 5 MΩ when filled with 3 M KCl. For dye injection, micropipettes were filled at the tip with 1 μl of 10 mM fura-2 dissolved in distilled water and then backfilled with 150 mM KCl. The potential at the tip of the micropipette was monitored to confirm fibre impalement. Upon impalement, indicated by a sudden drop in potential, a current of −15 nA was passed for 4–5 min. One fibre was treated with saponin (50 μg ml^{-1}) after injection of fura-2, and its fluorescence response to excitation at the isosbestic wavelength for fura-2 (358 nm in our system) was monitored. The saponin solution was exchanged every 2 min and fluorescence fell to background levels immediately after the fourth exchange, indicating that the dye had not entered membrane-enclosed intracellular compartments such as mitochondria or sarcoplasmic reticulum (Endo & Iino, 1980). This is in contrast to the response of fibres loaded via the AM form of fura-2, in which saponin reduced isosbestic fluorescence by only 50–80% (Morgan *et al.* 1997).

The fura-2 was alternately excited at two wavelengths, centred on 380 and 344 nm with 1·5 nm bandwidth, set by a pair of diffraction grating monochromators. Emitted fluorescence was passed through an interference filter centred at 510 nm with a bandwidth of 40 nm (Omega Optical, Brattleboro, VT, USA) and then detected by counting photons collected by a photomultiplier tube. Fluorescence data were acquired at a rate of 10 ratios s^{-1}, where a ratio (R) is the fluorescence collected during excitation at 344 nm divided by that collected during excitation at 380 nm, after removal of background and autofluorescence. Further details of the fluorescence measurement techniques as well as a discussion and demonstration of the advantages of using ratiometric dyes such as fura-2 for correction of motion artifact are given in Morgan *et al.* (1997).

The $[Ca^{2+}]_i$ was estimated according to the formula of Grynkiewicz, Poenie & Tsien (1985), $[Ca^{2+}] = K_D\beta(R - R_{min})/(R_{max} - R)$, where R_{min} and R_{max} are the values for R in zero and saturating $[Ca^{2+}]$, respectively, K_D is the dissociation constant and β is the ratio of the fluorescence at 380 nm excitation at zero $[Ca^{2+}]$ to that at saturating $[Ca^{2+}]$. R_{max} was determined using freshly loaded fibres that had not been stimulated by continuously monitoring R while rapidly replacing the normal Ringer solution in the chamber with a Ringer solution to which 10 mM caffeine had been added. The purpose of the caffeine was to cause a large and rapid release of Ca^{2+} from the

sarcoplasmic reticulum into the myoplasm (Konishi, Kurihara & Sakai, 1985). An R_{max} value of 8·43 ± 0·67 (mean ± S.E.M., $n = 4$) was obtained using this technique. Several attempts were made to measure R_{min} using the calcium ionophore 4-bromo-A23187 as described in Morgan *et al.* (1997), but none resulted in an R that was less than the R of a resting fibre (R_{rest}). Thus R_{rest} (0·56 ± 0·01, $n = 14$) was used as an estimate for R_{min}. The value $K_D\beta = 2{\cdot}51$ μM was taken from an *in vitro* calibration performed on the same optical set-up used for the fibre experiments.

Tension–$[Ca^{2+}]_i$ curves

The relationship between tension and $[Ca^{2+}]_i$ was characterized by fitting the Hill equation (Hill, 1913) to plots of tension against estimates of $[Ca^{2+}]_i$. Two measurements were obtained from each fitted curve: $[Ca^{2+}]_{50}$, the $[Ca^{2+}]_i$ at which 50% maximal tension is developed; and ΔpCa, the difference in pCa ($-\log_{10}[Ca^{2+}]$) between 90 and 10% maximal tension. Details of the fitting procedure and a discussion of the merits of using ΔpCa instead of the usual Hill coefficient, N (= $\log_{10}(81)/\Delta pCa$), can be found in Morgan *et al.* (1997).

Experimental protocol

For dye injection, fibres were stretched moderately (SL ≈ 2·8 μm) in the dissection dish and supported from below by a section of glass capillary tubing, 1 mm square, placed on its side. The fibre was impaled near the point where it met the capillary tubing, always within 2 mm of one of the tendons. Injection took place at room temperature. After injection of the dye, the fibre was tied into the experimental chamber and cooled to 3·0 °C. Autofluorescence was measured 4–5 mm from the site of injection where there was no contribution from the injected dye. After R_{rest} was determined, the fibres were soaked for 2 h in Ringer solution containing 1 μM CPA.

A typical experiment consisted of several 10 min series of contractions, each contraction elicited by a pair of stimuli separated by 50 ms and separated from succeeding contractions by 30–120 s. The reason for the variable inter-contraction interval is discussed in Results. Each 10 min series was separated from the next by a 10 min rest period. The SL was set to either 2·2 or 2·9 μm for an entire 10 min series and was then set to the other SL for the following series. Fluorescence was collected from a region 1·16 mm long by 0·3 mm wide at the fibre, defined by an adjustable rectangular mask in the light path. Typical fibre length was 7 mm at SL = 2·2 μm. The position of the mask along the fibre was chosen such that fluorescence intensity during excitation at 358 nm (isosbestic) was maximized. Care was taken to collect fluorescence from the same segment of the fibre regardless of SL. A video image of that segment was displayed continuously during each experiment. Striations remained visible throughout the contractions and showed no evidence that the segment of the fibre that was loaded with dye was being stretched by the dye-free segment.

Only contractions that required more than 10 s for tension relaxation (from 90 to 10%) were considered slow enough to enable the assumption of a steady-state relation between tension and $[Ca^{2+}]_i$ throughout relaxation. This criterion was based on previous results showing that $[Ca^{2+}]_{50}$ is quite sensitive to relaxation time for relaxations requiring less than 10 s, but is relatively constant for relaxation times of 10 s or more (Morgan *et al.* 1997; Fig. 6). Contractions with tension relaxation times greater than 30 s were avoided due to concerns about the metabolic effects of exceedingly long contractions; within a series, stimulation was stopped if relaxation time exceeded 30 s. Only contractions with relaxation times between 10 and 30 s were analysed.

Statistical procedures

Statistical analyses were performed using SigmaStat (Jandel Scientific). Means are accompanied by their standard error. Significance was determined mainly using Student's t test. However, when a test for the normality of the underlying population failed, the Mann–Whitney rank-sum test was used instead. This was the case for the pooled $[Ca^{2+}]_{50}$ results, the pooled relaxation times and the $[Ca^{2+}]_{50}$ results for individual fibres 970619 and 970625 (see Table 1).

RESULTS

Raw fluorescence ratio and tension records from a representative experiment are shown in Fig. 1. The records shown in Fig. 1*A* were obtained first, with the fibre held at SL = 2·9 μm. Note that, due to the presence of CPA, the duration of the tension response increases with each contraction. Of the four contractions shown in Fig. 1*A*, the final three met the criteria of having tension relaxation times greater than 10 s but not greater than 30 s. After a 10 min rest, the records shown in Fig. 1*B* were obtained at a SL of 2·2 μm; the final five contractions met the selection criteria. After a 10 min rest, the fibre was lengthened once again to SL = 2·9 μm and the records shown in Fig. 1*C* were obtained. The final two contractions met the selection criteria, with the tension relaxation time of the final contraction equal to 29·3 s. The series was stopped at that point because it was clear that additional stimuli would result in contractions with relaxation times greater than 30 s. Finally, the records shown in Fig. 1*D* were obtained at SL = 2·2 μm after a 10 min rest and the final five contractions met the criteria for analysis. Note that, for this fibre, the contractions at SL = 2·9 μm were separated by 120 s whereas those at 2·2 μm were separated by 60 s. The time separating contractions was determined by trial-and-error early in an experiment, and was designed to maximize the number of contractions having relaxation times between 10 and 30 s within a 10 min series. If contraction intervals were too short, the relaxation time would exceed 30 s after only a few; if they were not short enough, the minimum relaxation time of 10 s would not be reached for any contraction. It was consistently observed that the relaxation times at SL = 2·9 μm were much longer for a given contraction interval than those at SL = 2·2 μm. Consequently, the contraction interval used at SL = 2·9 μm was usually longer than that used at SL = 2·2 μm.

For each fibre, the individual contractions meeting the relaxation time criteria were identified and their fura-2 ratios transformed into an estimate of $[Ca^{2+}]_i$ as described in Methods. Tension was then plotted against estimated $[Ca^{2+}]_i$ for those contractions to form the tension–$[Ca^{2+}]_i$ relation for each contraction. Figure 2 shows, superimposed, all fifteen of the eligible contractions (5 at SL = 2·9 μm and 10 at SL = 2·2 μm) identified in Fig. 1 plotted in this way. Note that the plotted curves cluster according to SL, with the SL = 2·9 μm curves falling to the left of those obtained at SL = 2·2 μm, indicating an increase in sensitivity to

$[Ca^{2+}]_i$ at the longer length. Note also that the effect on the tension–$[Ca^{2+}]_i$ relation of changing SL is both reversible and repeatable. The Hill equation (Hill, 1913) was fitted to each individual eligible plot and the resulting $[Ca^{2+}]_{50}$ and ΔpCa were obtained as described in Methods. For the fibre shown in Figs 1 and 2, the mean $[Ca^{2+}]_{50}$ for all eight contractions (3 not shown) at SL = 2·9 μm was 0·61 ± 0·02 μM; for all fourteen contractions (4 not shown) at SL = 2·2 μm, mean $[Ca^{2+}]_{50}$ was significantly greater: 1·09 ± 0·04 μM ($P < 0·001$). The mean ΔpCa values for the same contractions were 0·14 ± 0·02 (corresponding to a Hill coefficient, *N*, of 13·5) and 0·12 ± 0·01 ($N = 16·2$) for SL = 2·9 and 2·2 μm, respectively.

The compiled results for all six fibres studied are given in Table 1. For each of the four fibres in which more than one contraction met the tension relaxation time criteria at both SLs tested, the $[Ca^{2+}]_{50}$ was significantly reduced at SL = 2·9 μm compared with 2·2 μm ($P < 0·001$). When all eligible contractions from all six fibres were pooled, the mean $[Ca^{2+}]_{50}$ was 0·69 ± 0·02 μM ($n = 22$) at SL = 2·9 μm and significantly greater, 1·09 ± 0·02 μM ($n = 61$), at SL = 2·2 μm ($P < 0·001$). Although the mean values for ΔpCa at SL = 2·9 μm were always larger than those at 2·2 μm for the four fibres in which more than one contraction was analysed at both SLs, statistical analysis indicated that this difference was significant for only one fibre (P was $< 0·001$ for that fibre and 0·127, 0·383 and 0·329 for the other three). However, when the results from all six fibres were pooled, a statistically significant ($P < 0·001$) increase in ΔpCa was indicated at SL = 2·9 μm (ΔpCa = 0·17 ± 0·01, $n = 22$) compared with SL = 2·2 μm (ΔpCa = 0·12 ± 0·01, $n = 61$). These ΔpCa values correspond to $N = 10·9$ and $N = 15·4$, respectively. Thus the relationship between tension and $[Ca^{2+}]_i$ as reported by the injected K^+ salt form of fura-2 is very steep, similar to that reported from a study in which fura-2 was loaded via the membrane permeant AM form (Morgan *et al.* 1997). The tension relaxation times at

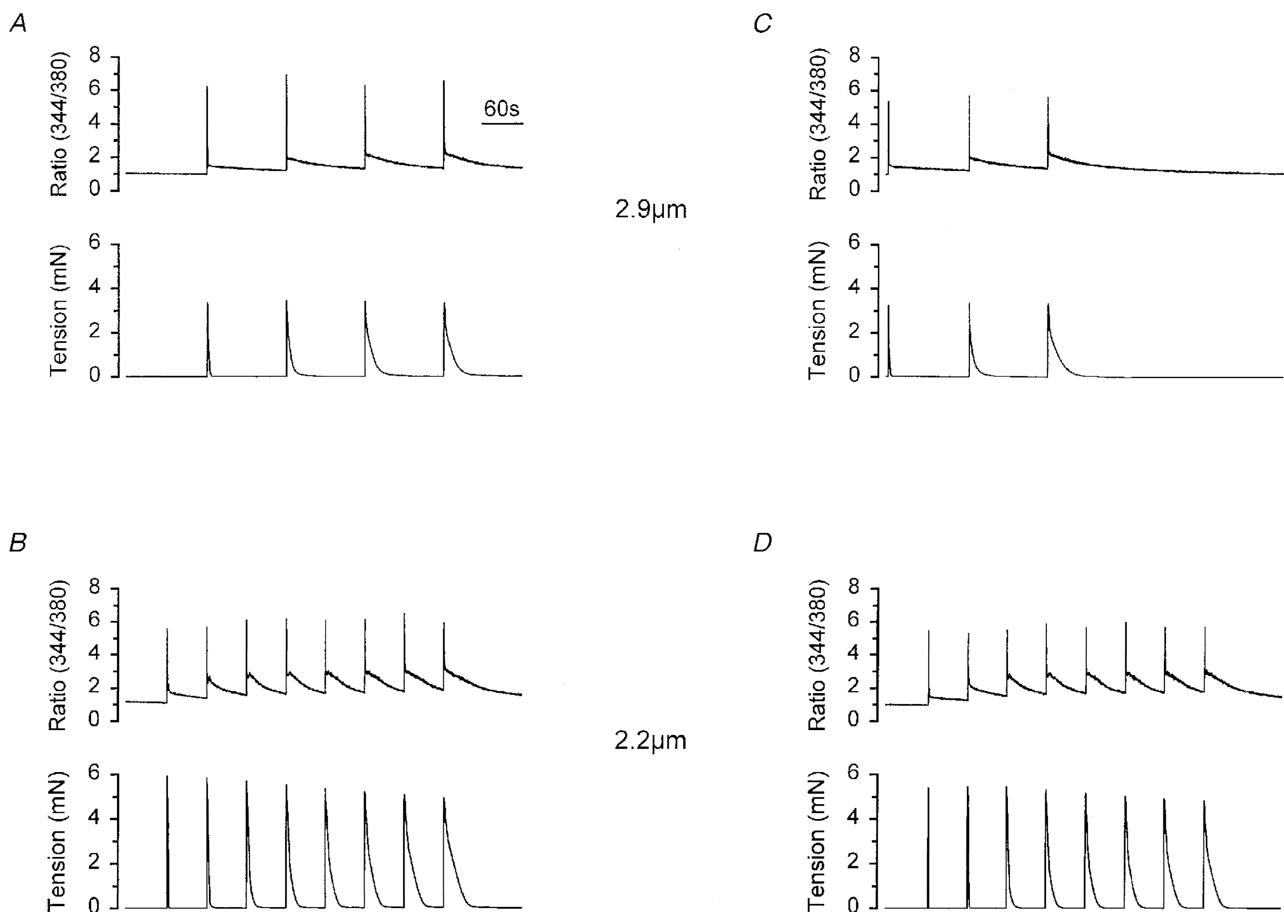

Figure 1. Fura-2 ratio and tension records at mean sarcomere lengths (SL) of 2·2 μm and 2·9 μm from a representative fibre

The records were obtained in the order *A*, *B*, *C* and then *D*, with 10 min rest between each set. *A*, fura-2 ratio (upper trace) and tension (lower trace) records resulting from pairs of stimuli, with 50 ms separation between stimuli within a pair and 120 s rest between pairs. SL was 2·9 μm, temperature was 3·0 °C. The time calibration bar applies to all traces in all panels of the figure. *B*, records as in *A* except that SL has been reduced to 2·2 μm and contractions are separated by 60 s. *C*, repeat at SL = 2·9 μm. *D*, repeat at SL = 2·2 μm. Fibre 970625.

Table 1. Summary of relaxation time, $[Ca^{2+}]_{50}$ and ΔpCa results for all fibres tested

	SL = 2·2 μm				SL = 2·9 μm			
Fibre no.	Relaxation time (s)	$[Ca^{2+}]_{50}$ (μM)	ΔpCa	No. of contractions	Relaxation time (s)	$[Ca^{2+}]_{50}$ (μM)	ΔpCa	No. of contractions
970416	17·6 ± 2·0	0·98 ± 0·04	0·10 ± 0·01	9	—	—	—	—
970425	24·0 ± 1·0	1·25 ± 0·07	0·16 ± 0·01	8	27·7	0·92	0·22	1
970507	19·5 ± 2·3	1·10 ± 0·02	0·10 ± 0·02	8	21·6 ± 2·4	0·70 ± 0·05*	0·16 ± 0·01	3
970515	20·0 ± 2·1	0·99 ± 0·01	0·17 ± 0·01	7	15·1 ± 1·1	0·80 ± 0·01*	0·19 ± 0·01	3
970619	15·8 ± 1·3	1·09 ± 0·05	0·11 ± 0·01	15	12·5 ± 0·5	0·70 ± 0·01*	0·21 ± 0·01*	7
970625	17·6 ± 1·4	1·09 ± 0·04	0·12 ± 0·01	14	18·5 ± 2·5	0·61 ± 0·02*	0·14 ± 0·02	8
Pooled	18·5 ± 0·7	1·09 ± 0·02	0·12 ± 0·01	61	17·0 ± 1·3	0·69 ± 0·02*	0·17 ± 0·01*	22

* Significantly different from the value obtained at mean sarcomere length (SL) of 2·2 μm.

SL = 2·2 μm were not different from those at 2·9 μm for either individual fibres or for the pooled data, thus ruling out differences in relaxation times as a confounding factor in the analyses of differences in $[Ca^{2+}]_{50}$ and ΔpCa. The consistency of the tension relaxation times was due to the careful manipulation of contraction intervals described above.

DISCUSSION

Effect of length on Ca^{2+} sensitivity of tension

The results show clearly that the sensitivity of tension generation to $[Ca^{2+}]_i$ in intact twitch skeletal muscle fibres from frogs increases as SL is increased over the descending limb of the length–tension relation. Such an increase has been reported to occur in skinned twitch fibres from both amphibian (Endo, 1972, 1973; Moisescu & Thieleczek, 1979; Stephenson & Williams, 1983) and mammalian (Stephenson & Williams, 1982) skeletal muscle, but has not before been demonstrated in intact fibres. Balnave & Allen (1996) reported no increase in the sensitivity of tension to $[Ca^{2+}]_i$ with increasing fibre length over the descending limb of the length–tension relation in intact mammalian fibres. In that study, no attempt was made to slow the rate of change of $[Ca^{2+}]_i$ in order to improve the likelihood that tension and $[Ca^{2+}]_i$ were near steady state. Morgan *et al.* (1997) presented evidence suggesting that, in frog fibres at 3·0 °C, such a condition required tension relaxation times that were at least 10 s in duration, which could not be achieved in a fibre that had not been treated with a sarcoplasmic reticulum Ca^{2+}-uptake pump inhibitor such as CPA. Thus the apparent discrepancy between intact frog and mammalian fibres could be due to a difference in the degree to which a steady state between tension and $[Ca^{2+}]_i$ was maintained; alternatively there could be a real difference in the way fibres from the two species respond.

In tetanized intact skeletal muscle fibres, tension generation is proportional to the degree of overlap of thick and thin filaments, and this property is responsible for the linear 'descending limb' of the length–tension relation (Gordon *et al.* 1966). Filament overlap does not, however, appear to be the sole determinant of tension generation under conditions expected to result in submaximal activation in intact skeletal muscle preparations. Such conditions include twitch contractions (Rack & Westbury, 1969; Close, 1972),

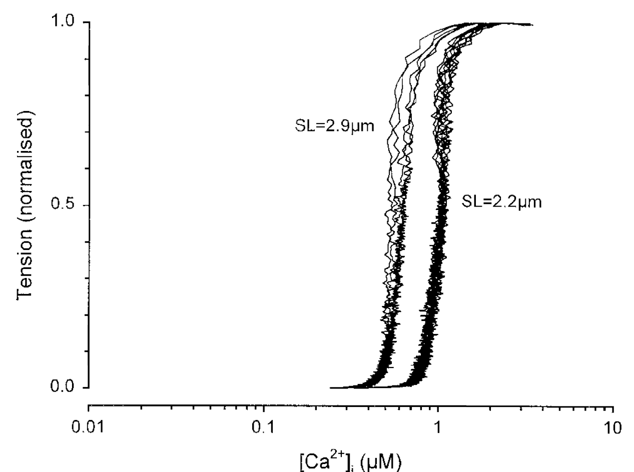

Figure 2. Tension plotted against $[Ca^{2+}]_i$ at mean sarcomere lengths (SL) of 2·2 μm and 2·9 μm from a representative fibre.

The tension records shown in Fig. 1 are normalized to 1·0 for each individual contraction and plotted here against $[Ca^{2+}]$ values calculated from the corresponding fura-2 ratio records. All contractions with tension relaxation times (90 to 10 %) between 10 and 30 s are superimposed. This includes the final 3 contractions at SL = 2·9 μm from Fig. 1*A*, the final 5 contractions at SL = 2·2 μm from Fig. 1*B*, the final 2 contractions at SL = 2·9 μm from Fig. 1*C* and the final 5 contractions at SL = 2·2 μm from Fig. 1*D* for a total of 10 plots at SL = 2·2 μm and 5 plots at SL = 2·9 μm. Note that the plots cluster by SL, with those from SL = 2·9 μm falling to the left (lower $[Ca^{2+}]_i$) of those from SL = 2·2 μm. Temperature was 3·0 °C. Fibre 970625.

contractions resulting from stimulation at relatively low rates (Balnave & Allen, 1996), and contractions in the presence of dantrolene sodium (Wendt & Barclay, 1980). Under these circumstances, the optimum of the length–tension relation is shifted to longer lengths such that, over part of the relation, tension generation is increasing as overlap is decreasing. This apparent paradox could be explained, at least in part, by the findings confirmed here. That is, as fibre length is increased under conditions resulting in submaximal activation, the attendant reduction in filament overlap could be more than compensated for by the increase in sensitivity of tension to $[Ca^{2+}]_i$ that results.

The mechanism responsible for the apparent increase in Ca^{2+} sensitivity with increasing sarcomere length in twitch fibres has not yet been identified. One possibility often proposed is the increased effective concentration of myosin heads in the vicinity of the thin filament due to the reduction in interfilament distance that results from stretch in skinned fibres (e.g. Endo, 1972, 1973). Since the volume of an intact skeletal muscle fibre remains constant with stretch, the distance between filaments decreases as the inverse square root of fibre length (Huxley, 1953). Hence explanations based upon the reduction in interfilament distance resulting from stretch are equally applicable to intact fibres. An alternative explanation is based on our previous report that, for models with strongly co-operative binding of Ca^{2+} and myosin to thin filaments, the tension–Ca^{2+} relation shifts towards lower $[Ca^{2+}]$ as the myosin off-rate is decreased (Morgan *et al.* 1997). It is reasonable to expect that the stabilizing effects of passive stiffness would tend to reduce internal movement and, consequently, the myosin off-rate at long fibre lengths. Thus the apparent increase in Ca^{2+} sensitivity at long length could be a manifestation of myosin–Ca^{2+} co-operativity brought about by increased passive stiffness. Neither of these possible mechanisms, however, can be reconciled with the finding of Stephenson & Williams (1983) that, contrary to results from twitch fibres from amphibians and both fast and slow fibres from mammals, amphibian slow fibres exhibit a *decrease* in Ca^{2+} sensitivity with stretch.

Another factor that must be considered in intact preparations is the effect of sarcomere length on the intracellular Ca^{2+} transient (ICT) that results from stimulation. We have shown previously that increasing SL from 2·2 to 2·8 μm does not affect the peak amplitude of the ICT, but does prolong significantly its duration in intact fibres (Claflin, Vandenboom, Morgan & Julian, 1997). Others have shown that twitch tension generation is closely correlated with ICT duration; longer duration results in greater tension (Jiang, Johnson & Rall, 1996; Sun, Lou & Edman, 1996; Johnson, Jiang & Flynn, 1997). Thus the increase in ICT duration observed with increased fibre length over the descending limb of the length–tension relation could be contributing to the reported shift in the optimum towards longer lengths in intact fibres that are less than fully activated.

It is likely that the long contractions required for these experiments are associated with an accumulation of metabolites in the myoplasm. Furthermore, the accumulation might be expected to be greater at SL = 2·2 μm than at SL = 2·9 μm due to increased opportunity for cross-bridge interaction with the thin filament at the shorter length, and this difference could be contributing to the results reported here. For example, phosphate has been shown to shift the tension–Ca^{2+} relation towards higher $[Ca^{2+}]$ in skinned skeletal muscle fibres (Millar & Homsher, 1990). We do not believe such accumulations are affecting our results for the following reasons. We have shown (Fig. 4 in Morgan *et al.* 1997) that the tension–$[Ca^{2+}]_i$ curve obtained during a slow rise in tension closely coincides with that obtained during the fall. This is evidence against effects due to accumulation during a single contraction. The consistency of the results shown in Figs 1 and 2 are evidence against effects due to accumulation during the course of a 10 min series of contractions.

Steepness of the tension–$[Ca^{2+}]$ relation

Morgan *et al.* (1997) reported that the tension–$[Ca^{2+}]_i$ relation in intact skeletal muscle fibres is much steeper than those reported in skinned fibres. In that study, $[Ca^{2+}]_i$ was monitored using fura-2 loaded via the membrane permeant AM form and 20–50 % of the loaded fluorescence remained after treatment with saponin, suggesting that membrane-enclosed intracellular compartments had been loaded in addition to the myoplasm. Several potential consequences of this saponin-resistant fluorescence were considered and it was concluded that, even making worst-case assumptions, the tension–$[Ca^{2+}]_i$ relation was still very steep (Morgan *et al.* 1997, Appendix II). In the present study, fura-2 was loaded in the K^+ salt form by iontophoretic injection and all added fluorescence was released within minutes by treatment with saponin. The finding in the present study that the tension–$[Ca^{2+}]_i$ relation is very steep, uncomplicated by considerations of dye-loaded intracellular compartments, confirms our previous finding and supports the hypothesis that tension generation is a highly co-operative process in intact skeletal muscle fibres.

Effect of length on tension–$[Ca^{2+}]$ steepness

The results suggest that the tension–$[Ca^{2+}]_i$ relation is less steep at SL = 2·9 μm than at SL = 2·2 μm. If so, this could be due to a true decrease in co-operativity at the longer length, perhaps due to reduced myosin–Ca^{2+} or myosin–myosin co-operativity due, in turn, to reduced overlap. Alternatively, the extreme steepness of the relation at full overlap could be due to increasing internal movement as relaxation proceeds, as suggested by Morgan *et al.* (1997). According to this model-based explanation, more internal movement causes a greater mean cross-bridge detachment rate which, due to myosin–Ca^{2+} co-operativity, causes a shift in the tension–$[Ca^{2+}]$ relation to the right (towards higher $[Ca^{2+}]$ – see Fig. 7 in Morgan *et al.* 1977). If internal movement increases *during* relaxation, then a single tension–$[Ca^{2+}]$ curve can no longer be used to describe the

relationship. Instead, a hybrid relationship would be more appropriate, constructed by beginning on the curve corresponding to relatively little internal movement and moving continuously to curves corresponding to more internal movement as relaxation proceeds. Because the curves shift to the right with increasing internal movement, the hybrid relationship will be steeper than any of the constituent curves. Extending this, reduced steepness at SL = 2·9 μm could be due to a reduced rate of increase of internal motion during relaxation due, in turn, to the stabilizing effects of the increased passive stiffness present at long lengths. That is, the steepness of the tension–$[Ca^{2+}]_i$ relation at SL = 2·9 μm would be closer to indicating the co-operativity of the tension generating process of a truly isometric fibre.

Fura-2 calibration

Estimates of the parameters required to interpret fura-2 fluorescence changes in terms of changes in absolute Ca^{2+} concentrations are subject to considerable uncertainty in intact skeletal muscle fibres (see Morgan *et al.* 1997 for a discussion of some of the difficulties). For the present study, the value used for $K_D\beta$ was that obtained from an *in vitro* calibration of fura-2 performed using the same optical set-up used for the fibre studies. The value used for R_{min} was R_{rest}, the R in a freshly loaded fibre before any stimulation. Although the K_D value measured *in vitro* is likely to be lower than that measured in myoplasm (Baylor & Hollingworth, 1988), any inaccuracy in estimates of $[Ca^{2+}]_i$ that result from this approximation do not affect our conclusions regarding the effect of SL on $[Ca^{2+}]_{50}$. This is because $K_D\beta$ simply scales the estimates of $[Ca^{2+}]_i$ and would have the same effect at both SLs. Furthermore, because the steepness of the tension–$[Ca^{2+}]_i$ relation is a function of the ratio of two $[Ca^{2+}]_i$ values, it is not affected by the value of $K_D\beta$. R_{rest} (0·56) represents an upper limit for R_{min}. To determine the sensitivity of our conclusions to inaccuracies in this estimate, sample calculations were performed using a very conservative estimate of 0·30 for the lower limit of R_{min}. With $R_{min} = 0·30$, the difference between the $[Ca^{2+}]_{50}$ values at the two SLs tested was reduced by only 8·6% (in terms of pCa units) compared with the values obtained with $R_{min} = 0·56$. The sensitivity of steepness (ΔpCa) to R_{min} was even smaller.

Conclusions

We conclude that the increase in sensitivity of tension to $[Ca^{2+}]$ with increasing fibre length that is a feature of skinned twitch skeletal muscle fibres also occurs under the more physiological conditions found in intact fibres from frogs. In addition, we conclude that the very steep tension–$[Ca^{2+}]$ relation that is reported to exist in intact skeletal muscle fibres from frogs is not an artifact attributable to the technique used to load the $[Ca^{2+}]$ reporter, fura-2. Finally, our results suggest that the steepness of the tension–$[Ca^{2+}]$ relation is reduced at long sarcomere lengths, consistent with hypotheses that include strong co-operativity between Ca^{2+} and myosin binding to thin filaments.

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