Total and sarcoplasmic reticulum calcium contents of skinned fibres from rat skeletal muscle

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1. The Ca^{2+} content of single mammalian skeletal muscle fibres was determined using a novel technique. Mechanically skinned fibres were equilibrated with varying amounts of the Ca^{2+} buffer BAPTA and were then lysed in a detergent–paraffin oil emulsion. The subsequent myofilament force response was used to estimate the additional amount of Ca^{2+} bound to BAPTA following lysis of intracellular membranes.

2. The total endogenous Ca^{2+} content (corrected for endogenous Ca^{2+} buffering) of fast-twitch (FT) and slow-twitch (ST) fibres at a myoplasmic pCa ($-\log[Ca^{2+}]$) of 7·15 was $1·32 \pm 0·02$ and $1·35 \pm 0·08$ mM per fibre volume, respectively. The sarcoplasmic reticulum (SR) component of these estimates was calculated as 1·01 and 1·14 mM, respectively, which normalized to SR volume corresponds to resting SR Ca^{2+} contents of 11 and 21 mM, respectively.

3. Equilibration of 'resting' fibres with low myoplasmic $[Ca^{2+}]$ (pCa 7·67–9·00) elicited a time-dependent decrease in Ca^{2+} content in both fibre types. Equilibration of resting fibres with higher myoplasmic $[Ca^{2+}]$ (pCa 5·96–6·32) had no effect on the Ca^{2+} content of ST fibres but increased the Ca^{2+} content of FT fibres. The maximum steady-state total Ca^{2+} content (3·85 mM) was achieved in FT fibres after 3 min equilibration at pCa 5·96. Equilibration at higher myoplasmic $[Ca^{2+}]$ was less effective, probably due to Ca^{2+}-induced Ca^{2+} release from the SR.

4. Exposure of fibres to either caffeine (30 mM, pCa ~8, 2 min) or low myoplasmic $[Mg^{2+}]$ (0·05 mM, pCa ~9, 1 min) released approximately 85% of the resting SR Ca^{2+} content. The ability of caffeine to release SR Ca^{2+} was dependent on the myoplasmic Ca^{2+} buffering conditions.

5. The results demonstrate that the SR of ST fibres is saturated with Ca^{2+} at resting myoplasmic $[Ca^{2+}]$ while the SR of FT fibres is only about one-third saturated with Ca^{2+} under equivalent conditions. These differences suggest that the rate of SR Ca^{2+} uptake in FT fibres is predominantly controlled by myoplasmic $[Ca^{2+}]$ while that of ST fibres is more likely to be limited by the $[Ca^{2+}]$ within the SR lumen.

The time course of the contraction–relaxation cycle of skeletal muscle is greatly influenced by the function of the sarcoplasmic reticulum (SR), which acts to both release and to re-sequester intracellular calcium ions. Some aspects of SR function in mammalian skeletal muscle have been ascertained from the simultaneous measurement of the myoplasmic free calcium ion concentration ($[Ca^{2+}]_i$) and force output of intact fibres (Fryer & Neering, 1986; Westerblad & Allen, 1991). However, the $[Ca^{2+}]_i$ changes measured in these studies represent only a small fraction of the total Ca^{2+} that is released and re-sequestered by the SR during any given cycle. The total Ca^{2+} content of the SR under 'resting' myoplasmic conditions has been estimated as ~1–2 mM per fibre volume in a number of different studies. The methods used include radiotracer (^{45}Ca) techniques in skinned frog fibres (Ford & Podolsky, 1972), mathematical modelling of $[Ca^{2+}]_i$ transients in cut fibres (Klein, Kovacs, Simon & Schneider, 1991), and electron probe microanalysis of snap-frozen whole muscles (Somlyo, McClellan, Gonzales-Serratos & Somlyo, 1985). One important parameter that has not been clearly defined in these studies is the degree of Ca^{2+} saturation of calsequestrin inside the SR lumen at 'rest' and during activity. An assessment of this parameter under different myoplasmic conditions is important because it will ultimately influence the level of free calcium in the SR

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lumen ($[Ca^{2+}]_{SR}$), which is a key determinant of: (i) the rate of Ca^{2+} loss through SR Ca^{2+} release channels (Feher & Briggs, 1982; Sitsapesan & Williams, 1995), and (ii) the activity of the SR Ca^{2+} pump (Inesi & De Meis, 1989).

The aim of the present study was to investigate changes in the Ca^{2+} content of single skinned mammalian skeletal muscle fibres that have been exposed to myoplasmic calcium levels similar to those seen in intact fibres during rest and activity. A novel technique is described for the direct measurement of fibre Ca^{2+} content that utilizes the myofilament force response as a Ca^{2+} indicator during intracellular membrane lysis within a restricted volume. The results obtained with this technique reveal fundamental differences in SR Ca^{2+} handling between fast-twitch (FT) and slow-twitch (ST) fibres that are related to differences in their Ca^{2+} storage capacity.

Some aspects of the present work have been previously communicated in abstract form (Fryer & Stephenson, 1993*a*, *b*; 1994).

METHODS

Skinned muscle fibre preparation

Skinned fibres were prepared using methods previously decribed in detail (Fink, Stephenson & Williams, 1986). Male Long–Evans hooded rats (*Rattus norvegicus*; 5–12 months old) were killed by diethyl ether overdose. The soleus (ST) and extensor digitorum longus (EDL, FT) muscles were rapidly excised, blotted on filter paper and pinned out on a bed of Sylgard 184 (Dow Chemicals, Midland, MI, USA) under paraffin oil (Ajax Chemicals, Sydney, Australia). In most experiments, a single fibre was isolated and mechanically skinned using fine forceps. One end of the fibre was attached to a force transducer (AME 875; Horten, Norway) using fine braided silk thread (10/0 gauge; Deknatel, Fall River, MA, USA) while the free end was clamped in fine platform-ended forceps. In some experiments, two or three segments from the same skinned fibre were isolated and used to assess the reproducibility of the Ca^{2+} content technique within the same fibre. Isometric force was recorded on a chart recorder. Prior to the experiment, the diameter (D) and length (L) of each fibre was measured under oil after setting the sarcomere length to ~3·1–3·2 μm. At this sarcomere length the preparation is under tension in the relaxed state. It is therefore possible to ascertain, with great sensitivity, when Ca^{2+}-activated force develops anywhere in the fibre segment (Stephenson & Williams, 1982). All experiments were performed at a temperature of 22 ± 1 °C.

Fibre types

The rat soleus muscle is comprised 85% ST fibres (Close, 1972). These fibres characteristically displayed force oscillations of myofibrillar origin during submaximal contractions and gave near-maximal tension responses in a Sr^{2+}-buffered solution of pSr ($-\log[Sr^{2+}]$) 5·5 (Stephenson, O'Callaghan & Stephenson, 1994). The remaining fibres (~15%) have intermediate Ca^{2+}-activation properties between FT and ST fibres (Stephenson & Williams, 1981), express both FT and ST isoforms (Stephenson *et al.* 1994) and can be easily identified by the lack of force response when exposed to a Sr^{2+}-buffered solution of pSr 5·5. Results from this type of fibre were not included in the present study. The Sr^{2+}-buffered solution (pSr 5·5) was also used to identify the rare occurrence (< 5%) of ST fibres in the EDL muscle. Results from these fibres were also excluded. The EDL is composed (> 95%) of roughly equal numbers of type II A and II B fibres (Close, 1972) that are unresponsive to pSr 5·5 solution.

BAPTA solutions

In initial experiments, solutions containing EGTA as the Ca^{2+} buffer were used in an attempt to quantify the total fibre Ca^{2+} content. However, use of these solutions yielded unstable force responses during experimental protocols similar to that shown in Fig. 1. In particular, problems associated with pH changes and rigor development required a new set of solutions to be designed. These new solutions ('B' solutions) contained a relatively pH-insensitive Ca^{2+} buffer (BAPTA) and a high concentration of creatine phosphate (CP).

Table 1 shows the composition of the BAPTA-based solutions used for determining the total fibre Ca^{2+} content. Three main types of solution were used: B1 (relaxing), B2 (Ca^{2+} activating) and B3 (low relaxing). Fibres were fully relaxed in B1 solution and fully activated by Ca^{2+} in B2 solution (unless otherwise noted). B3 solution was used for diluting the total [BAPTA] to below 10 mM in B solutions and also as a pre-activating solution prior to recording force–$[Ca^{2+}]$ calibration curves. In the latter case, 50 μM BAPTA was added to the B3 solution.

Solutions of different BAPTA and Ca^{2+} concentrations (pCa ($-\log[Ca^{2+}]$) ≥ 5·5) were obtained by mixing B1, B2 and B3 solutions in appropriate proportions. Solutions of pCa < 5·5 were obtained by adding small amounts of $CaCl_2$ (< 1·5 mM) and readjusting the pH to 7·10. The concentration of ionic equivalents and the osmolality of solutions varied within narrow ranges: 185 ± 10 mM and 300 ± 15 mosmol kg^{-1}, respectively. In some experiments the SR was depleted of Ca^{2+} using a solution composed of caffeine (30 mM) dissolved in a B1–B3 (1 : 20) solution. In other experiments SR depletion was achieved using a low $[Mg^{2+}]$ B3 solution (B3LM, Table 1; total $[Mg^{2+}]$, 2 mM; free $[Mg^{2+}]$, ~50 μM).

The ratio of total $[Ca^{2+}]$ to total [BAPTA] was determined by back-titrating B solutions with Ca^{2+} using a Ca^{2+}-sensitive microelectrode (Model 93–20, Orion, Cambridge, MA, USA). The Ca^{2+} electrode output was Nernstian between pCa 3·0 and 7·5 with BAPTA-buffered solutions (slope, 28 mV (pCa unit)$^{-1}$). Alternatively, the concentration of Ca^{2+}-free BAPTA in solution was determined by a potentiometric method where known amounts of EGTA were added to chelate excess Ca^{2+} not bound by BAPTA after addition of known amounts of total Ca^{2+} (Bakker, Head, Williams & Stephenson, 1993). Once the total [BAPTA] was determined, the apparent affinity constant (K_{app}) of BAPTA for Ca^{2+} was calculated by either: (i) adding Ca^{2+} corresponding to half the known amount of BAPTA and measuring the free $[Ca^{2+}]$ (3 measurements), or (ii) preparing various mixtures of B1 and B2 and measuring their free $[Ca^{2+}]$ (4 measurements). The mean (± S.E.M.) K_{app} from these seven determinations was $7·1 \times 10^6 \pm 0·3 \times 10^6$ M^{-1}.

Experimental protocol for determining fibre Ca^{2+} content

Figure 1 illustrates the use of BAPTA-based solutions in determining the total Ca^{2+} content of an ST (*A*) and an FT fibre (*B*). In both preparations the fibre was initally transferred from paraffin oil into an equilibration solution (for 1 min) containing 0·8 mM total Ca^{2+} and 2·3 mM total BAPTA (pCa 7·15). The fibre was then plunged into a freshly triturated emulsion of 90%

Table 1. Composition of BAPTA-based solutions

Solution	BAPTA (mM)	CP (mM)	Total [Ca^{2+}] (mM)	Free [Mg^{2+}] (mM)	K^+ (mM)	Na^+ (mM)	Sucrose (mM)
B1	10·5	40	—	1	99	97	80
B2	10·5	40	10·0	1	78	97	80
B3	—	50	—	1	58	117	80
B3LM	—	50	—	0·05	58	117	80

All solutions contained 8 mM total ATP and 1 mM NaN_3, and were buffered to a pH of 7·10 ± 0·01 at 23 °C with piperazine-*N*,*N*′-bis(2-ethanesulphonic acid).

paraffin oil and 10% of the non-ionic detergent Triton X-100 (Sigma-Aldrich, Sydney, Australia) (Triton–oil emulsion). The detergent entered the aqueous phase of the preparation, lysed all intracellular membranes, and liberated Ca^{2+} from intracellular stores. As a result, [Ca^{2+}] rose to a plateau within the volume occupied by the preparation and active force developed (~16 and 13% of maximum Ca^{2+}-activated force in Fig. 1*A* and *B*, respectively) up to a level determined by the free [Ca^{2+}] achieved at the myofilaments. Once a force plateau was achieved the fibre was transferred to a relaxing solution (B1), which served to both relax the fibre and to wash off the Triton–oil emulsion. Finally, the fibre was sequentially activated in BAPTA solutions containing progressively higher percentages of total Ca^{2+} (expressed as a percentage of total [Ca^{2+}]/total [BAPTA], %CaBAPTA), in order to establish the relationship between %CaBAPTA and force. This relationship was established for each individual fibre in each experiment. The mean data from twenty to sixty-five fibres of each muscle type are shown later in Fig. 2*D* and *E* (filled triangles).

Calibration of force responses

In order to calibrate the force response obtained during lysis in Triton–oil emulsion it was necessary to take into account the effects of this emulsion on the maximum force generating capacity (F_{max}) and the Ca^{2+} sensitivity of the myofilaments. The effect of Triton X-100 on F_{max} was determined by either: (i) moving the fibre from a maximally activating CaBAPTA solution into the Triton–oil emulsion and observing the change in force development (Fig. 2*A*), or (ii) equilibrating the fibre with a very low total [BAPTA] and then lysing it in the Triton–oil emulsion to elicit a saturating response (Fig. 2*B*). Both methods consistently revealed a depression of F_{max} of ~40% in both ST and FT fibres,

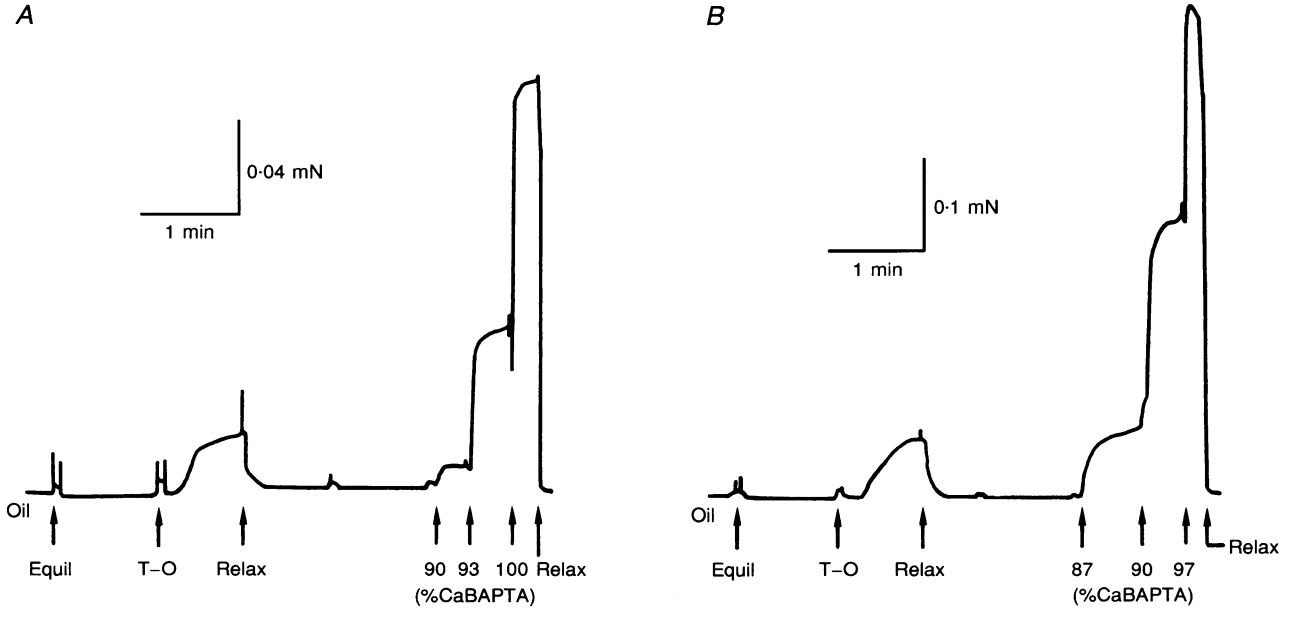

Figure 1. Protocol for determining Ca^{2+} content using BAPTA-based solutions

A, ST fibre (*L*, 1·5 mm; *D*, 33 μm) was transferred from paraffin oil into the following sequence of solutions: (i) B1–B2 mixture (2·3 mM total BAPTA, 0·8 mM total Ca^{2+}) for 1 min equilbration (Equil); (ii) emulsion of 10% Triton X-100 and 90% oil (T–O) to lyse the SR; (iii) B1 solution to wash and relax the fibre (Relax); (iv) B3 solution containing ~50 μM BAPTA for pre-activation; (v) force calibration in a series of type 'B' solutions containing 10·5 mM BAPTA with increasing percentages (values shown beneath arrows) of total Ca^{2+} saturation (%CaBAPTA). *B*, FT fibre (*L*, 1·6 mm; *D*, 33 μm) subjected to the same protocol as the ST fibre in *A* except that lower %CaBAPTA solutions were required for the force calibration procedure.

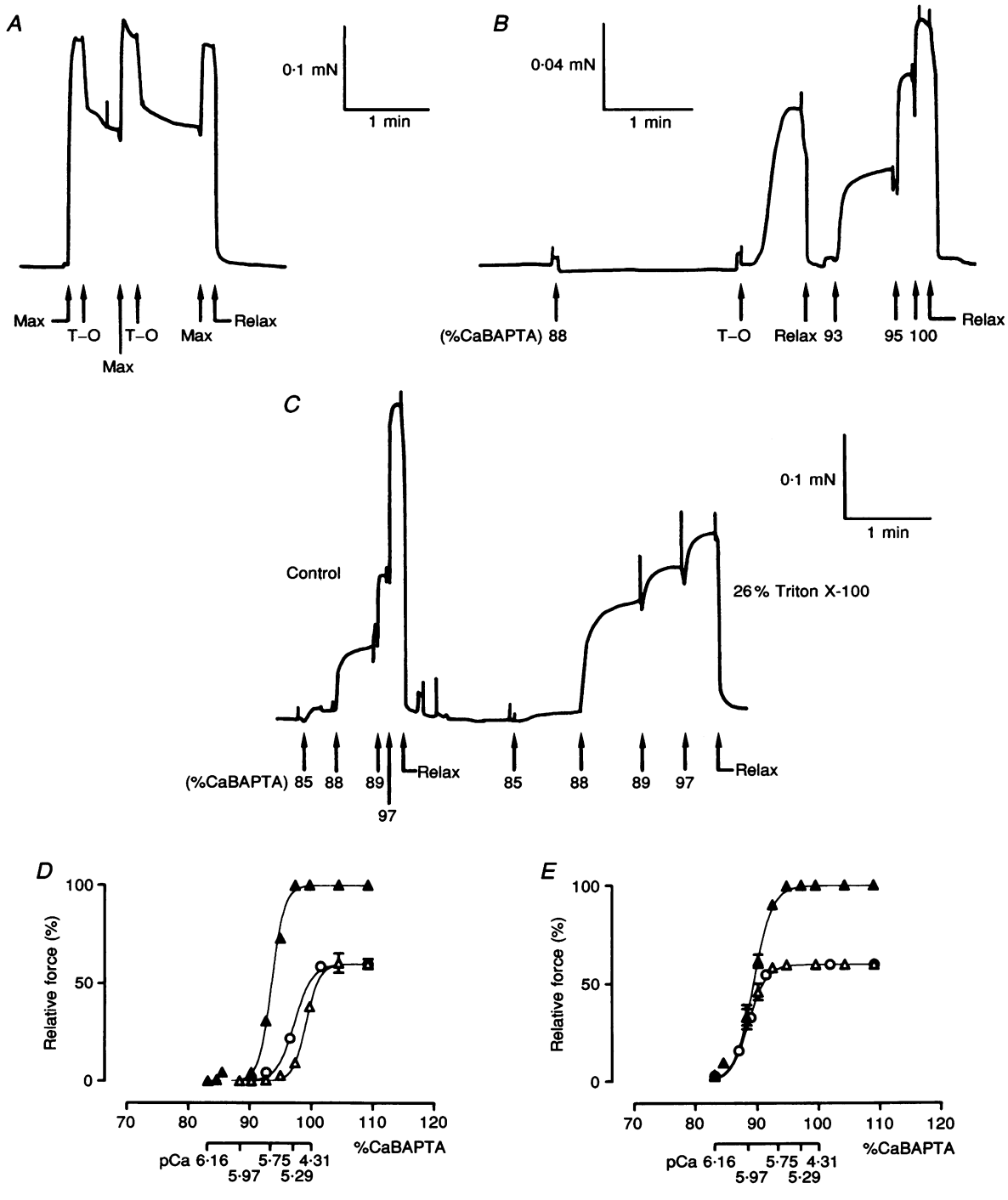

Figure 2. Effects of Triton–oil treatment on maximum Ca^{2+}-activated force (F_{max}) and myofilament Ca^{2+} sensitivity

A, Triton–oil treatment depresses F_{max}. A single FT fibre (*L*, 1·4 mm; *D*, 38 μm) was transferred between an aqueous maximal Ca^{2+}-activation solution (Max; 10·5 mM total BAPTA, 10·2 mM total Ca^{2+}) and the Triton–oil emulsion (T–O). *B*, a typical saturated force response recorded during Triton–oil lysis. A single ST fibre (*L*, 1·2 mm; *D*, 33 μm) was equilibrated for 2 min in a B1–B2 solution (1 mM total BAPTA, 0·88 mM total Ca^{2+}) before fibre lysis in Triton–oil. *C*, protocol showing the change in myofilament Ca^{2+} sensitivity of a single FT fibre (*L*, 1·8 mm; *D*, 38 μm) between control solutions (type B) and paired solutions containing 26% Triton X-100 (v/v). *D* and *E*, effect of Triton X-100 on the force–[Ca^{2+}] relation in ST fibres (*D*) and FT (*E*) fibres. ▲, mean (± S.E.M.) normalized force response obtained from 20–65 fibres under control conditions (type B solutions, no added Triton X-100). This data came from experiments like that shown in *C* and also from the force–[Ca^{2+}] calibration curves performed at the end

which we ascribe to the presence of a high Triton X-100 concentration in the fibre. To elicit an equivalent reduction in F_{max} in aqueous solution it was necessary to add 26% Triton X-100 (v/v).

Two methods were used to assess the effects of the Triton–oil emulsion on submaximal force responses. In the first method, force was recorded in a series of submaximal activation solutions, with one set containing 26% Triton X-100 in aqueous solution and the other set serving as control. A typical set of force responses obtained in this manner is shown in Fig. 2*C*, while the mean results obtained from six ST and six FT fibres are shown as open triangles in Fig. 2*D* and *E*, respectively.

The second method involved the initial activation of a fibre in a submaximal %CaBAPTA solution followed by transfer to the Triton–oil emulsion (protocol similar to that shown for maximum force in Fig. 2*A*). The relative force produced at various %CaBAPTA using this protocol is shown as open circles in Fig. 2*D* and *E*. It can be seen that both submaximum calibration techniques yielded similar results in FT fibres (Fig. 2*E*). In ST fibres, both calibration methods revealed a Triton X-100-induced decrease in myofilament Ca^{2+} sensitivity; however, the second method showed a slightly smaller shift than the first (Fig. 2*D*). For the present study the open triangle curve was used for calibrating the ST force responses during Triton X-100 lysis. Use of the alternative curve lead to Ca^{2+} content estimations that were only negligibly different (< 5%).

In summary, the calibration curves (Fig. 2*D* and *E*) show that the Triton X-100 treatment: (i) depressed F_{max} in both fibre types by ~40%, (ii) had virtually no effect on Ca^{2+}-activated force below 40% of F_{max} in FT fibres, and (iii) had an inhibitory effect on Ca^{2+} sensitivity in ST fibres. Finally, in separate control experiments, it was shown that immersion of a relaxed SR-destroyed fibre in either paraffin oil or Triton X-100 detergent alone did not lead to any observable force response, indicating the lack of any significant Ca^{2+} contamination in these fluids.

Criteria for data acceptance

The following criteria were set for accepting the validity of a Triton X-100 lysis response being 'on-scale'.

(i) Active force had to develop within 90 s of immersion in the Triton–oil emulsion (to negate the possibility of rigor development). Given that the myofibrillar ATPase activities measured during maximum activation of ST soleus and FT EDL fibres are approximately 0·1 and 0·8 mM s^{-1}, respectively (Stewart, Wilson & Stephenson, 1987), we can safely assume that after 90 s activation at less than 50% F_{max}, the [ATP] within all fibres is expected to remain essentially unchanged because the large CP pool (40 mM) would not have been completely depleted.

(ii) The amplitude had to be < 50% of the F_{max} obtained in control solutions (to ensure that the force response was not saturated near 60% F_{max}).

(iii) The fibre had to be relaxed fully and rapidly once immersed in relaxing solution (to test for the absence of damage to the myofibrillar proteins).

Calculation of Ca^{2+} content

The Ca^{2+} released during fibre lysis (e.g. the FT fibre in Fig. 1*A*) can be estimated in a first approximation from the amplitude of the force plateau as follows. The FT fibre attained 13% F_{max} during Triton X-100 lysis (Fig. 1*B*), which corresponds to a %CaBAPTA of 87% (Fig. 2*E*, open symbols). Since the original equilibration solution had a %CaBAPTA of 35% then the difference is 87% − 35% = 52%. The total [BAPTA] in the equilibration solution was 2·3 mM. Therefore, the Ca^{2+} released from intracellular stores during Triton X-100 lysis was 0·52 × 2·3 = 1·2 mM Ca^{2+}.

Corrections for fibre volume changes and BAPTA distribution

In the mechanically skinned fibre preparation, only the sarcolemma is removed, leaving behind sarcoplasmic reticulum (SR) that is functionally intact. The SR retains its integrity in the present experiments until it is lysed in a Triton–oil emulsion for the determination of the SR Ca^{2+} content. Knowledge of the total [BAPTA] present at the myofilaments during Triton X-100 lysis is important for calculating the SR Ca^{2+} content using our technique. This parameter will be affected by: (i) changes in the fibre volume, (ii) electrical potential differences between the myofibrillar space and the rest of the sarcoplasm, and (iii) the total [BAPTA] in the equilibration solution.

Consideration of these three factors requires the nominal [BAPTA] in the equilibration solution to be multiplied by a factor of 1·1 in both FT and ST fibres (for details of the corrections see Appendix). Thus, the Ca^{2+} content of the FT fibre shown in Fig. 1*B* must be multiplied by 1·1 to give a final value of 1·32 mM.

Cellular components of the Ca^{2+} content estimation

The present estimates of the total Ca^{2+} content in mammalian fibres are probably composed of several components representing the release of Ca^{2+} (onto BAPTA and the myofilaments) from one or more intracellular compartments as well as various endogenous Ca^{2+} binding proteins.

Ca^{2+} of non-SR origin could come from Ca^{2+} sinks such as the sealed-off transverse tubular (t)-system and mitochondria. In the mechanically skinned fibre preparation the t-system seals off, forming a compartment that could potentially bind significant amounts of Ca^{2+} (Lamb & Stephenson, 1991). The contribution of Ca^{2+} in the t-system to the total [Ca^{2+}] estimated by the present technique has been assessed in a separate set of experiments in our laboratory (V. J. Owen, G. D. Lamb, D. G. Stephenson & M. W. Fryer, unpublished observations) in which the t-tubules were permeabilized with saponin leaving the SR intact. This treatment decreased the total Ca^{2+} content by about 0·15 mM in FT fibres, suggesting that this amount of total [Ca^{2+}] resides in the sealed t-system. ST fibres have only half the t-system volume of FT fibres (Eisenberg, 1983) and thus should contain roughly half this amount of t-system Ca^{2+}.

The endogenous Ca^{2+} content of mitochondria under normal myoplasmic conditions (pCa 7–6) has been shown to be only 2% of that measured in the terminal cisternae of the SR (Somlyo & Somlyo, 1986). This observation, along with the pCa dependence

of each SR lysis experiment (e.g. end of *B*). △, mean (± S.E.M.) force response (normalized to F_{max} obtained under control conditions) in the presence of 26% aqueous Triton X-100 (6 fibres; protocol as in *C*). ○, force response obtained after moving from a solution of a given %CaBAPTA into the Triton–oil mixture (similar protocol to that in *A*). In most cases the S.E.M. is smaller than the symbol size.

of Ca^{2+} loading measured in FT fibres (pCa 7·2–5·96; Fig. 4), strongly suggests that mitochondria cannot effectively compete with the SR in the physiological range of $[Ca^{2+}]_i$. In addition, the presence of azide (1 mM) in all equilibration solutions should have minimized any mitochondrial Ca^{2+} loading at the higher end of the Ca^{2+} range. Based on the electron probe results, a terminal cisternae Ca^{2+} content of 1·5 mM per fibre volume should yield a mitochondrial Ca^{2+} content of ~0·03 mM Ca^{2+} per fibre volume in FT fibres and ~0·04 mM Ca^{2+} per fibre volume in ST fibres.

Soluble myoplasmic Ca^{2+} buffers such as calmodulin and the parvalbumins should have little effect on the Ca^{2+} content estimation because they are rapidly washed out during equilibration of the skinned fibre in an aqueous environment. This conclusion is supported by the results shown in Fig. 7 where it can be seen that there is little difference in Ca^{2+} content between initially Ca^{2+}-loaded preparations and those reloaded after a depletion protocol (where the preparations were exposed to the bathing solutions for at least 5 min before being tested).

Other experimental protocols

The standard experimental protocol for determining the Ca^{2+} content of FT and ST fibres is shown in Fig. 1. Fibres were normally equilibrated for between 30 s and 3 min at a given pCa and were then lysed in freshly triturated Triton–oil emulsion. The minimum equilibration period used was 30 s, which allowed full equilibration of BAPTA with the myofilaments. At pCa < 7·15 the standard protocol was slightly altered to avoid complications due

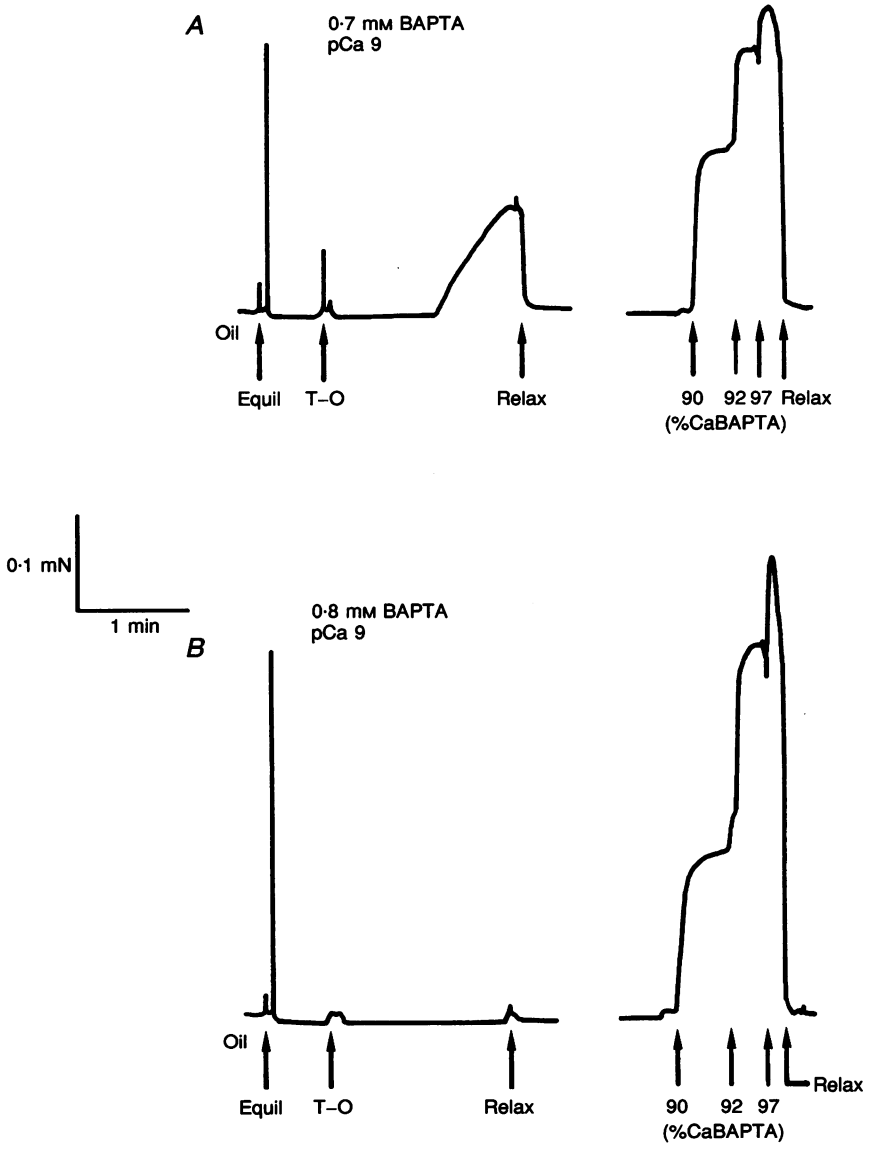

Figure 3. Sensitivity of the Ca^{2+} content technique

Total Ca^{2+} content determination was attempted in two segments of the same skinned FT fibre (*D*, 45 μm) using the basic protocol outlined in the figure. *A*, first segment of an FT fibre (*L*, 2·1 mm) equilibrated for 30 s in a type B solution containing 0·7 mM BAPTA solution and no added Ca^{2+} (pCa ~9). *B*, second segment of the same FT fibre (*L*, 1·5 mm) equilibrated for the same amount of time in a similar solution but containing 0·8 mM BAPTA (pCa ~9). Note the absence of force response from this fibre segment during fibre lysis in Triton–oil emulsion.

to myosin light chain phosphorylation, which can be triggered by an initial exposure of the skinned fibre to higher Ca^{2+} levels (Stephenson & Stephenson, 1993). In these experiments the fibre was first kept for 60 s in a solution in which little net loss or gain of total Ca^{2+} content occurred (pCa 7·15) in order to wash out potential enzymes involved in the phosphorylation reaction such as the Ca^{2+}–calmodulin-dependent protein kinase and the cyclic AMP-dependent protein kinase. The fibre was then exposed to the solution of pCa < 7·15 for a given equilibration time (30 s to 3 min) followed by 30 s back in pCa 7·15 before lysis in Triton–oil emulsion.

Fibres that were exposed to SR-depleting solutions containing caffeine and/or low Mg^{2+} were not plunged directly into the Triton–oil emulsion after their treatment. This was because these fibres would carry caffeine and/or low Mg^{2+} into the lysis emulsion and potentially change the force–$[Ca^{2+}]$ calibration curves (e.g. it is well known that caffeine can enhance the sensitivity of the myofilaments; Wendt & Stephenson, 1983). To avoid such complications, fibres were rinsed for 30 s in B1 solution (pCa ~9) prior to Triton–oil treatment, in order to wash out any contaminating caffeine and/or low Mg^{2+}.

Statistical analysis

Data are expressed as means ± S.E.M. throughout. The number of observations (n) used for statistical purposes did not include segments taken from the same muscle fibre. Statistical significance between results was determined at the 95% confidence level using Student's t test for either paired or unpaired observations.

RESULTS

Sensitivity of the Ca^{2+} content technique

The degree of sensitivity of the Ca^{2+} content technique is demonstrated in Fig. 3, where two segments of the same skinned FT fibre were equilibrated in solutions that contained no added Ca^{2+} (pCa > 9) and varied by only 0·1 mM in total [BAPTA]. When the total [BAPTA] was 0·7 mM in the equilibrating solution (Fig. 3*A*) a force response was obtained (35% of F_{max}), leading to an estimated Ca^{2+} content of $0{\cdot}886 \times 0{\cdot}7 \times 1{\cdot}1 = 0{\cdot}68$ mM without considering the endogenous Ca^{2+} buffers. However, when the total [BAPTA] was increased to 0·8 mM in the equilibrating solution, the $[Ca^{2+}]$ achieved at the myofilaments was subthreshold for force development (Fig. 3*B*) indicating that the amount of Ca^{2+} liberated during lysis was less than 83% of the total [BAPTA] (see Fig. 2*E*). This shows that less than 0·06 mM Ca^{2+} ((0·83 × 0·8 × 1·1 mM) − 0·68 mM) is bound to endogenous sites to cause a rise in force from subthreshold level to 35% of F_{max}. This value is only a small fraction of the total amount of Ca^{2+} bound to BAPTA.

Ca^{2+} content of FT and ST fibres

The total Ca^{2+} content of freshly skinned FT and ST fibres was determined in experiments in which the fibres were equilibrated in solutions of different pCa values for different lengths of time before exposure to the Triton–oil emulsion (Fig. 4). Either single exponential curves or straight lines provided good fits ($r^2 > 0{\cdot}98$) to the various data sets (Graphpad Prism, San Diego, CA, USA). These experiments not only revealed time-dependent changes in Ca^{2+} content at different pCa values, but also allowed the determination of the amount of Ca^{2+} bound to endogenous Ca^{2+} buffers at different pCa values. A detailed analysis of the latter may be found in the Appendix.

Endogenous Ca^{2+} content of FT and ST fibres at resting $[Ca^{2+}]_i$. The total, endogenous Ca^{2+} content of FT and ST fibres at resting myoplasmic pCa (pCa 7·15 = 71 nM $[Ca^{2+}]$) can be calculated by back-extrapolation of the data points of the pCa 7·15 curve to the y-intercept in Fig. 4*A* and *B*. The resting Ca^{2+} content estimated in this manner was 1·19 ± 0·02 mM for FT fibres and 1·25 ± 0·08 mM for ST fibres. After correction for Ca^{2+} binding to endogenous Ca^{2+}

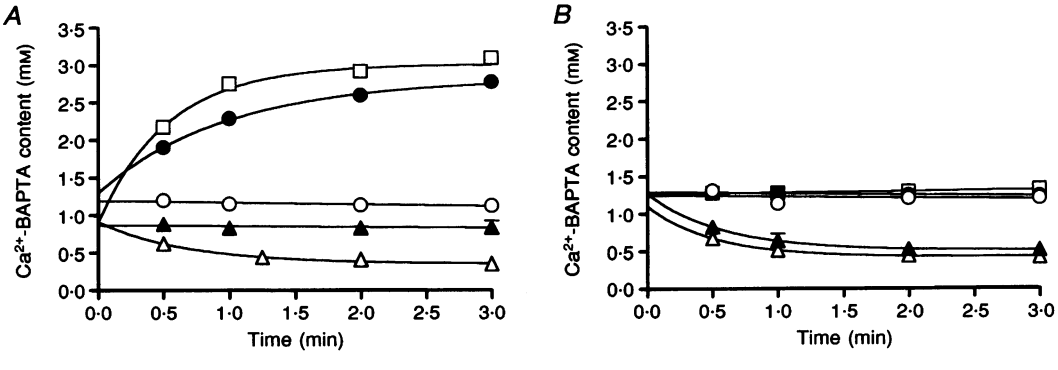

Figure 4. Time-dependent changes of Ca^{2+} content in skinned fibres equilibrated at different myoplasmic pCa values

A, FT fibres from EDL muscles. *B*, ST fibres from soleus muscles. Points represent means ± S.E.M. from 3–10 fibres. For most points the S.E.M. is smaller than the symbol size. Equilibration pCa: 6·14 (□), 6·32 (●), 7·15 (○), 7·67 (▲) and 9·00 (△). The continuous lines are computer-generated least-squares fits of the data to either a straight line or a single exponential function. Each fit was extrapolated to zero time in order to yield estimates of the resting Ca^{2+} content prior to equilibration. Differences in the y-intercept of the fitted curves in *A* and *B* were used to estimate the capacity of endogenous Ca^{2+} buffering at different myoplasmic pCa values (see Appendix).

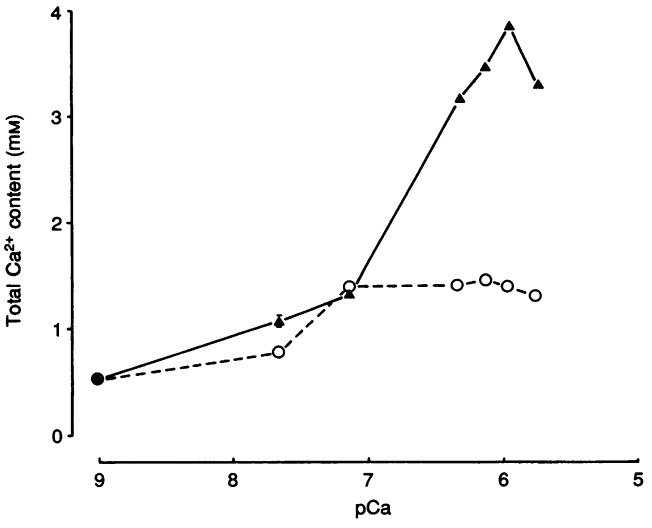

Figure 5. Changes in total Ca^{2+} content after 3 min equilibration at different myoplasmic pCa values
Points represent the mean total Ca^{2+} content (± S.E.M.) from 3–6 FT fibres (▲) and ST fibres (○). Fibres were equilibrated for 3 min at a given pCa and then the Ca^{2+}-BAPTA content was determined. Data was then corrected for endogenous Ca^{2+} buffering as described in text. For all points the S.E.M. is smaller than the symbol size. Lines have been drawn to connect the points to facilitate data comparison.

binding sites at pCa 7·15 (0·13 mM for FT, 0·10 mM for ST; see Appendix) these values become 1·32 mM and 1·35 mM, respectively, which are only ~10% larger than the uncorrected values. Taking into consideration all of the non-SR Ca^{2+} components, we can calculate the approximate value of the SR Ca^{2+} component at myoplasmic pCa 7·15 as follows: FT fibres: 1·32 mM (total) − 0·15 mM (t-system) − 0·13 mM (endogenous Ca^{2+} buffers) − 0·03 mM (mitochondria) = 1·01 mM (SR); ST fibres: 1·35 mM (total) − 0·075 mM (t-system) − 0·10 mM (endogenous Ca^{2+} buffers) − 0·04 mM (mitochondria) = 1·14 mM (SR). Based on these calculations, the SR Ca^{2+} component is 77% of the total FT fibre Ca^{2+} and ~84% of the total ST fibre Ca^{2+}. The SR is therefore the major source of Ca^{2+} contributing to our resting Ca^{2+} content estimate. Given that FT fibres have approximately twice the volume of SR as ST fibres (9·3% *vs.* 5·5%; Eisenberg, 1983) the endogenous SR Ca^{2+} component of the fibre (expressed per SR volume) at resting $[Ca^{2+}]_i$ may be calculated as $1·01/0·093 \approx 11$ mM for FT fibres and $1·30/0·055 \approx 21$ mM for ST fibres. These figures indicate that the SR of ST fibres contains about twice as much Ca^{2+} as the SR of FT fibres at resting myoplasmic pCa.

Myoplasmic $[Ca^{2+}]$ dependence of Ca^{2+} content. When FT fibres were equilibrated at pCa 7·15–7·67 for 0·5–3 min there was little net change in Ca^{2+} content (Fig. 4*A*). At pCa 9 the Ca^{2+} content declined with first-order kinetics to a steady-state level of 0·36 ± 0·02 mM (4 fibres, 7 segments) with a rate constant of 25 ± 5 $\mu M\ s^{-1}$. In

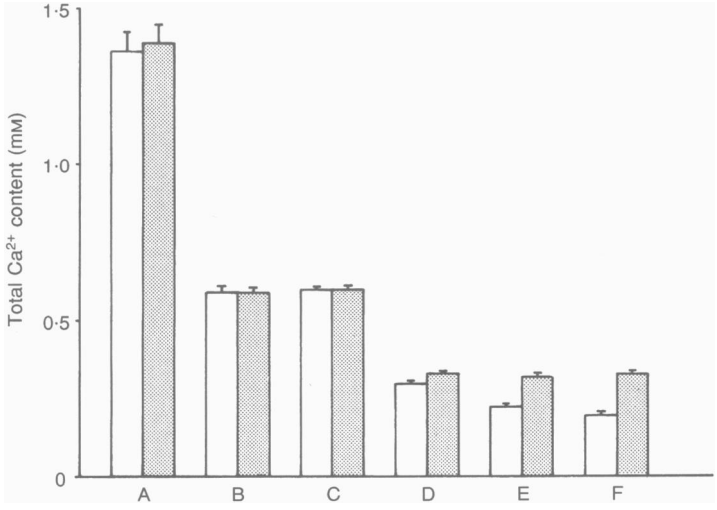

Figure 6. Caffeine and low myoplasmic Mg^{2+} reduce the total Ca^{2+} content
Fibres were equilibrated under the following conditions: A, 1 min at pCa 7·15; B, 2 min in 0·5 mM BAPTA (pCa ~9); C, 2 min in 30 mM caffeine plus 0·5 mM BAPTA (pCa ~9); D, 2 min in 30 mM caffeine plus 0·2 mM BAPTA (pCa ~8); E, 1 min in 0·05 mM Mg^{2+} (0·1–0·2 mM BAPTA; pCa ~8)); F, 1 min in 30 mM caffeine plus 0·05 mM Mg^{2+} (0·1–0·2 mM BAPTA; pCa ~8). Fibres in C to E were rinsed for 30 s in B1 solution (pCa 9) just prior to Triton–oil treatment. Bars represent the means ± S.E.M. from 3–10 fibres. □, ST soleus fibres; ▩, FT EDL. All data has been corrected for endogenous Ca^{2+} buffering as described in the Appendix.

contrast, equilibration at pCa 6·32 and 6·14 produced large increases in Ca^{2+} content that attained steady-state concentrations at 3 min of $2·77 \pm 0·03$ mM (6 fibres, 9 segments) and $3·10 \pm 0·03$ mM (4 fibres, 5 segments), respectively. The rate constants for the net Ca^{2+} uptake at these pCa levels were 17 ± 4 and 31 ± 6 $\mu M\ s^{-1}$, respectively.

ST fibres maintained a relatively constant Ca^{2+} content at pCa 7·15 but at pCa 7·67 and 9 the Ca^{2+} content declined to a constant steady-state level of ~0·4 mM within 3 min (Fig. 4*B*). The rate constant for the net leakage of Ca^{2+} in this pCa range was 33 ± 2 $\mu M\ s^{-1}$. A striking, and unexpected result was that ST fibres equilibrated at higher than resting [Ca^{2+}] (< pCa 7·15) failed to increase their Ca^{2+} content relative to pCa 7·15 (Fig. 4*B*).

Some of the results from Fig. 4*A* and *B* were corrected for Ca^{2+} binding to endogenous sites as described earlier, and in the Appendix, and are plotted in Fig. 5 to illustrate the relationship between total Ca^{2+} content and myoplasmic pCa at a fixed equilibration time of 3 min. At pCa 9, both FT and ST fibres had reduced Ca^{2+} contents relative to their resting level, indicating a net Ca^{2+} leakage under these conditions. ST fibres were somewhat leakier than FT fibres at pCa 7·67. At pCa < 7·15 the two fibre types diverged in their Ca^{2+} content profiles. The Ca^{2+} content of FT fibres increased to a maximum level of $3·85 \pm 0·02$ mM ($n = 5$) at pCa 5·96 and declined thereafter. This indicated a substantial net Ca^{2+} uptake in this pCa range. The results from ST fibres were markedly different, showing no significant change in Ca^{2+} content ($P > 0·05$) when equilibrated at pCa between 7·15 and 5·96.

Releasable SR Ca^{2+} content

The technique for measuring Ca^{2+} content was next used to determine the effects of two interventions that are known to elicit Ca^{2+} release from the SR in rat skeletal muscle fibres: (i) treatment with caffeine (Fryer & Neering, 1989) and (ii) exposure to low myoplasmic Mg^{2+} (Lamb & Stephenson, 1991). Results from these experiments are shown in Fig. 6. All Ca^{2+} content estimates discussed in relation to Fig. 6 refer to values corrected for endogenous Ca^{2+} buffering as mentioned earlier in the text.

A 2 min exposure to a solution containing 30 mM caffeine and 0·5 mM BAPTA (pCa ~9) reduced the Ca^{2+} content to ~0·6 mM in both FT and ST fibres (Fig. 6, C). However, the results obtained after an equivalent period of exposure to 0·5 mM BAPTA (pCa ~9) alone (Fig. 7*B*) were not significantly different ($P > 0·05$) indicating that caffeine had failed to augment the effect of Ca^{2+} leakage alone. When the fibres were exposed to caffeine in the presence of lower BAPTA concentrations (0·2 mM, pCa ~8; Fig. 6*D*) the total Ca^{2+} content was reduced to $0·33 \pm 0·01$ mM ($n = 6$ FT fibres) and $0·30 \pm 0·01$ mM ($n = 7$ ST fibres). Calculation of the Ca^{2+} left in the SR after this treatment requires the subtraction of Ca^{2+} from alternative sinks such as the sealed t-system and mitochondria (see earlier text; FT, ~0·18 mM; ST, 0·12 mM), yielding values of 0·15 mM

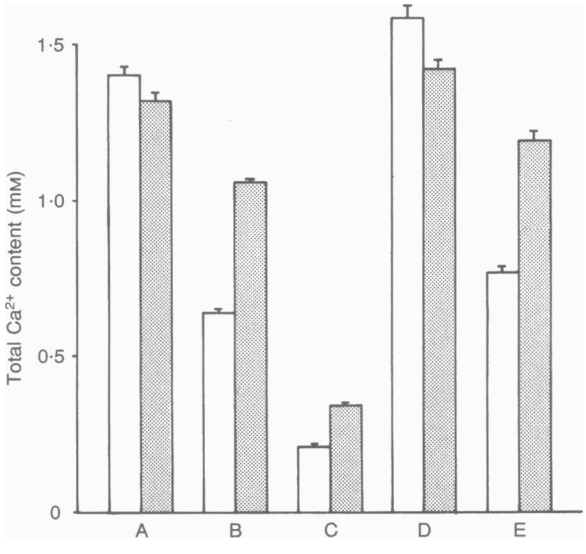

Figure 7. Ca^{2+} stores reload following depletion in a caffeine–low Mg^{2+} solution

The bars in the centre (C) represent the total Ca^{2+} content of fibres 'depleted' by 1 min exposure to a solution containing 30 mM caffeine, 0·05 mM Mg^{2+} and 0·1–0·2 mM BAPTA (pCa ~8). The bars to the right of centre (D and E) show the Ca^{2+} content measured after 'depleted' fibres were exposed to reloading solutions at pCa 7·67 and 7·15, respectively, for 3 min. For comparison, the bars to the left of centre (A and B) show the Ca^{2+} content (redrawn from Fig. 5) obtained after equilibrating resting fibres for 3 min at pCa 7·15 and 7·67, respectively. Note the similarity between the left- and right-hand sides of the figure. Bars represent the mean ± S.E.M. from 3–8 fibres. □, ST soleus fibres; ▩, FT EDL. All data have been corrected for endogenous Ca^{2+} buffering as described in text.

and 0·18 mM for FT and ST fibres, respectively. Given that the initial SR Ca^{2+} content was 1·01 mM (FT) and 1·14 mM (ST), then it can be estimated that caffeine released 85% and 84% of the SR Ca^{2+} content, respectively, at pCa 8.

A 1 min exposure to a solution containing low Mg^{2+} (0·05 mM, Fig. 6*E*) and 0·1–0·2 mM BAPTA (pCa ~8) was as effective as the caffeine–0·2 mM BAPTA (pCa ~8) treatment in reducing the Ca^{2+} content of FT fibres (0·33 ± 0·01 mM, $n = 3$) but was more effective than the latter treatment in ST fibres (0·23 ± 0·01 mM, $n = 3$). Simultaneous exposure of the fibres to caffeine and low Mg^{2+} (Fig. 6, F; 5 FT and 5 ST fibres) had no greater effect on Ca^{2+} content than that produced by low Mg^{2+} alone.

Reloading of Ca^{2+} content in depleted fibres

In a final set of experiments the Ca^{2+} content technique was used to determine the extent of reloading of Ca^{2+} stores (Fig. 7, D and E) following their initial depletion by a 1 min exposure to a caffeine–Low Mg^{2+} solution (Fig. 7, C). All results were corrected for Ca^{2+} binding to endogenous sites as described earlier in the text. For comparison, Fig. 7, A and B show the Ca^{2+} content (data redrawn from Fig. 5) of fibres that had been exposed to a 3 min equilibration at pCa 7·15 and 7·67. The similarity between Fig. 7, A and B and D and E suggests that Ca^{2+} depletion is totally reversible in both fibre types.

DISCUSSION

Advantages and limitations of the Ca^{2+} content technique

A new technique has been described for the measurement of the total Ca^{2+} content of single, skinned skeletal muscle fibres. The technique allows good control over the myoplasmic milieu, it is quick and simple to perform, and offers a high degree of reproducibility and sensitivity. The use of the endogenous myofibrillar force response as a Ca^{2+} indicator in the present setting is preferable to using an exogenous Ca^{2+} indicator (e.g. fluorescent dye) for several reasons. Firstly, the force–%CaBAPTA curve is very steep (Fig. 2*D* and *E*), which yields very small errors in estimating %CaBAPTA after lysis. Secondly, because the fibre diameter is much smaller than its length, and the fibre is stretched, it will report threshold levels of Ca^{2+}-activated force as soon as any part of the fibre becomes activated in response to local Ca^{2+} release. In contrast, fluorescent indicators will report only the spatial average of the overall fluoresence change. This change in fluorescence may not be readily translated into $[Ca^{2+}]$ changes as the affinity for Ca^{2+} of these indicators may alter when bound to myofibrillar components. Finally, the equipment required for the present technique is simple and inexpensive when compared with fluorescence excitation and detection methodology.

The present technique gives an accurate, quantitative indication (to within ~10% of the total equilibrating [BAPTA]) of the total Ca^{2+} content of single muscle fibres under a variety of myoplasmic conditions and thus represents a simple alternative to other methods such as: (i) the measurement of ^{45}Ca efflux from multiple intracellular compartments in skinned fibres (Ford & Podolsky, 1972), (ii) electron probe cryo-microanalysis of whole muscles with the necessary conversion of units from $[Ca^{2+}]$ per kilogram wet weight to millimoles per litre fibre volume (Somlyo *et al.* 1985), and (iii) complex mathematical modelling that relies on several starting assumptions obtained from the literature (Klein *et al.* 1991). Unlike the other methods, the present technique has readily revealed fundamental differences between FT and ST fibres with respect to SR Ca^{2+} loading.

There are several limitations of the technique that should be noted. One practical disadvantage is that the procedure is irreversible, so it cannot be used for repeated experimental cycles. This limitation may be compensated in part by using several segments from the same fibre. Another limitation is the assessment of Triton X-100 effects on myofilament force generation, which require careful calibration and appear to be quite temperature sensitive (M. W. Fryer & D. G. Stephenson, unpublished observations). This latter observation is probably a result of differences in micelle species formed by Triton X-100 at different temperatures, and should be kept in mind if different experimental temperatures are going to be used. This limitation can be minimized by adjusting the [BAPTA] in solutions in order to obtain responses below 20% of F_{max}.

Estimating changes in SR Ca^{2+} content

We have estimated that the SR contribution to the total Ca^{2+} content measured at rest is approximately 75–85%, indicating that the greater part of our Ca^{2+} content estimate is SR derived. In addition, a number of the results suggest that most of the change in total fibre Ca^{2+} content is SR related. Firstly, the Ca^{2+} content of FT fibres was dramatically increased when the fibres were equilibrated at myoplasmic pCa values of between 7·15 and 5·96 (Figs 4 and 5). This dynamic range is consistent with the Ca^{2+} activation range of the SR Ca^{2+} ATPase pump (Hasselbach & Oetliker, 1983) but is somewhat outside the range required for activating any significant mitochondrial Ca^{2+} uptake (Rasgado-Flores & Blaustein, 1987). Secondly, a large proportion of the estimated resting Ca^{2+} content was depleted by interventions (Fig. 6) known to specifically trigger Ca^{2+} release from the SR of mammalian fibres such as caffeine (Fryer & Neering, 1989) and low myoplasmic Mg^{2+} (Lamb & Stephenson, 1991).

Endogenous Ca^{2+} content at resting $[Ca^{2+}]_i$

A novel result from the present study was the direct determination of the endogenous, total Ca^{2+} content of single fibres at resting myoplasmic pCa (pCa 7·15), yielding values (per litre fibre volume) of 1·32 ± 0·02 mM (FT fibres) and 1·35 ± 0·08 mM (ST fibres). The SR contribution to

these values was estimated as 1·01 and 1·14 mM, respectively. As far as we are aware, this is the first reported estimate of the SR Ca^{2+} content for ST mammalian skeletal muscle fibres. Previous estimates of SR Ca^{2+} content in other fibre types have been made for chemically skinned human skeletal muscle fibres (0·79 mM; Salviati, Sorenson & Eastwood, 1982) and skinned frog skeletal muscle fibres (0·9 mM; Ford & Podolsky, 1972). If these previous values are corrected to the volume of intact fibre as in the present study (i.e. multiplied by a factor of 1·1), they become 0·87 and 0·99 mM, respectively, which are close to our estimate.

Taking the present estimate of total SR Ca^{2+} in FT fibres (11 mM) at pCa 7·15, and our previously calculated value for the free $[Ca^{2+}]$ inside the SR ($[Ca^{2+}]_{SR}$) of FT fibres at a similar pCa (~1·2 mM; Fryer, Owen, Lamb & Stephenson, 1995), we estimate that approximately 10% of the total calcium within the SR of FT fibres is free at myoplasmic pCa 7·15.

Ca^{2+} content at elevated $[Ca^{2+}]_i$

The effects of changing $[Ca^{2+}]_i$ on the fibre Ca^{2+} content revealed striking differences in SR Ca^{2+} handling between FT and ST mammalian fibres. A surprising result was the observation that the steady-state Ca^{2+} content of ST fibres at resting myoplasmic pCa (7·15) was maximal, indicating that these fibres could load no more than 1·35 mM total Ca^{2+} (per fibre volume), even when the $[Ca^{2+}]_i$ was elevated. One possible explanation is that the free $[Ca^{2+}]_{SR}$ of ST fibres is so high that all of the available calsequestrin is already saturated with Ca^{2+} at resting $[Ca^{2+}]_i$. In this situation any further attempts to increase SR Ca^{2+} content would lead to a rapid rise in $[Ca^{2+}]_{SR}$, which would oppose further Ca^{2+} uptake by increasing both the rate of Ca^{2+} leak from the SR as well as the degree of back-inhibition of the SR Ca^{2+}-ATPase pump (Inesi & De Meis, 1989).

The results from FT fibres were markedly different in that their steady-state total Ca^{2+} content at rest (1·32 mM) was only about one-third of the maximal SR Ca^{2+} content achievable at pCa 5·96 (3·85 mM, Fig. 5). This ability of FT fibres to increase their steady-state SR Ca^{2+} content by at least 3-fold (Figs 4 and 5) is consistent with their greater calsequestrin content compared with ST fibres (Leberer & Pette, 1986). Under optimum loading conditions the limiting factor to the maximal SR Ca^{2+} content achieved in FT fibres is a concomitant increase in SR Ca^{2+} leakage that becomes greater at pCa < 6 (Fig. 6) where Ca^{2+}-induced Ca^{2+} release starts to occur (Miyamoto & Racker, 1981).

The different relaxation speeds of FT and ST fibres probably results from differences in SR Ca^{2+} pump density (Dulhunty, Banyard & Medvecsky, 1987) and crossbridge kinetics. It is also possible that differences in the degree of calsequestrin saturation with Ca^{2+} might also contribute to this phenomenon as follows: in FT fibres, the increase in $[Ca^{2+}]_i$ following SR Ca^{2+} release activates the SR Ca^{2+} pump to well above its resting rate, initiating a rapid resequestration of Ca^{2+} into the SR lumen. Ca^{2+} uptake is rapid and substantial because FT fibres have a large endogenous calsequestrin capacity that is only partially saturated with Ca^{2+} at rest (Fig. 4*A*). This means that only small rises in $[Ca^{2+}]_{SR}$ occur during SR Ca^{2+} loading. Thus, the SR Ca^{2+} pump activity of FT fibres may be primed by an elevated $[Ca^{2+}]_i$ with little interference from back-inhibition by an elevated $[Ca^{2+}]_{SR}$. In contrast, the rate of SR Ca^{2+} uptake of ST fibres in response to an elevated $[Ca^{2+}]_i$ will be rapidly curtailed as the limited calsequestrin store becomes saturated with Ca^{2+}, $[Ca^{2+}]_{SR}$ starts to rise, and the pump is retarded by back-inhibition (Inesi & De Meis, 1989). Thus, SR Ca^{2+} uptake in ST fibres appears to be controlled more by $[Ca^{2+}]_{SR}$ rather than by the free $[Ca^{2+}]_i$.

Ca^{2+} content at reduced $[Ca^{2+}]_i$

The Ca^{2+} content results at reduced $[Ca^{2+}]_i$ revealed valuable information regarding the relative activity of Ca^{2+} leakage and Ca^{2+} uptake processes. For example, the loss of Ca^{2+} content from the endogenous level at pCa > 7·15 (Fig. 4) indicated the presence of a Ca^{2+} leakage process that was occurring at a greater rate than SR Ca^{2+} uptake. However, comparison of the results between resting and depleted fibres (Fig. 7) revealed that the relative balance between these two processes clearly changed with the degree of SR Ca^{2+} loading. Thus, the response of resting fibres to myoplasmic pCa 7·67 was a net Ca^{2+} loss (Fig. 4), while the response of depleted fibres to the same solution was a net Ca^{2+} gain (Fig. 7). The latter result clearly showed that the SR Ca^{2+} pump was active at pCa 7·67 while other experiments at pCa 7·15 revealed an even faster rate of SR Ca^{2+} reloading (Fig. 7), demonstrating that the SR Ca^{2+} pump is further stimulated by myoplasmic Ca^{2+} in this range.

At pCa 9, where the SR Ca^{2+} pump does not operate, a rapid (rate constant, 28–33 $\mu M\ s^{-1}$) leakage of Ca^{2+} was observed in both FT and ST fibres (Fig. 4). This rate of SR Ca^{2+} leak is approximately one order of magnitude greater than that estimated for frog skeletal muscle fibres at resting myoplasmic pCa (~2 $\mu M\ s^{-1}$; Klein *et al.* 1991). This type of Ca^{2+} leakage has been previously observed in skinned mammalian fibres (Su & Hasselbach, 1984) and occurs at least partially through the SR Ca^{2+} release channels because a portion of it can be blocked by 1 μM Ruthenium Red (M. W. Fryer & D. G. Stephenson, unpublished observations). Given that ST fibres have about half the number of SR Ca^{2+} release channels of FT fibres and that their release channels have a lower open probability (Lee, Ondrias, Duhl, Ehrlich & Kim, 1991), we might expect ST fibres to display less SR Ca^{2+} leakage than FT fibres. However, our results show the opposite trend (compare Fig. 4*A* and *B*). This greater leakage rate of Ca^{2+} from the SR of ST fibres is consistent with our finding that the SR of

ST fibres is more highly loaded with Ca^{2+} at rest (Sitsapesan & Williams, 1995).

Depletion of Ca^{2+} content using caffeine and low Mg^{2+}

Two minutes treatment with 30 mM caffeine was found to deplete the SR Ca^{2+} content by about 85% in both FT and ST fibres at pCa ~8. This effect was much greater than previously reported for mammalian skeletal muscle. Su (1988) estimated that 25 mM caffeine (in 50 μM EGTA) released 20–25% of the SR Ca^{2+} content in skinned ST and FT fibres from rabbit skeletal muscle. The difference in results may be ascribed either to a difference between preparations or, alternatively, might point to quantitative inaccuracies in the measurement of Ca^{2+} content changes by the integration of caffeine-induced force responses. The present results may be more directly compared to electron probe microanalysis studies on frog skeletal muscle where 2 min treatment with 5 mM caffeine released ~85% of the total Ca^{2+} content (Yoshioka & Somlyo, 1984).

Reducing the myoplasmic free [Mg^{2+}] from 1 to 0·05 mM was also extremely effective at depleting the total fibre Ca^{2+} content (Fig. 6). This intervention, like caffeine at pCa 8, depleted about 85% of the endogenous SR Ca^{2+} content in both ST and FT fibres. Lamb & Stephenson (1991) have suggested that this type of SR Ca^{2+} release is a consequence of relieving the resting inhibition of the SR Ca^{2+} release channel by myoplasmic Mg^{2+}.

Conclusions

The present method for determining the total Ca^{2+} content of single mechanically skinned skeletal muscle fibres can be used to gain insight into SR Ca^{2+} handling under a variety of myoplasmic conditions. Fundamental differences in SR Ca^{2+} handling between FT and ST muscles have been discerned using this technique that were previously undetected using other methods. In particular, the results indicated that the SR of ST fibres was 'full' at resting myoplasmic [Ca^{2+}] while the SR of FT fibres was only about one-third full under the same conditions. It is suggested that differences in SR Ca^{2+} handling such as these may contribute to the differences in the speed of relaxation of the two muscle types.

APPENDIX

Correcting Ca^{2+} content estimates for fibre volume changes and BAPTA distribution

Calculation of the Ca^{2+} content using the present technique relies on knowledge of the total [BAPTA] present at the myofilaments during Triton X-100 lysis. This in turn depends upon several factors: (i) changes in the fibre volume, (ii) electrical potential differences between the myofibrillar space and the rest of the sarcoplasm, and (iii) the total [BAPTA] in the equilibration solution.

(i) There is considerable swelling in the diameter of the skinned fibres upon transfer from paraffin oil to an aqueous solution. We have measured the change in diameter of fibres after transferring them from paraffin oil to B3 solution as a 1·29 (± 0·05)-fold increase for six FT fibres.

(ii) Previous measurements have indicated that the average [EGTA] within skinned muscle fibres devoid of internal membraneous compartments was 77% of the [EGTA] in the bathing solution at pH 7·10, and at a concentration of ionic equivalents of 207 mM (Stephenson, Wendt & Forrest, 1981), which is close to that in the current experiments. Given that both EGTA and BAPTA are negatively charged at pH 7·10 and that the Ca^{2+} content estimates were similar using EGTA or BAPTA solutions (M. W. Fryer & D. G. Stephenson, unpublished observations) it is assumed that the average concentration of both Ca^{2+} buffers is similar at the myofilaments. Therefore, if the amount of BAPTA carried in a skinned fibre preparation is expressed in terms of BAPTA concentration per volume of intact fibre, then a value of $77\% \times 1{\cdot}29 \times 1{\cdot}29 = 128{\cdot}1\%$ of the [BAPTA] in the bathing solution may be calculated.

A further correction is required for the fractional volume occupied by the intracellular membraneous compartments to which EGTA is not accessible (sarcoplasmic reticulum: 9·3% in EDL, 5·5% in soleus; mitochondria: 7·1% in EDL, 10% in soleus; nuclei: 1·1% in EDL, 2·6% in soleus; and t-system: ~0·4% in EDL and ~0·2% in soleus; Eisenberg, 1983). The total amount of inaccessible internal membrane compartments equals 17·9 and 18·3% of the total fibre volume for EDL and soleus fibres, respectively. Since these internal membraneous compartments are not expected to swell or shrink in our solutions compared with their respective volume in the intact fibres, one can subtract these values to estimate the amount of BAPTA carried by our skinned fibre preparation when it is transferred between solutions. The final corrected values correspond to a [BAPTA] expressed per volume of intact fibre of about 110·2% (128·1–17·9%) and 109·8% (128·1–18·3%) of the [EGTA] in the bathing solution for the EDL and soleus fibres, respectively.

Estimation of endogenous Ca^{2+} buffering in FT and ST fibres

Extrapolation of the curves obtained at different pCa values (Fig. 4) to the moment of immersion of the freshly skinned fibre segment in the equilibration solution indicated that in FT fibres there was no difference in the y-axis intercept whether the pCa in the initial equilibration solution was 9·0 or 7·67 (Fig. 4*A*). This implied the presence of a very weak endogenous Ca^{2+} buffer in this pCa range. When the incubation was at pCa 7·15 the curve intercepted the y-axis at 0·36 mM above the pCa 9 and pCa 7·67 curves, indicating that in FT fibres, the endogenous Ca^{2+} buffers bind about 0·35 mM Ca^{2+} in the pCa range 7·67–7·15. The pCa 6·32 curve intersects the y-axis at 0·47 mM above the pCa 9 curve, indicating that the endogenous Ca^{2+} buffers bind about 0·1 mM Ca^{2+} when pCa decreases from 7·15 to 6·32. The curve at pCa 6·14

intersects at a lower level which is consistent with some initial loss of Ca^{2+} from the SR (via Ca^{2+}-induced Ca^{2+} release) at this elevated [Ca^{2+}]. From several experiments like that shown in Fig. 3 it appears that less than 0·1 mM Ca^{2+} is bound by endogenous myoplasmic sites between subthreshold and 10–40% of F_{max} responses where most measurements were done. This is in full agreement with direct measurements of Ca^{2+} binding to Triton X-100-treated FT mammalian muscle fibres, in which about 0·45 μmol Ca^{2+} per gram myofibrillar protein was bound over the pCa range corresponding to a force response rise from below threshold to 40% of F_{max} (Fuchs, 1985). The value of 0·45 μmol Ca^{2+} per gram myofibrillar protein corresponds to about 90% of the troponin C concentration in the fibre (Fuchs, 1985), which in turn represents about 0·078 mM expressed per total intact fibre volume (troponin C = 0·07 mmol per kilogram whole muscle; 1 kg whole muscle occupies 0·934 l assuming a density of 1·07 kg l^{-1} and 0·125 l is extracellular fluid; see Baylor, Chandler & Marshall, 1983: therefore, 90% × 0·07 × 0·809 = 0·078 mM). The total amount of Ca^{2+} bound to the detergent-treated fibres when force reaches 40% F_{max} is close to 0·8 μmol Ca^{2+} per gram myofibrillar protein (Fuchs, 1985) which translates to about 0·14 mM Ca^{2+} expressed per intact fibre volume. The difference between the concentration of Ca^{2+} bound to endogenous Ca^{2+} buffers in mechanically skinned fibres over the pCa range 7·67–7·15 (about 0·5 mM) and that bound to the Triton X-100-treated fibres (about 0·1 mM) over the same pCa range is likely to be mainly due to the SR Ca^{2+} pump sites which were estimated to bind Ca^{2+} up to an equivalent concentration of 0·3 mM expressed per volume of intact frog skeletal muscle fibre (Baylor *et al.* 1983). In the lysed fibre the SR Ca^{2+} pump is not likely to bind Ca^{2+} with high affinity and parvalbumin is likely to have diffused out of the mechanically skinned fibre during the exposure period to an aqueous solution which was at least 30 s. Therefore, the results in Fig. 4*A* would have to be corrected only for Ca^{2+} binding to the high Ca^{2+} affinity sites still present in the Triton X-100-lysed preparations, which most probably are the Ca^{2+} binding sites associated with troponin C (Fuchs, 1985).

Assuming that the troponin C Ca^{2+} binding sites have a K_D value of 2 μM (see Baylor *et al.* 1983), that the maximal value for Ca^{2+} bound to the detergent treated fibres is 0·28 mM (i.e. 1·6 μmol Ca^{2+}per gram myofibrillar protein, Fuchs, 1985) and that 0·14 mM Ca^{2+} was bound to these sites at the time when the measurements were made (10–40% of F_{max}), it follows that the points on the FT curves (Fig. 4*A*) for pCa 9, 7·67, 7·15, 6·32 and 6·14 would need to be increased by 0·14, 0·14, 0·13, 0·085 and 0·065 mM, respectively.

A similar analysis of data points from ST fibres indicates that the endogenous Ca^{2+} buffering capacity has a quite different pCa relationship from that seen in FT fibres. In this case there was a clear difference in the *y*-axis intercept between the pCa 9 and pCa 7·67 curves, indicating that the endogenous Ca^{2+} buffering sites bind about 0·19 mM Ca^{2+} in this pCa range. There was, however, very little further change in the *y*-axis intercept when the initial pCa was decreased from 7·67 to 6·14, indicating a very small endogenous Ca^{2+} buffering capacity in this pCa range.

As with the FT fibres a correction for Ca^{2+} binding to ST troponin C must be considered. Since ST troponin C has only three Ca^{2+} binding sites, while the FT troponin C has four Ca^{2+} binding sites (for review see Goodman, 1987), the maximal amount of Ca^{2+} bound to ST troponin C would be 0·75 × 0·28 = 0·21 mM. Scaling down by the same factor the concentration of Ca^{2+} bound to ST troponin C when the measurements were done (0·75 × 0·14 = 0·105 mM) and assuming the same average K_D value (2 μM) as for the FT fibres, it follows that the correction for Ca^{2+} binding to ST troponin C amounts to 0·105 mM for the pCa points on the pCa 9 and pCa 7·67 curves, 0·10 mM for the points on the pCa 7·15 curve, 0·065 mM for the pCa 6·32 curve and 0·05 mM for the pCa 6·14 curve. Ca^{2+} binding sites on the SR Ca^{2+} pump are likely to account for the 0·19 mM rise in the amount of Ca^{2+} bound to endogenous Ca^{2+} binding sites when the pCa decreased from 9·0 to 7·67 in the skinned fibres. However, the SR Ca^{2+} pump sites are not likely to bind Ca^{2+} with high affinity when the SR is lysed.

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