Raised intracellular [Ca^{2+}] abolishes excitation–contraction coupling in skeletal muscle fibres of rat and toad

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1. Raising the intracellular [Ca^{2+}] for 10 s at 23 °C abolished depolarization-induced force responses in mechanically skinned muscle fibres of toad and rat (half-maximal effect at 10 and 23 μM, respectively), without affecting the ability of caffeine or low [Mg^{2+}] to open the ryanodine receptor (RyR)/Ca^{2+} release channels. Thus, excitation–contraction coupling was lost, even though the Ca^{2+} release channels were still functional. Coupling could not be restored in the duration of an experiment (up to 1 h).
2. The Ca^{2+}-dependent uncoupling had a $Q_{10} > 3{\cdot}5$, and was three times slower at pH 5·8 than at pH 7·1. Sr^{2+} caused similar uncoupling at twenty times higher concentration, but Mg^{2+}, even at 10 mM, was ineffective. Uncoupling was not noticeably affected by removal of ATP or application of protein kinase or phosphatase inhibitors.
3. Confocal laser scanning microscopy showed that the transverse tubular system was sealed in its entirety in mechanically skinned fibres and that its integrity was maintained in uncoupled fibres. Electron microscopy revealed distorted or severed triad junctions and Z-line aberrations in uncoupled fibres.
4. Only when uncoupling was induced at a relatively slow rate (e.g. over 60 s with 2·5 μM Ca^{2+}) could it be prevented by the protease inhibitor leupeptin (1 mM). Immunostaining of Western blots showed no evidence of proteolysis of the RyR, the α_1-subunit of dihydropyridine receptor (DHPR) or triadin in uncoupled fibres.
5. Fibres which, whilst intact, were stimulated repeatedly by potassium depolarization with simultaneous application of 30 mM caffeine showed reduced responsiveness after skinning to depolarization but not to caffeine. Rapid release of endogenous Ca^{2+}, or raised [Ca^{2+}] under conditions which minimized the loss of endogenous diffusible myoplasmic molecules from the skinned fibre, caused complete uncoupling. Taken together, these results suggest that Ca^{2+}-dependent uncoupling can also occur in intact fibres.
6. This Ca^{2+}-dependent loss of depolarization-induced Ca^{2+} release may play an important feedback role in muscle by stopping Ca^{2+} release in localized areas where it is excessive and may be responsible for long-lasting muscle fatigue after severe exercise, as well as contributing to muscle weakness in various dystrophies.

Action potentials trigger contraction in skeletal muscle by a sequence of events known as excitation–contraction (E–C) coupling (Ashley, Mulligan & Lea, 1991; Rios & Pizarro, 1991). The action potentials travel into the muscle fibre along the transverse-tubular (T-) system, which for much of its length is sandwiched between the terminal cisternae (TC) of two sections of the sarcoplasmic reticulum (SR), forming the 'triad' junction (see Fig. 7*H*). Depolarization of the T-system is detected by voltage-sensor molecules, now known to be dihydropyridine receptors (DHPRs) (see Lamb, 1992), which cause the ryanodine receptors (RyRs) in the adjacent SR to release Ca^{2+} into the myoplasm, thereby activating the contractile apparatus. It is still not known whether the DHPRs activate the RyRs by some direct physical interaction, by a linking protein such as 'triadin' (Brandt, Caswell, Brunschwig, Kang, Antoniu & Ikemoto, 1992), or via a diffusible second messenger. Moreover, it is not known which macromolecules are responsible for holding the triad junction together, although it is clear that the integrity of the triad junction is essential for E–C coupling. Here we demonstrate that raised myoplasmic [Ca^{2+}] abolishes depolarization-induced Ca^{2+} release, apparently by interfering with the coupling between the DHPR and the RyR. Electron microscopy

revealed that this uncoupling is accompanied by alterations in the structure of the triad junction.

Some of these results have been presented briefly previously (Lamb, Junankar & Stephenson, 1994, 1995).

METHODS

Long–Evans hooded rats (*Rattus norvegicus*) were anaesthetized by halothane inhalation (2% v/v) and killed by suffocation before removing the extensor digitorum longus (EDL) muscles. Cane toads (*Bufo marinus*) were double pithed and the iliofibularis muscles were removed. As described previously (Lamb & Stephenson, 1990*a*), muscle fibres were mechanically skinned under paraffin oil, mounted on a force transducer (AME875, SensoNor, Norway), stretched to 120% of rest length and bathed (at 23 ± 2 °C, unless otherwise stated) in a high-[K^+] solution with 0·1 μM free Ca^{2+} at pH 7·1 (mM: K^+, 117; Na^+, 36; hexamethylene-diamine-tetra-acetic acid (HDTA), 50; ATP, 8; total magnesium, 8·6; creatine phosphate, 10; NaN_3, 1; EGTA, 0·05; Hepes buffer, 60 for toad and 90 for rat). Free [Mg^{2+}] was 1 mM in all solutions, unless indicated. The T-system was depolarized by substituting a similar, isosmotic solution in which all K^+ and $HDTA^{2-}$ were replaced with choline chloride or simply all the K^+ was replaced with Na^+; both methods gave similar results (see Lamb & Stephenson, 1994). In rigor solutions, ATP and creatine phosphate were replaced isosmotically with HDTA. Potassium solutions with 50 mM Ca-EGTA replacing all HDTA (8·12 mM total magnesium) and a free [Ca^{2+}] of either 20 μM ('max' solution) or 1 mM (max + 1 mM Ca^{2+}) were used to induce maximum force. The Ca^{2+} sensitivity of the contractile apparatus was studied using appropriate mixtures of the max solution and a corresponding solution with 50 mM EGTA. The SR was loaded, where indicated, in standard K^+ solution (50 μM EGTA) with a [Ca^{2+}] of 2 μM. [Ca^{2+}] was measured with a Ca^{2+}-sensitive electrode (Orion, Cambridge, MA, USA).

Confocal imaging

For confocal fluorescence imaging, a droplet of Ringer solution (with 2·5 mM $CaCl_2$) containing 1 mM fluo-3 (membrane-impermeable K^+ salt) was applied for 1·5 min to an intact fibre under paraffin oil. Confocal images were obtained by placing the fibre on the stage of a Leitz confocal laser scanning microscope (laser wavelength, 488 nm; emission at 510 nm) with a ×63 or ×40 oil immersion objective lens, and averaging 16 scans. Other fibres were exposed to fluo-3 as described above and then mechanically skinned under paraffin oil before being mounted on the force transducer, depolarized if required and then transferred to the confocal microscope stage. Where appropriate, fibres were uncoupled whilst on the force transducer by exposure to 100 μM Ca^{2+} for 10 s (see Results).

Immunostaining of proteins

Each skinned fibre sample consisted of four fibre segments (each approximately 5 mm long) which were mounted in parallel on the transducer and either exposed to 100 μM Ca^{2+} for 15 s during rigor (and shown to be uncoupled as in Fig. 3*A*) or treated identically but without Ca^{2+} exposure (and shown to retain normal coupling). Each sample was then immediately frozen with liquid N_2, in 10 μl of the K-HDTA solution with 1 mM EGTA and 1 mM leupeptin, and stored on dry ice or at −70 °C. Samples were later thawed with additional protease inhibitors (1 μM pepstatin A, 1 mM benzamidine, 2 mM dithiothreitol, 0·5 mM phenylmethylsulphonyl fluoride) and solubilized by vortexing in electrophoresis sample preparation buffer (final concentrations: 5% glycerol, 1·25% SDS, 2·5% 2-mercaptoethanol, 0·1% Bromphenol Blue, 32·5 mM Tris–HCl, pH 6·8). Samples for RyR and triadin staining were boiled for 4 min and run on a 2–10% gradient SDS–polyacrylamide gel using the buffer system of Laemmli (1970) and then transferred using a BioRad Mini Trans-Blot cell to Immobilon P (Millipore) at 150 V for 30 min and 100 V for 2 h in 10 mM 2-[*N*-cyclohexylamino]ethanesulphonic acid (Ches), 10% ethanol, 0·5% SDS, pH 9·6. Samples for DHPR staining contained 50 mM dithiothreitol and were incubated at room temperature for 20 min before being run on a 4–15% gradient gel and transferred to Immobilon P at 100 V for 2·5 h in 10 mM Ches, pH 9·6. Immunostaining of blots was performed as in Junankar & Dulhunty (1994), using antibodies to the RyR (5C3; Dulhunty, Junankar & Stanhope, 1992), triadin (Brandt *et al.* 1992) or α_1-subunit of DHPR (mAb 1A; Morton & Froehner, 1987). Blots were blocked with 5% skimmed milk in 20 mM Tris–HCl, 500 mM NaCl, pH 7·5 (TBS), incubated with monoclonal antibodies in 1% bovine serum albumin in TBS for 16 h, followed by anti-mouse IgG–alkaline phosphatase conjugate (Silenius, Hawthorn, Victoria, Australia) for 2–3 h. Colour was developed by the method of Blake, Johnson, Russell-Jones & Gotschlich (1984). Rabbit SR vesicles were prepared as in Saito, Seiler, Chu & Fleischer (1984), TC corresponding to R4 and T-tubules/light SR corresponding to R1.

Electron microscopy

After verifying that a skinned fibre was uncoupled after Ca^{2+} treatment (see Results) or still coupled after identical treatment without Ca^{2+} exposure, each skinned fibre was slackened and fixed with 2·5% gluteraldehyde in the standard K-HDTA solution with 0·15 mM (rat) or 0·5 mM EGTA (toad) for >24 h (initial 10 min until rigid, on the transducer). Samples were washed (3 × 20 min in 100 mM sodium cacodylate buffer with 120 mM sucrose), post-fixed in 1% osmium tetroxide (in wash solution for 60 min at room temperature), dehydrated in graded acetone (30%, 50%, 70%, 95%, dry absolute) and infiltrated with Spurr's epoxy resin (which was polymerized overnight at 60 °C). Thin sections (90 nm) were cut, mounted on hexagonal nickel grids, stained with 1% aqueous potassium permanganate and examined with a Joel 100S electron microscope (Tokyo, Japan). Scale bars were calibrated assuming a myosin length of 1·6 μm.

Force traces and data presentation

In force traces, unless otherwise indicated, the muscle fibre was bathed in the standard potassium solution (0·1 μM Ca^{2+}, 50 μM EGTA, pCa 7·1). In the text, mean values ± standard error of the mean are given; *n* is the number of fibres. The statistical probability (*P*) was determined with Student's paired and unpaired *t* tests, as appropriate.

RESULTS

E–C coupling in a skinned fibre with a sealed T-system

It has been previously inferred that the T-system of a skeletal muscle fibre seals off when the fibre is skinned by microdissection, based on the responses of such fibres to solution changes designed to 'depolarize' the T-system (Stephenson, 1985; Fill & Best, 1988; Lamb & Stephenson, 1990*a*, 1994). We established this more directly using confocal laser scanning microscopy (Stephenson & Lamb, 1993). First, we examined *intact* muscle fibres of the toad, bathed in a Ringer solution (with 2·5 mM Ca^{2+}) containing

the membrane-impermeable salt of the Ca^{2+}-sensitive fluophore fluo-3 (1 mM). Confocal optical sections through such fibres revealed a regular, banded fluorescence pattern (not shown), which was absent if the fluo-3 was omitted. Imaging the same region with transmitted light revealed that the fluorescent bands occurred in the middle of the I-band in each fibre, where the T-system is located. When a fibre exposed to fluo-3 was mechanically skinned and placed in a low-[Ca^{2+}] solution (2 mM EGTA, pCa > 8, in K-HDTA intracellular solution), the same banded fluorescence pattern was present and persisted for more than 1 h (Fig. 1*A*). In fibres which were exposed to fluo-3 over a large region and then skinned for only part of their length before being placed in a low-[Ca^{2+}] solution, in every case the banded pattern was visible in the skinned region (e.g. Fig. 1*D*), but not in the unskinned region (e.g. Fig. 1*C*), with a clear transition at the 'cuff' region where the skinning stopped (not shown).

This clearly shows that the skinning procedure prevented loss of the fluorescence signal, presumably because it stopped both the influx of the EGTA–low-[Ca^{2+}] solution

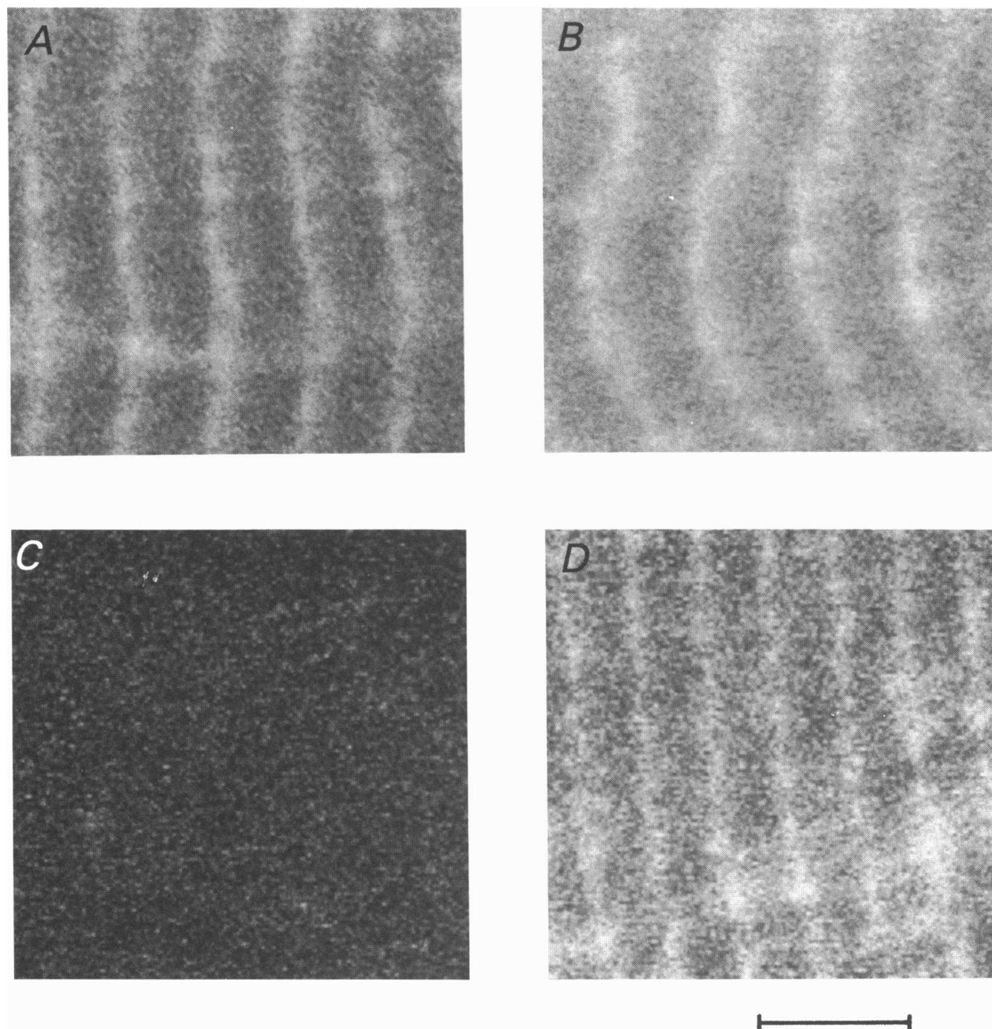

Figure 1. The T-system seals off when a muscle fibre is mechanically skinned and remains sealed after uncoupling

A, confocal laser scanning microscopy of a toad skinned fibre that had been exposed to fluo-3 before skinning. The banded fluorescence pattern, which persisted for more than 1 h with < 10 nM Ca^{2+} (2 mM EGTA) in the bathing solution, indicates that the fluo-3 was trapped in the sealed T-system. *B*, confocal image of a toad skinned fibre that had been uncoupled by Ca^{2+} exposure (see text) (recording settings identical to *A*). *C* and *D*, confocal images of a toad fibre that was exposed to fluo-3 and then skinned for only part of its length; fluorescent bands were present in the skinned region (*D*), but not in the unskinned region (*C*) (uncoupled fibre bathed in 50 mM EGTA/Ca-EGTA with 0·1 μM Ca^{2+}). Note that the resolution limit of this confocal microscopy is only approximately 0·3 μm in the *X–Y* plane and 0·8 μm in the *Z*-plane. Scale bar: 3·2 μm in *A* and *B*, 5 μm in *C* and *D*.

into the T-system and the efflux of Ca^{2+} and some or all of the fluo-3 from the T-system. Thus, the solution in the T-system in the skinned region of the fibre was isolated from the surrounding solution, indicating that the T-system was indeed sealed. In agreement, we also found that treatment of skinned fibres with saponin (0·5 mg ml^{-1}, 2 min), which is thought to selectively permeabilize the T-system (Endo & Iino, 1980), abolished the fluorescence signal (2 fibres, not shown). The complete absence of the fluorescence signal in the unskinned regions of fibres that were exposed to fluo-3 and then imaged within 2 min of exposure to the low-[Ca^{2+}] solution indicates that having only the outer end of the T-system open is sufficient for rapid loss/change of the T-system contents. This emphasizes that the T-system in the skinned region of a fibre must be well sealed over its entire length.

As described previously (Lamb & Stephenson, 1990*a*, 1994), if a mechanically skinned fibre is bathed in a high-[K^+] solution mimicking the normal intracellular environment, the sealed T-system apparently becomes polarized and can be rapidly depolarized by substituting a solution with low [K^+] (see Methods), thereby triggering Ca^{2+} release from the SR and a large force response (e.g. Fig. 2). Depolarization-induced responses in fibres skinned with fluo-3 in the T-system were not noticeably different from those in fibres without fluo-3. Significantly, the banded fluorescence pattern was not lost even after a large number of depolarization-induced responses, implying that the contracture of the fibre did not reopen the sealed T-system, and this is consistent with the reproducibility of the responses themselves.

High myoplasmic [Ca^{2+}] disrupts E–C coupling

E–C coupling in skinned fibres is quite robust and unaffected by large changes in intracellular pH (Lamb & Stephenson, 1994) or ionic strength (Lamb, Stephenson & Stienen, 1993). Furthermore, 'myoplasmic' [Ca^{2+}] can be raised sufficiently to produce maximum force for about 30 s without interfering with subsequent E–C coupling (Fig. 2). However, when the free [Ca^{2+}] was raised to 1 mM, depolarization lost its ability to induce any force response in all ten toad and four rat fibres examined, even though caffeine (2 mM) and low [Mg^{2+}] (see Lamb & Stephenson, 1991) could each still trigger Ca^{2+} release (e.g. Fig. 2). Thus, depolarization-induced responses were abolished, even though the SR contained a large amount of releasable Ca^{2+} and the Ca^{2+} release channels were still functional. Clearly, this loss of E–C coupling was not caused simply by force-induced damage, because (a) maximum force could be induced for a similar period by exposure to the max solution (20 μM Ca^{2+}) without causing the effect, and (b) the effect occurred even though the rate of force development in the high-[Ca^{2+}] solution was greatly slowed (rise time >3 s) by initially bathing the fibre in a heavily buffered low-[Ca^{2+}] solution (see Fig. 2). Such Ca^{2+}-dependent 'uncoupling' could not be reversed over the duration of the experiment (>30 min) in any of the fibres examined in this way, irrespective of how low the myoplasmic [Ca^{2+}] was made (e.g. pCa > 9 with 50 mM EGTA, for > 5 min).

Ca^{2+} dependence of uncoupling

In the above experiments, the [Ca^{2+}] within the muscle fibre was probably not well controlled, because the SR Ca^{2+} pump continued to take up Ca^{2+} into the SR (hence accounting for the larger depolarization-induced response after exposure to the max solution in Fig. 2) and because Ca^{2+}-induced Ca^{2+} release (Endo, 1985), although greatly dampened, is not completely blocked at 1 mM Mg^{2+} (Lamb & Stephenson, 1990*b*). Thus, in order to quantify better the Ca^{2+} dependence of the uncoupling, we examined the effect

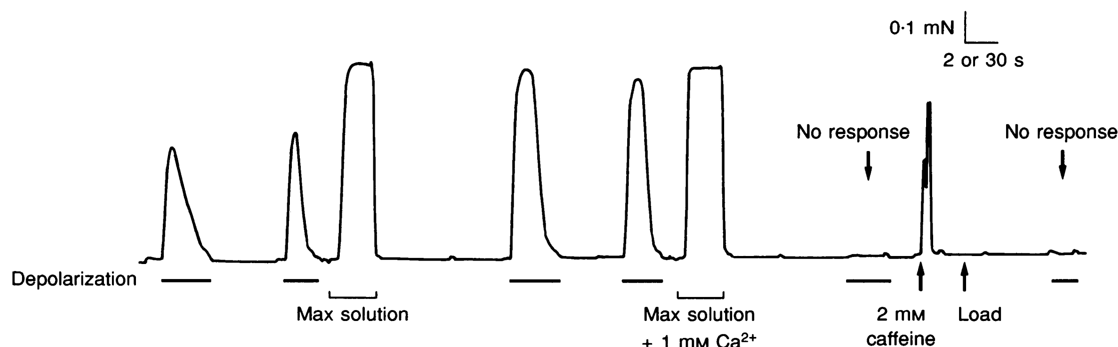

Figure 2. Exposure to high [Ca^{2+}] abolishes depolarization-induced force responses in a skinned muscle fibre

Depolarization (bars) of a toad iliofibularis fibre induced a fast transient force response, and myoplasmic [Ca^{2+}] could be raised sufficiently to produce maximum force (Max solution: 20 μM free Ca^{2+}, 50 mM Ca-EGTA, 30 s), without interfering with subsequent E–C coupling. However, after exposure to a similar solution with 1 mM free Ca^{2+}, depolarization was unable to elicit any response, although caffeine (2 mM) could still do so. The fibre was exposed to a solution with 50 mM EGTA (pCa > 10) for 2 s before exposure to max and max + 1 mM Ca^{2+} solutions, to reduce the rate of force rise, and again afterwards for 2 s, to rapidly lower the [Ca^{2+}]. Time scale: 2 s during depolarization and 30 s elsewhere.

of raised $[Ca^{2+}]$ in the absence of ATP, as this both stops active Ca^{2+} uptake and further suppresses Ca^{2+}-induced Ca^{2+} release (Endo, 1985). The absence of ATP caused rigor in each fibre, but this had no effect on E–C coupling after ATP had been restored (Fig. 3*A*). In contrast, raising the $[Ca^{2+}]$ for 10 s during rigor abolished subsequent E–C coupling with half-maximal effects at approximately 10 and 23 μM in toad and rat muscle fibres, respectively (Fig. 3). Fibres partially or completely uncoupled in this manner showed no recovery over the duration of the experiment (up to 1 h).

After complete uncoupling, neither the maximum force production of the contractile apparatus nor its Ca^{2+} sensitivity was altered (maximum force: 99 ± 1 % of initial and shift of pCa_{50} (= $-\log_{10}[Ca^{2+}]$ at half-maximum force)

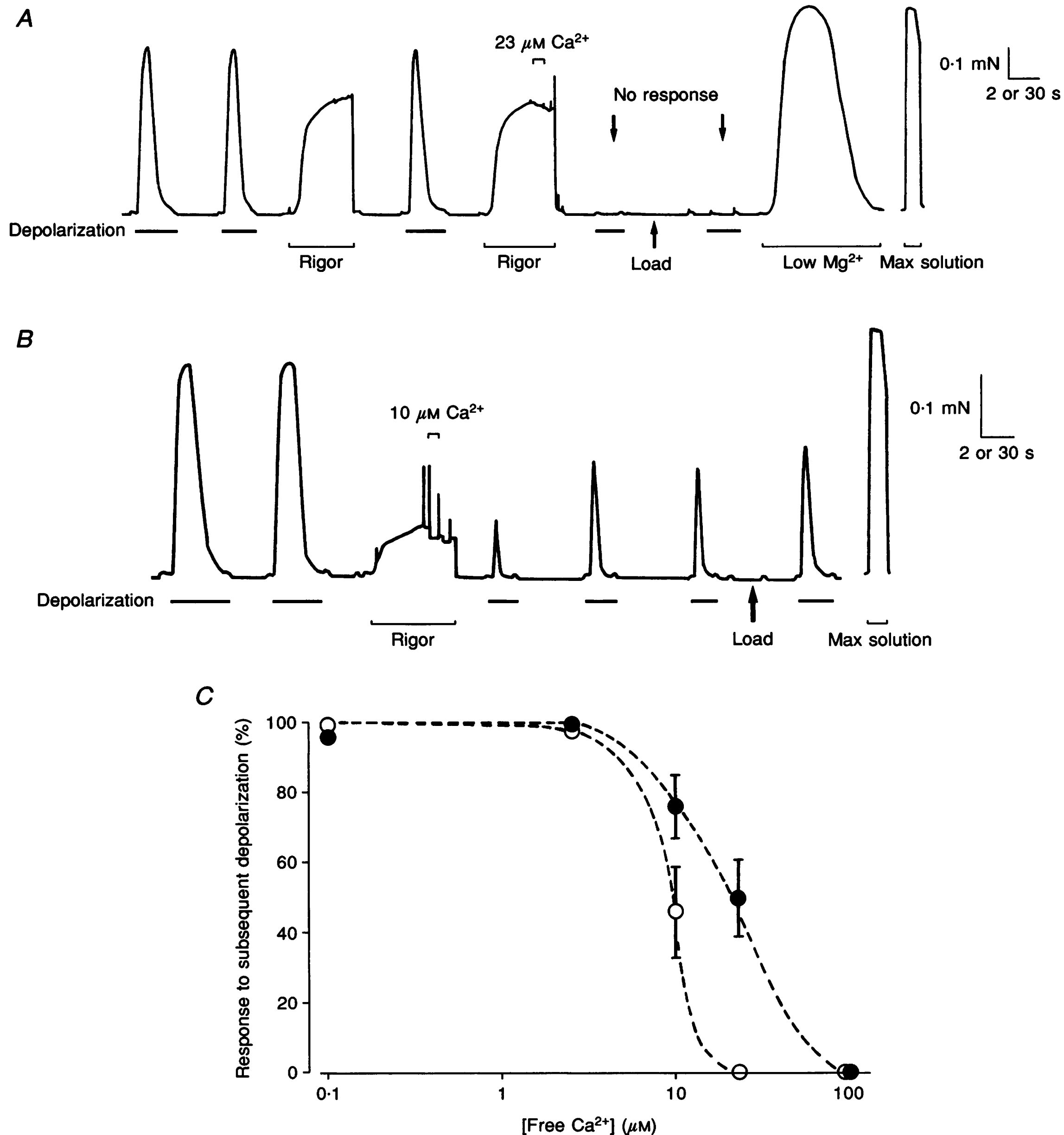

Figure 3. Effect of $[Ca^{2+}]$ on depolarization-induced force responses in skinned muscle fibres

A, raising the $[Ca^{2+}]$ to 23 μM for 10 s during rigor (no ATP) abolished the response of a toad fibre to depolarization, but lowering $[Mg^{2+}]$ from 1 to 0·05 mM (Low Mg^{2+}) still induced Ca^{2+} release. *B*, partial uncoupling in a rat EDL fibre after 10 s exposure to 10 μM Ca^{2+}. Horizontal scale in *A* and *B*: 2 s during depolarization and exposure to low $[Mg^{2+}]$ and 30 s elsewhere. *C*, mean response to depolarization (± S.E.M.) as a percentage of the initial response, after raising the $[Ca^{2+}]$ to the indicated level for 10 s during rigor in toad iliofibularis fibres (○) and rat EDL fibres (●). $[Ca^{2+}]$ and number of fibres for toad and rat, respectively: 0·1 μM, 4 and 7; 2·5 μM, 5 and 3; 10 μM, 9 and 5; 23 μM, 11 and 6; 100 μM, 3 and 21. Curves fitted by eye.

Table 1. Effects of pH, temperature, exposure duration and leupeptin on the extent of uncoupling caused by raised [Ca^{2+}] in skinned fibres

A. Rat fibres exposed to 100 μM Ca^{2+}					B. Toad fibres exposed to 2·5 μM Ca^{2+}		
pH 5·8, 23 °C	pH 7·1, 23 °C		pH 7·1, 3 °C		pH 7·1, 23 °C		
10 s	3 s	10 s	10 s	40 s	10 s	60 s	60 s + Leup
25 ± 12 ($n = 7$)	30 ± 9 ($n = 4$)	0 ± 0 ($n = 21$)	57 ± 16 ($n = 7$)	46 ± 17 ($n = 3$)	98 ± 2 ($n = 5$)	54 ± 13 ($n = 7$)	92 ± 9 ($n = 5$)

Mean (± S.E.M.) of the maximum force response to depolarization following exposure, during rigor, to raised [Ca^{2+}] for the times indicated (e.g. Fig. 3). Values expressed as a percentage of the response in the same fibre before treatment. In A, rigor was initially induced in each fibre at pH 7·1 and 23 ± 2 °C and then the fibre was equilibrated in a rigor solution of appropriate temperature and pH for at least 30 s before, and 10 s after, raising the [Ca^{2+}] (see Figs 5 and 6). 100 μM Ca^{2+} (pH 7·1, 23 °C) was shown to cause complete uncoupling in at least one fibre from every muscle used to examine the other conditions. In B, treatments for 60 s with and without leupeptin (Leup; 1 mM) were examined in alternate fibres from the same muscles and the means are significantly different ($P < 0·01$, Student's t test).

by 0·00 ± 0·01 in three rat fibres, determined with heavily buffered Ca^{2+} solutions – see Methods). Furthermore, as with uncoupling in the presence of ATP (e.g. Fig. 2), Ca^{2+} release could still be induced by low concentrations of caffeine (2 mM) or by lowering the [Mg^{2+}] (e.g. Fig. 3*A*). We quantified the ability of the SR to load and release Ca^{2+} after such uncoupling, using a solution with 30 mM caffeine, low [Mg^{2+}] and 0·5 mM EGTA to potently induce Ca^{2+} release and completely deplete the SR of releasable Ca^{2+}, before reloading it and repeating the procedure (Fig. 4). The resulting force responses, which are indicative of the level of Ca^{2+} loading in the SR (Endo, 1985), were highly reproducible and the time integral of the response was linearly related to the SR loading time (for the range of loading times used, e.g. Fig. 4). In the four rat fibres examined, the time integral of the force response after uncoupling was 97 ± 9% of that before uncoupling in the same fibre for the same loading time, indicating that the ability of the SR to take up and store Ca^{2+} and to release it again upon stimulation with caffeine–low [Mg^{2+}], was unaffected by the uncoupling. We also found that uncoupling was not dependent on the Ca^{2+} content of the SR, as it occurred (with the same Ca^{2+} dependence) when the SR had first been depleted of Ca^{2+} (not shown).

Dependence of uncoupling on duration of Ca^{2+} exposure, temperature and pH

As might be expected, the extent of uncoupling was dependent on the duration of the Ca^{2+} exposure as well as on the [Ca^{2+}]. For example, whereas uncoupling in rat fibres was complete with 10 s exposure to 100 μM Ca^{2+} at 23 °C, exposure for 3 s caused only partial uncoupling (Table 1A). Similarly, a low [Ca^{2+}] (2·5 μM), which had little or no effect with 10 s exposure, caused substantial uncoupling with 60 s

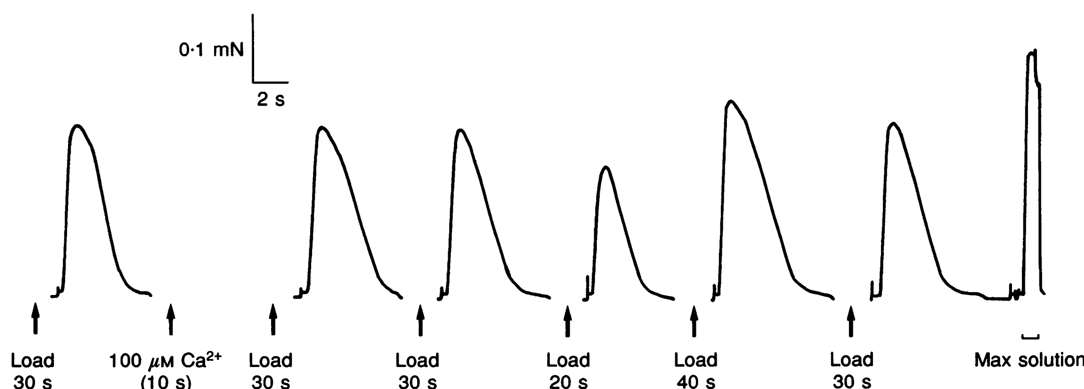

Figure 4. Caffeine-induced Ca^{2+} release is unchanged after uncoupling

Responses were induced in a rat EDL fibre by exposure to a solution with 30 mM caffeine (potassium solution with 0·02 mM Mg^{2+} and 0·5 mM EGTA) and the fibre was left in this solution for 2 min to completely deplete the SR of Ca^{2+} before reloading (in a solution with 2 μM Ca^{2+}, 50 μM EGTA) for the time indicated. The fibre was uncoupled by 10 s exposure to 100 μM Ca^{2+} during rigor, where indicated. The time integral of the responses after uncoupling as a percentage of the response before uncoupling were: 113% (load time: 30 s), 101% (30 s), 64% (20 s), 136% (40 s), 105% (30 s). Time scale 30 s in max solution.

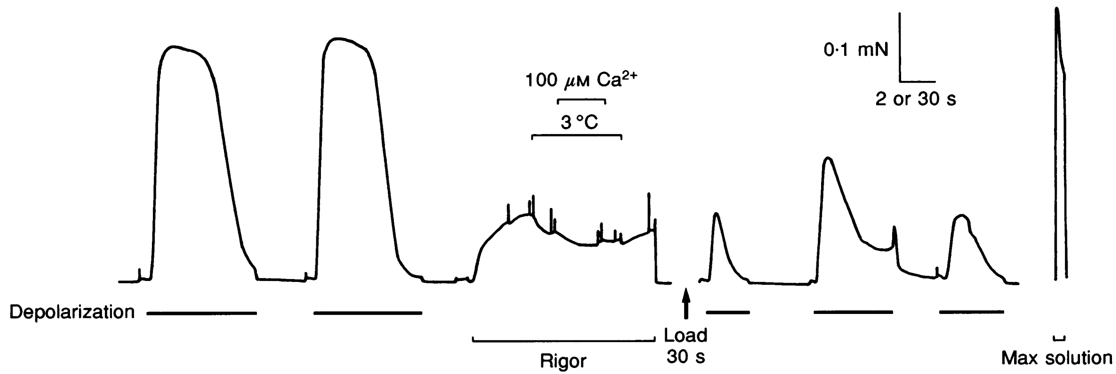

Figure 5. Ca^{2+}-dependent uncoupling was slower at 3 °C

Exposure of a rat EDL fibre to 100 μM Ca^{2+} for 40 s at 3 °C only caused partial uncoupling. Temperature, 23 °C unless specified. Time scale: 2 s during depolarizations and 30 s elsewhere.

exposure (Table 1B). The rate of uncoupling was also dependent on the temperature during the period of Ca^{2+} exposure; a 40 s exposure to 100 μM Ca^{2+} at 3 °C (e.g. Fig. 5) caused less uncoupling than a 3 s exposure at 23 °C (see Table 1A), i.e. the uncoupling was more than 13-fold slower at 3 than at 23 °C, indicating a Q_{10} for the process of more than 3·5. Uncoupling was also about 3-fold slower at pH 5·8 than at pH 7·1; a 10 s exposure to 100 μM Ca^{2+} at pH 5·8 (e.g. Fig. 6) only caused approximately the same degree of uncoupling as a 3 s exposure at pH 7·1 (see Table 1A). Sr^{2+} also caused uncoupling, although at approximately 20-fold higher concentration than Ca^{2+}: half-maximal effect at approximately 500 μM in rat fibres (mean response after 10 s exposure, as percentage of that before exposure: 92 ± 8% ($n = 3$) and 11 ± 6% ($n = 6$) for 330 and 770 μM Sr^{2+}, respectively). In contrast, high [Mg^{2+}] neither induced uncoupling, even at 10 mM (e.g. see Lamb & Stephenson, 1994), nor prevented the effect of Ca^{2+} (all 3 rat fibres examined were completely uncoupled by exposure to 100 μM Ca^{2+} for 10 s in the presence of 7 mM Mg^{2+}).

Evidence for uncoupling in intact fibres

Three series of experiments indicated that Ca^{2+}-dependent uncoupling probably also occurs in intact fibres. Firstly, in freshly skinned fibres which had only the endogenous level of Ca^{2+} loading, rapid and extensive *release of SR Ca^{2+}* (induced with 30 mM caffeine–low-[Mg^{2+}] solution) caused complete abolition of E–C coupling if the [Ca^{2+}] was

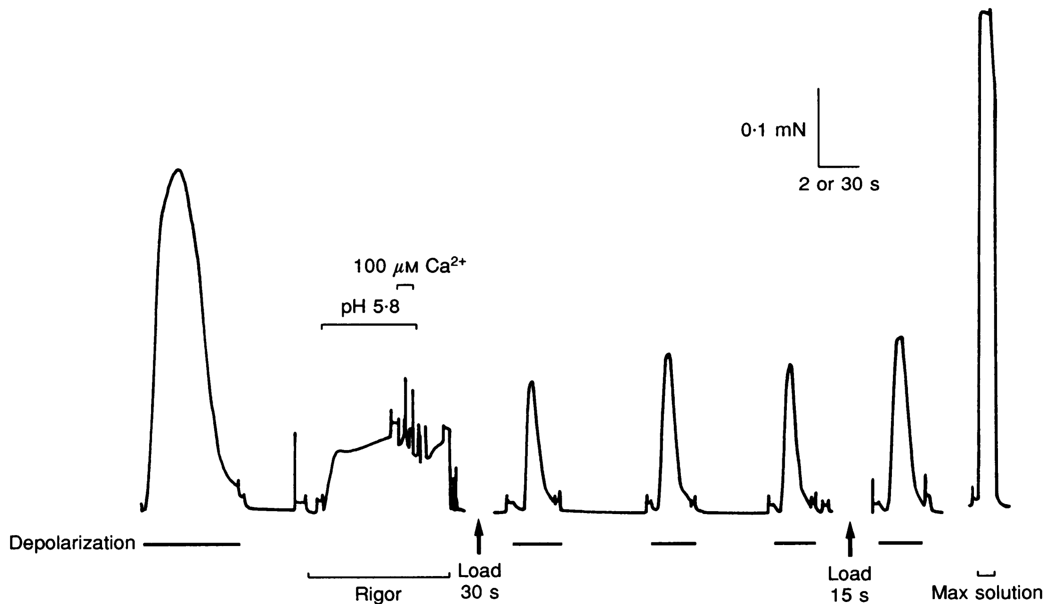

Figure 6. Uncoupling was slower at pH 5·8

Exposure of a rat EDL fibre to 100 μM Ca^{2+} for 10 s at pH 5·8 only caused partial uncoupling. pH 7·1 unless specified. Time scale: 2 s during depolarizations and 30 s elsewhere.

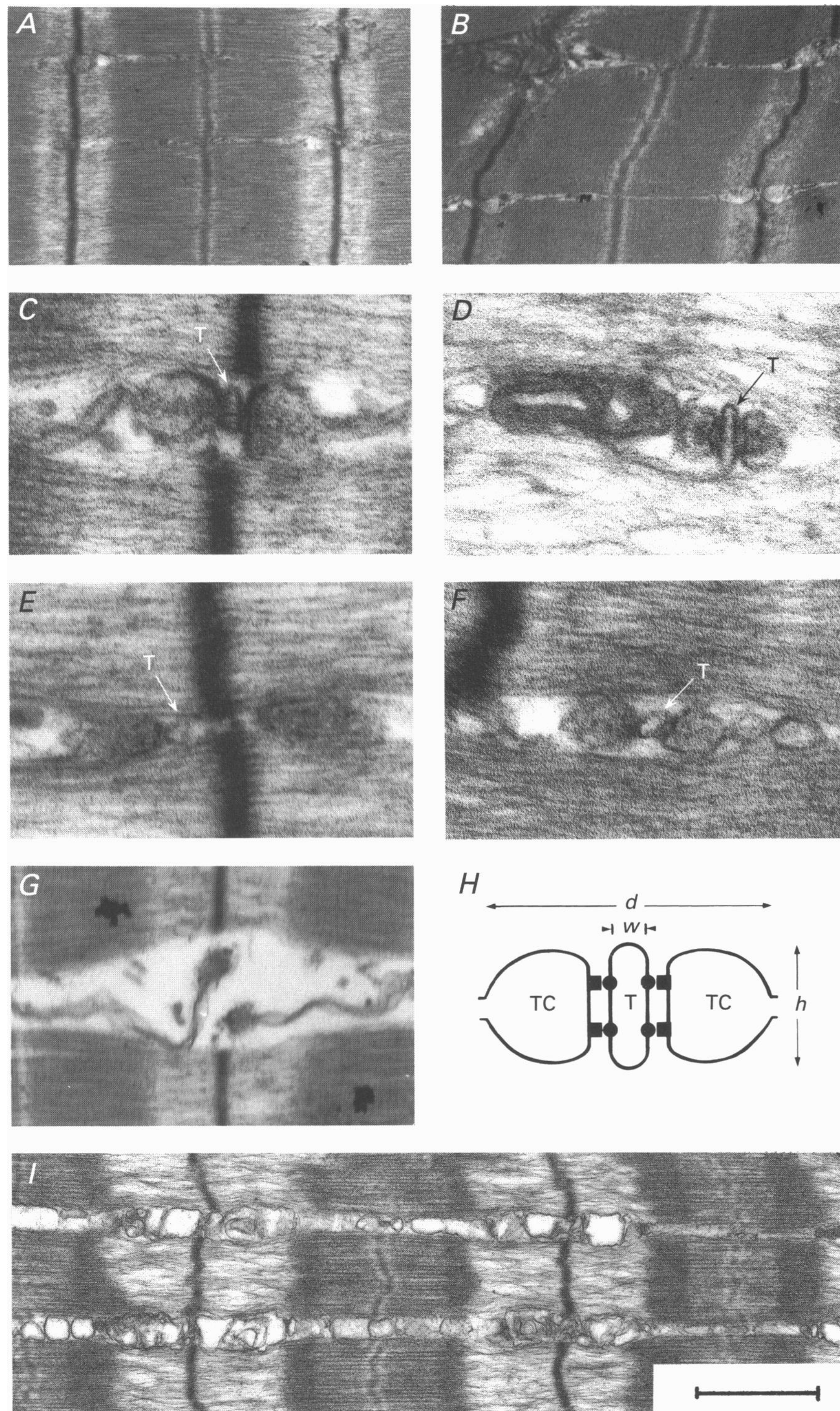

Figure 7. Electron micrographs of uncoupled fibres reveal structural alterations at the triad

Z-line organization in a coupled fibre (*A*) and uncoupled fibre (100 μM Ca^{2+}, 10 s; *B*), from toad. Triads in coupled fibre of toad (*C*) and rat (*D*, adjacent to mitochondrion); note that the T-system (T) is elongated perpendicular to the TC–TC axis. Triads in uncoupled fibres, noticeably distended along the TC–TC axis (*E*, toad; *F*, rat) or severed (*G*, toad). *H*, schematic diagram of the triad, with the squares and circles representing RyRs and DHPRs, respectively. *I*, swelling and vesiculation of SR and disintegration of mitochondria in a rat fibre exposed to 100 μM Ca^{2+} for 60 s. All longitudinal sections. Scale bar: 1·0 μm in *A*, 0·95 μm in *B*, 230 nm in *C–F*, 740 nm in *G* and 1·05 μm in *I*. Sarcomere lengths: 2·2–2·4 μm in both coupled and uncoupled toad fibres and 3·0–3·2 μm and 2·8–3·2 μm in coupled and uncoupled rat fibres, respectively.

permitted to rise unchecked (only 50 μM EGTA, 3 fibres), but did not cause any uncoupling if the [Ca^{2+}] was kept very low during the release (with 2 mM EGTA, 2 fibres). Depolarization-induced release of Ca^{2+} also caused substantial uncoupling in both rat and toad fibres, when Ca^{2+} uptake by the SR was blocked by 2,5-di-(tert-butyl)-1,4-hydroquinone (TBQ) (Bakker, Lamb & Stephenson, 1995). These results show that release of the amount of Ca^{2+} stored in an intact fibre can be sufficient to cause uncoupling.

Secondly, we found that raised [Ca^{2+}] caused uncoupling in skinned fibres under conditions which minimized the loss of large, diffusible myoplasmic molecules. Myoplasmic [Ca^{2+}] was rapidly raised by transferring a freshly skinned fibre from paraffin oil to a solution with 2 mM free Ca^{2+} and 30 mM caffeine for only 0·5 s (which is insufficient time for the full equilibration of even small molecules) and then returning it to the oil for 10 s to prevent any further diffusion. In each fibre, the myoplasmic [Ca^{2+}] remained high enough to maintain maximum force for the period in oil, despite the presence of endogenous Ca^{2+} buffers and Ca^{2+} uptake by the SR, and E–C coupling was completely absent afterwards (3 rat fibres). When fibres were treated in an identical fashion, but without the additional Ca^{2+} in the caffeine solution, the myoplasmic [Ca^{2+}] only rose enough to produce less than 10% of maximum force and E–C coupling was totally functional afterwards (2 fibres). This suggests that a large rise in the [Ca^{2+}] in the myoplasm of an intact fibre will cause uncoupling.

Thirdly, and most importantly, we found that gross stimulation of *intact fibres* also apparently caused uncoupling. Skinned fibres obtained from bundles of intact fibres which had been stimulated three times by a potassium contracture and simultaneous application of 30 mM caffeine (1 min episodes) produced significantly smaller responses to depolarization (45 ± 11% of maximum Ca^{2+}-activated force, $n = 11$) than did skinned fibres from the unstimulated contralateral muscles (79 ± 8%, $n = 11$, $P < 0{\cdot}05$), even though caffeine or low [Mg^{2+}] induced similar Ca^{2+} release in both cases. Thus, under conditions in which any differences in the myoplasmic environment of the stimulated and unstimulated fibres (e.g. metabolites, [ATP], etc.) had been eliminated, stimulated fibres showed reduced responsiveness to depolarization, presumably due to partial Ca^{2+}-dependent uncoupling. Together, these three series of experiments suggest that excessive Ca^{2+} release in intact muscle fibres can cause uncoupling.

Phosphorylation and oxidation

The high Q_{10} of the uncoupling suggests that an underlying enzymatic process may be responsible. Ca^{2+} can activate a calmodulin-dependent protein kinase in skeletal muscle, which phosphorylates many proteins of the junctional SR (Chu, Sumbilla, Inesi, Jay & Campbell, 1990), and might thus modify E–C coupling. However, it seems unlikely that a phosphorylation mechanism is responsible for uncoupling, because the uncoupling was not noticeably affected by (a) the presence or absence of ATP (including when 12 units ml^{-1} of the Ca^{2+}-independent ATPase, apyrase, was present in the rigor solution), (b) the non-specific protein kinase inhibitors, staurosporine (10 μM) and H7 (100 μM) or an inhibitory peptide of the Ca^{2+}–calmodulin-dependent protein kinase II (fragment 290–309, 100 μM), or (c) calmodulin (2 to 5 μM). Also, uncoupling was not reversed by acid phosphatase (20 units ml^{-1}, pH 6·2, 3 min). Similarly, Ca^{2+}-dependent dephosphorylation also appears unlikely, because the potent phosphatase inhibitor microcystin-LR (50 μM) (MacKintosh, Beattie, Klumpp, Cohen & Codd, 1990) did not prevent uncoupling and the catalytic subunit of protein kinase A did not reverse the uncoupling (> 3 min, 30 units ml^{-1}). (None of the above agents noticeably affected normal E–C coupling and each result was obtained in 3 fibres from rat.) Furthermore, the reducing agent dithiothreitol (10 mM) neither prevented nor reversed uncoupling (nor indeed noticeably affected E–C coupling), implying that uncoupling was not due to an oxidation reaction (3 rat fibres).

Ca^{2+}-dependent structural changes

Electron microscopy revealed specific structural changes in uncoupled fibres. Z-lines were markedly wavy in many sarcomeres in fibres uncoupled by 10 s exposure to 100 μM Ca^{2+} (3 toad, 3 rat fibres; e.g. Fig. 7*B*), whereas they were straight and perpendicular to the fibre axis in nearly all regions examined in coupled fibres (4 toad, 3 rat fibres; e.g. Fig. 7*A*). With much longer exposure (60 s) to 100 μM Ca^{2+}, there were severe Z-line abnormalities, SR swelling and vesiculation and mitochrondrial disruption throughout all four rat fibres examined (Fig. 7*I*).

Triads in coupled fibres (subjected to rigor) from both toad (Fig. 7*C*) and rat (Fig. 7*D*), closely resembled those observed in fibres processed directly for electron microscopy (Cullen, Hollingworth & Marshall, 1984; Dulhunty, 1984), with the T-tubule being 3–4 times longer in the dimension perpendicular to the TC–TC axis (i.e. $h \gg w$ in Fig. 7*H*). In contrast, in all uncoupled fibres the T-tubule was often difficult to visualize, due to distortion of the triads, but where reasonably defined it was elongated on the TC–TC axis, with $w \gg h$ in all cases (e.g. Fig. 7*E* and *F*). In agreement, where triads were sectioned along the TC–TC axis (i.e. into page in Fig. 7*H*), the width of the T-tubule (w) was about 2–5 times larger in uncoupled fibres than in coupled fibres, in both species (not shown). The distance across each triad (d in Fig. 7*H*) was very similar in all coupled fibres from toad muscle (grouped mean, 343 ± 10 nm, $n = 37$) and was significantly greater ($P < 0{\cdot}05$) in each of the three uncoupled fibres (grouped mean, 477 ± 24 nm, $n = 38$). Similar results were obtained with rat fibres (222 ± 5 nm, $n = 18$, and 284 ± 10 nm, $n = 21$, respectively). Also, in uncoupled fibres, a small percentage of triads were very obviously severed (e.g. Fig. 7*G*).

T-system integrity and voltage sensor inactivation

Disruptions at the level of the triad may compromise the integrity of the T-system, which would chronically depolarize the T-system and lead to the inactivation of the voltage sensors/DHPRs, thus abolishing E–C coupling. However, confocal microscopy of fibres exposed to fluo-3 before skinning showed that the banded fluorescence pattern persisted for more than 1 h after uncoupling (Fig. 1*B* and *D*), indicating that the fluo-3 remained trapped in the T-system and that the $[Ca^{2+}]$ in the T-tubules was much higher than in the myofibrillar environment. Hence, the integrity of the T-system was not compromised in uncoupled fibres.

The possibility exists that after exposure to Ca^{2+} the T-system becomes chronically depolarized by some irreversible, Ca^{2+}-dependent change in the ionic permeabilities of the T-system. However, we could find no evidence of this. We were not able to prevent or reduce uncoupling by any of a number of treatments designed to minimize various possible permeability changes (achieved by bathing the muscle in an appropriate solution before skinning fibres under oil): (a) blocking the T-system Na^+ channels with tetrodotoxin (3 μM), (b) replacing T-system Na^+ with the large impermeant cation, *N*-methyl-D-glucamine, (c) blocking the T-system Ca^{2+} channels with Co^{2+} (5 mM Co^{2+} and 5 mM Mg^{2+}), or (d) buffering the T-system $[Ca^{2+}]$ (with nitrilo-triacetic acid, 25 mM total Ca^{2+}) to prevent it dropping to levels where the Ca^{2+} channels become non-specific or the voltage sensors become inactive (Rios & Pizarro, 1991) (at least 3 rat fibres for each case).

We cannot entirely discard the possibility that the raised $[Ca^{2+}]$ rendered the K^+ channels in the T-tubules non-functional, thus chronically depolarizing the T-system, although this seems unlikely given that we could demonstrate that partial Ca^{2+}-dependent uncoupling (e.g. Fig. 3*B*) was not caused by partial depolarization of the whole T-system. Decreasing the myoplasmic $[K^+]$ to two-thirds for 20 s before a full depolarization, which should increase any submaximal level of steady-state voltage sensor inactivation (Lamb & Stephenson, 1990*a*), did not decrease the ensuing response (e.g. Fig. 8; mean responses in 4 partially uncoupled toad fibres, as percentage of initial coupled response: 22 ± 6% with normal $[K^+]$ and 29 ± 10% with 2/3 $[K^+]$). Thus, a chronic depolarizing effect could only account for partial uncoupling if some parts of the T-system remain fully polarized whilst other parts are completely depolarized, but this is difficult to reconcile both with the expected widespread effects of any proposed permeability change and with the relatively long electrical length constant of the T-system.

Actin depolymerization

The alteration in the triadic junction is analogous to the Ca^{2+}-dependent alteration of the postsynaptic cytoskeletal structure in hippocampal neurones (Rosenmund & Westbrook, 1993), which could be prevented by stabilizing the actin filaments with phalloidin. However, pretreatment with phalloidin (40 μM, labelled with rhodamine to verify widespread binding to actin filaments) neither prevented nor reduced the uncoupling induced by a 10 s exposure to 100 μM Ca^{2+} in any of the three rat fibres tested.

Proteolysis

The observed structural changes at the triad following exposure to Ca^{2+} could be because of Ca^{2+}-dependent proteolysis of key structural connections involved in

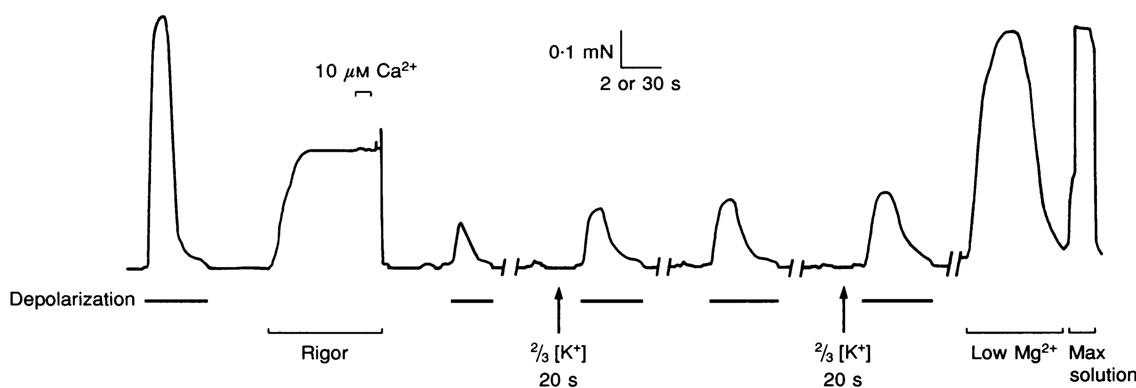

Figure 8. Partial uncoupling is not caused by partial depolarization of the T-system

In a toad muscle fibre that had been partially uncoupled by exposure to 10 μM Ca^{2+} during rigor, decreasing the $[K^+]$ in the bathing solution to two-thirds of standard for the 20 s before depolarization had little effect on the response. The inability of Ca^{2+} loading (for 30 s during first 3 breaks) to restore the response shows that the fibre was indeed partially uncoupled and not simply Ca^{2+} depleted; this is also indicated by the large response to low $[Mg^{2+}]$ (0·05 mM). This result indicates that the partial uncoupling was not caused by voltage sensor inactivation resulting from incomplete polarization of the T-system, because such inactivation would have been critically dependent on the myoplasmic $[K^+]$ (see text). Time scale: 2 s during depolarization and low $[Mg^{2+}]$ and 30 s elsewhere.

maintaining the normal shape of the T-tubule. Skeletal muscle contains high levels of Ca^{2+}-activated neutral proteases (CANP), which can cleave the RyR without destroying Ca^{2+} channel function (Gilchrist, Wang, Katz & Belcastro, 1992). Nevertheless, immunostaining of muscle proteins from uncoupled fibres showed no evidence of any proteolysis of the RyR (Fig. 9*A*), the DHPR α_1-subunit (Fig. 9*C*) or triadin (Fig. 9*B*); identical results were obtained three times for each protein. (We verified in two separate experiments that the anti-RyR monoclonal antibody, 5C3, could detect a proteolytic fragment of relative molecular mass (M_r) ≈ 400 000 (when present) produced by endogenous Ca^{2+}-dependent protease(s). Firstly, we showed that incubating a crude pellet of homogenized rat skeletal muscle with 1·5 mM free Ca^{2+} for 1–5 min at 25 °C gave bands for the cleaved and uncleaved proteins, which, respectively, became more and less intense with longer incubation periods, until they were of approximately equal intensity with 5 min incubation (4 μg protein per lane). Secondly, we could detect the band for the proteolytic fragment (approximately 10% of intensity of uncleaved band) when freshly skinned fibres mounted on the force transducer were briefly exposed (< 0·5 s) to a solution containing 5 mM Ca^{2+} and 30 mM caffeine and then returned to the paraffin oil (in order to retain most of the normal myoplasmic contents) for 1·5–3 min, at which time they completely broke under the resting tension, presumably due to proteolysis of titin.)

The rapid uncoupling occurring with 10 s exposure to high $[Ca^{2+}]$ (100 μM, rat; 23 μM, toad) was not prevented by the specific CANP inhibitor calpastatin (100 μM), nor by the non-specific inhibitors, leupeptin (1 mM), iodoacetamide (1 mM) or E-64 (100 μM) (3 fibres in each case). However, as the inhibitors only bind to the protease after it has been activated, even high concentrations of inhibitors may be insufficient to totally prevent the protease molecules from reaching nearby targets if many are simultaneously activated. When uncoupling was induced at a slower rate (2·5 μM Ca^{2+} for 60 s; mean response in toad fibres, 54 ± 13%, $n = 7$), it could be almost completely prevented by the presence of 1 mM leupeptin (Table 1B), although at such concentration leupeptin also caused reversible inhibition of depolarization-induced responses in some fibres. Thus, although Ca^{2+}-dependent uncoupling is not apparently

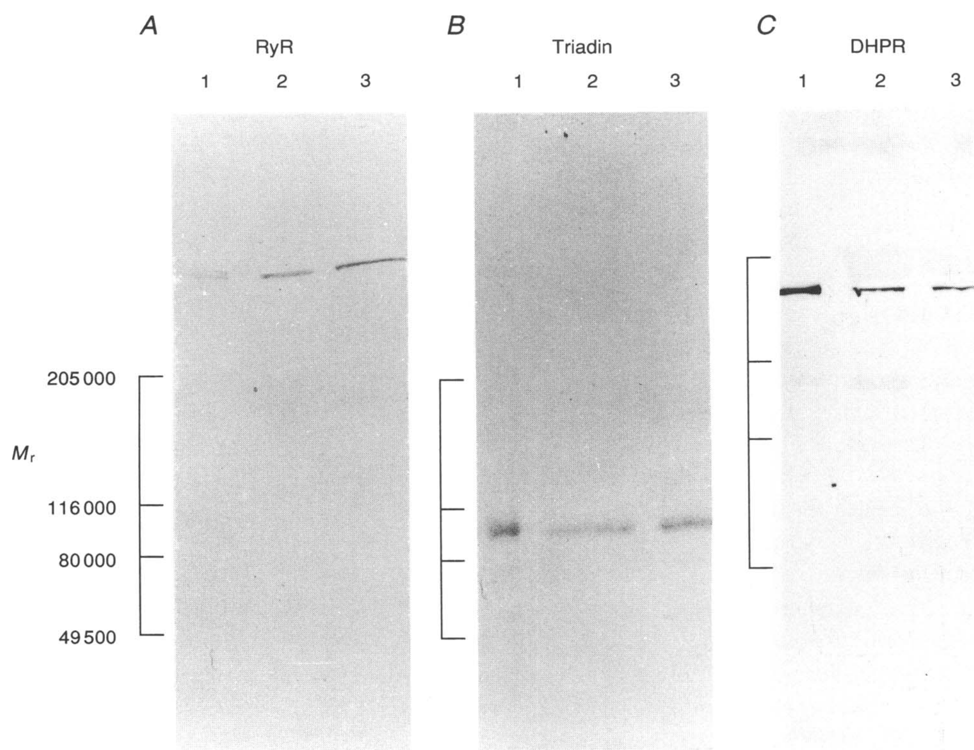

Figure 9. Ca^{2+}-dependent uncoupling does not involve proteolysis of the RyR, triadin or the α_1-subunit of the DHPR

Western blots of muscle proteins were immunostained with anti-RyR, 5C3 (*A*), anti-triadin (*B*) or anti-α_1-DHPR, mAb 1A (*C*). Proteins from coupled (lane 2) and uncoupled (lane 3) skinned fibres of rat (2 μg protein per lane) and TC (100 ng; lanes *A*1 and *B*1) and T-tubules/light SR (10 ng; lane *C*1) of rabbit. The antigenic polypeptides in rat muscle fibres showed the same apparent M_r as the rabbit antigens in fractionated SR vesicles. No loss of immunostaining intensity or formation of proteolytic fragments were observed in uncoupled fibres (see text).

caused by any significant level of proteolysis of RyR, α_1-DHPR or triadin, it is still possible that the uncoupling involves proteolysis of some other muscle protein. Our observation that the triad junction can be disrupted even though the RyR, α_1-DHPR or triadin are not proteolysed suggests that other structures at the triad play an important role in maintaining the integrity of the junction.

DISCUSSION

Physiological significance of Ca^{2+}-induced uncoupling

We show here that elevated myoplasmic [Ca^{2+}] abolishes depolarization-induced responses in skinned fibres from both rat and toad, in a concentration- and time-dependent manner, without affecting the ability of the other stimuli to directly activate the RyR/Ca^{2+} release channels in the SR. This Ca^{2+}-dependent effect also appears to occur in intact muscle fibres. Ca^{2+}-induced uncoupling occurs at the triad junction when the [Ca^{2+}] is maintained for 10 s at just above the average level reached in the myoplasm during a tetanus (Pape, Jong, Chandler & Baylor, 1993; Westerblad, Duty & Allen, 1993). Ca^{2+}-dependent uncoupling also occurs at much lower Ca^{2+} levels if those levels are maintained for longer times in the myoplasm (Table 1B), although in intact fibres this effect may be antagonized to some extent by endogenous factors, such as the CANP inhibitor, calpastatin. As the [Ca^{2+}] near the RyRs during peak Ca^{2+} release is probably considerably higher than the myoplasmic average, uncoupling could be expected to occur in localized regions of a functioning muscle wherever the [Ca^{2+}] remains too high for too long. Uncoupling should normally be self-limiting in intact fibres with action potential stimulation, and this is consistent with our observation that gross stimulation of bundles of intact fibres caused only partial uncoupling in the fibres. Uncoupling may play an important regulatory role in muscle fibres by stopping Ca^{2+} release where it is excessive, limiting the pool of depolarization-releasable Ca^{2+}, whilst still leaving all the SR able to sequester Ca^{2+}. Such focal uncoupling may be present to some extent in all active muscles, and may be important in preventing more severe Ca^{2+}-dependent damage to the fibres. Ca^{2+}-dependent uncoupling is quite possibly responsible for 'low frequency fatigue', where prolonged activity of a muscle fibre causes impairment of depolarization-induced Ca^{2+} release, which persists for more than a day, even though ATP and creatine phosphate stores recover in an hour (Edwards, Hill, Jones & Merton, 1977; Westerblad *et al.* 1993). Significantly, the same Z-line aberrations and SR swelling described here after Ca^{2+} treatment sufficient to cause uncoupling (Fig. 7) are also observed in about 20% of sarcomeres in muscles from rats run to exhaustion (Belcastro, Parkhouse, Dobson & Gilchrist, 1988). Ca^{2+}-dependent uncoupling may also contribute to the weakness in dystrophic muscle, where resting [Ca^{2+}] is permanently elevated severalfold (Mongini *et al.* 1988; Williams, Head, Bakker & Stephenson, 1990) and Z-line aberrations and swollen SR are observed (Mrak, 1985).

Ca^{2+}-dependent structural changes

Electron microscopy of uncoupled fibres revealed Z-line alterations and SR vesiculation (Fig. 7). The same structural changes have been observed in chemically skinned frog fibres treated with Ca^{2+} (or 10-fold higher [Sr^{2+}]), occurring even at 0·5 μM Ca^{2+} with a 10 min exposure, with the onset apparently slowed in the presence of high concentrations of protease inhibitors (Duncan, 1987); prolonged activity and caffeine treatment also caused the same changes in intact mammalian fibres (Duncan & Smith, 1980). Importantly, the electron microscopy in this study also showed that many of the triads in uncoupled fibres were distorted, and in some cases severed (Fig. 7). Dissociation of the T-tubule and TC membranes has been observed previously in intact mammalian fibres subjected to a prolonged caffeine contracture (Yoshioka, Nemoto, Yamada & Yamashita-Goto, 1993). Furthermore, the severing of the triads and SR vesiculation (Fig. 7) is consistent with the formation of large SR 'blebs' ('sarcoballs'), with accessible RyR/Ca^{2+} release channels, when skinned fibres are treated with high [Ca^{2+}] and allowed to supercontract (Stein & Palade, 1988; Lewis, Dulhunty, Junankar & Stanhope, 1992). It may be argued that structural changes seen in the uncoupled fibres with the electron microscope were caused by the fixation procedure. However, the fact that there are obvious electron-microscopic differences between coupled and uncoupled fibres indicates that there must have been significant structural differences between the two types of fibres before fixation. Moreover, fixation artifacts should be much less of a problem in single skinned fibres than in bundles of intact fibres, given the lack of sarcolemma to impede diffusion of gluteraldehyde and the smaller diffusion distances.

The observed changes at the triad could be caused by either Ca^{2+}-dependent proteolysis or dissociation of key structural connections involved in maintaining the normal shape of the T-tubule. The integrity of the triadic junction appears to become impaired, however, without proteolysis of the RyR, triadin or α_1-subunit of the DHPR, indicating that the simple interaction between these three molecules is not sufficient to maintain the integrity of the triad. This conclusion is consistent with recent observations made on skeletal muscle from dispedic mice that lack the RyR, where the triadic junctions appear even tighter than in the skeletal muscle from normal mice (Takekura, Nishi, Noda, Takeshima & Franzini-Armstrong, 1995).

Mechanism of Ca^{2+}-induced uncoupling

The molecular basis of the Ca^{2+}-induced uncoupling described here is not clear. It does not appear to be caused by either perforation (Fig. 1) or generalized depolarization of the T-system (see Fig. 8 and Results). It seems that the

voltage sensors lose their ability to activate the Ca^{2+} release channels. This is apparently not caused by proteolysis of the RyR, triadin or α_1-subunit of the DHPR (Fig. 9), nor by protein phosphorylation or dephosphorylation. The lack of effect of agents interfering with the phosphorylation/dephosphorylation state of the triadic components involved in E–C coupling is unlikely to be due to restricted access of these agents to the triadic junction, because we could demonstrate that many molecules such as Ruthenium Red, ryanodine and ATP diffuse rather quickly into the triadic junction when applied in the solution bathing the skinned fibre (Lamb & Stephenson, 1990*a*, *b*, 1991). Thus, even though exogenous kinases and phosphatases may not interact with phosphorylation sites in the same manner as their endogenous counterparts (Hain, Nath, Mayrleitner, Fleischer & Schindler, 1994), we would still expect ATP and the small inhibitory agents to interact appropriately with the sites. Uncoupling may be the result of high myoplasmic [Ca^{2+}] rendering the voltage sensors, or some linking protein, dysfunctional. Alternatively, because the distinctive elongated shape of the T-tubule in normal muscle (Fig. 7*C* and *D*) might be vital for ensuring proper communication between DHPRs and RyRs (Fig. 7*H*), the observed distortion of the T-tubule may be responsible for uncoupling; certainly normal coupling must be abolished where a triad is actually severed (Fig. 7*G*). Even if the Ca^{2+}-dependent structural changes reported here in uncoupled fibres are not responsible for the uncoupling, they occur under similar conditions and, consequently, may be a good physical indicator of some degree of uncoupling.

In conclusion, the Ca^{2+}-dependent uncoupling phenomenon and associated structural changes reported here seem likely to be of fundamental importance in basic muscle function, exercise and disease.

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