Role of calcium in triggering rapid ultrastructural damage in muscle: a study with chemically skinned fibres

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Summary

Agents (A23187, caffeine) believed to raise $[Ca]_i$ in vertebrate cardiac and skeletal muscles cause rapid and characteristic subcellular damage *in vitro* and *in vivo*. By using saponin-skinned amphibian pectoris cutaneous muscle and Ca–EGTA-buffered solutions it is shown that low [Ca] consistently triggers the same rapid (2–20 min), ultrastructural damage.

Electron micrographs reveal a close similarity between the damaged intact and skinned preparations, namely loss of myofilament organization, specific Z-line damage, dissolution and hypercontraction bands, characteristic mitochondrial swelling and division. Where both actin and myosin filaments were lost, an underlying cytoskeletal network frequently remained, still attached to the Z-line framework.

Ca was effective in skinned preparations from 5×10^{-7} M to 8×10^{-6} M, within the concentration range experienced by a contracting muscle. Damage was [Ca]- and time-dependent and it is suggested that it is probably the active movement of Ca ions across key membrane sites that is critical in triggering damage of the myofilament apparatus. Strontium can substitute for Ca at higher concentrations. The action of saponin suggests that the chemically skinned cell is partially activated. Ca-triggering can be bypassed experimentally by membrane-active agents or by sulphydryl agents.

Ruthenium Red and trifluoperazine indirectly cause damage in the intact cell by raising $[Ca]_i$.

Studies with saponin-skinned cells and protease inhibitors show that changes in pH_i, loss of ATP, Ca-activated neutral protease, or release of lysosomal enzymes (cathepsins B, D, L or H), are not involved in characteristic *rapid* myofilament damage.

Key words: calcium, muscle damage, lysosomes, calcium-activated neutral protease.

Introduction

There is considerable current interest in the triggering and regulation of cellular breakdown and death in a variety of tissues including brain, liver, muscle and kidney as well as in insects and amphibians undergoing metamorphosis. A range of different factors have been implicated, e.g. lysosomal and non-lysosomal proteases, energy-dependent and energy-independent proteases, cellular supply of high-energy phosphates, thyroid hormones, impaired mitochondrial functioning, prostaglandins, phospholipase A_2 activity, endotoxaemia, vitamin E deficiency, oxygen radicals and trauma.

The suggestion that an alteration in intracellular Ca homeostasis was an important step in the cascade of events that culminate in cellular damage (Duncan, 1978; Schanne *et al.* 1979; Katz & Reuter, 1979; Farber, 1981; Trump *et al.* 1981; Ishiura, 1981; Nayler, 1983) provided a unifying hypothesis with important medical implications for many degradative conditions. However, in opposition to this hypothesis, the following conclusions have recently been advanced: "chemical-induced hepatic cell death is not caused by an increase in total cellular Ca^{2+} resulting from an influx of extracellular Ca^{2+}" (Fariss & Reed, 1985), and "We report here that free Ca in metabolically-poisoned myocytes is remarkably stable and that severe injury to the cell occurs before the free Ca concentration rises above 1 to 3×10^{-7} M, hence cell damage seems to be a cause, not a consequence of a rise in free Ca" (Cobbold & Bourne, 1984).

It is well known that subcellular damage in both skeletal and cardiac muscle can be produced rapidly,

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i.e. with a time-course of minutes; e.g. the ischaemic mammalian heart and skeletal muscle during malignant hyperthermia. These clinical events can be replicated experimentally not only *in vivo*, as in studies of the calcium paradox and ischaemia of cardiac muscle, but also *in vitro*, as in ultrastructural studies of both skeletal and cardiac muscle. Many of these examples of rapid subcellular damage are triggered by agents that appear to raise $[Ca]_i$ in the muscle (both cardiac and skeletal) or to promote Ca fluxes: (1) the Ca paradox of cardiac muscle (Grinwald & Nayler, 1981; Opie, 1985); (2) the actions of the divalent cation ionophore A23187 (Statham *et al.* 1976; Publicover *et al.* 1978) where ultrastructural damage has now been linked to rises in $[Ca]_i$ (Yoshimura *et al.* 1986); (3) the action of Dantrolene sodium in preventing the release of Ca from the sarcoplasmic reticulum (SR) and protecting against malignant hyperthermia (Blanck & Gruener, 1983); (4) the effect of the withdrawal of extracellular Ca in protecting against protein efflux from isolated mouse muscle when challenged with excessive contractile activity in anoxia (Jones *et al.* 1984).

The conclusion from these studies is that abrupt rises in intracellular Ca are able to trigger rapid and characteristic ultrastructural damage and that there is a common degradative mechanism of cardiac and skeletal muscle in both amphibians and mammals.

However, we have, as yet, no direct measurements of intracellular [Ca] during the process of damage in skeletal muscle and thus the first objective of the present study was to develop a chemically skinned skeletal muscle fibre preparation in which intracellular Ca could be accurately controlled. It is shown that a [Ca] of 10^{-6} M can consistently trigger rapid and characteristic damage.

Second, the skinned fibre preparation was used to assess the importance of proteolytic enzymes in cellular damage. One hypothesis concerns the involvement of Ca-activated neutral proteases (CANP) and, alternatively, the Ca-activated damage process might involve lysosomal labilization and breakdown. Thus, DNP is one agent that is also known to cause release of lysosomal hydrolases only *via* mechanisms that operate in the intact cell (Martini, 1959), whilst an increase in lysosomal catheptic enzyme activity has been reported in various muscular dystrophies (Pearson & Kar, 1979). It is reported here that a variety of protease inhibitors failed to prevent rapid damage in the skinned muscle cell. Finally, other mechanisms have been suggested concerning the triggering of damage in muscle cells, such as a fall in pH_i or a severe depletion of high-energy phosphates. The skinned-fibre preparation was used to adjust and control the intracellular environment and to test these hypotheses.

Materials and methods

Frogs

Rana pipiens were maintained in the laboratory at 23 °C and fed on blowfly larvae; *R. temporaria* were maintained at 9 °C. Pectoris cutaneous muscles were isolated and pinned to dental wax in ice-cold Ringer solution lacking Ca^{2+}.

Skinning

The preparations were transferred to one of the two following media of composition (mM): (1) sucrose (250), piperazine-*N*,*N*′-bis[2-ethanesulphonic acid] (4), magnesium acetate (4), EGTA (0·5), ATP (Na salt) (3), brought to pH 7·1 with NaOH and KOH so that $[K^+] = 2{\cdot}5$ and $[Na^+] = 10$. (2) Potassium glutamate (100), piperazine-*N*,*N*′-bis[2-ethanesulphonic acid] (10), magnesium acetate (4), EGTA (0·5), ATP (Na salt) (3), pH 7·1 with KOH. Temperature, 18 °C. Saponin was added to the skinning solution immediately before use and briskly stirred.

Exposure of skinned preparations to test solutions: skinned preparations were rinsed in ice-cold potassium glutamate solution (medium (2)) and then transferred to fresh potassium glutamate medium (18 °C). Samples of 1 M-$CaCl_2$ (AnalaR) or of $SrCl_2$ were added to produce calculated fixed free divalent cation concentrations in the EGTA buffer systems, using the appropriate p*K* values as given by Bjerrum *et al.* (1957). Protease inhibitors were dissolved in potassium glutamate medium immediately before use; chymostatin was solubilized in DMSO (dimethyl sulphoxide), pepstatin in ethanol.

Unskinned preparations

Cutaneous pectoris muscles were pinned out in Ringer's solution containing 1·8 mM-Ca and 5 $\mu g\,ml^{-1}$ A23187, which was initially solubilized in ethanol. Temperature, 20 °C; pH 7·1.

Electron microscopy

Fixation was by one of three methods: (1) Karnovsky fixative at 20 °C for 30 min, with transfer to fresh fixative for a further 3–5 h, followed by two washes with 0·1 M-sodium cacodylate, pH 7·2, each of 30 min. The tissue was then cut into smaller pieces and postfixed in OsO_4 for 2 h at room temperature. The pieces of muscle were then cut into small blocks for washing in cacodylate buffer (two changes). (2) Glutaraldehyde (3 %) with a protocol as in method (1). (3) OsO_4 (1 %) in venonal acetate buffer, pH 7·2 at 20 °C for 3–5 h, followed by two washes in 0·1 M-sodium cacodylate, pH 7·2, each of 30 min. All preparations were dehydrated through a graded ethanol series, embedded in Spurr's resin and sections were cut at 60–90 nm and stained with uranyl acetate and lead citrate.

Assessment of ultrastructural changes

At least three separate samples were taken from each muscle and the sections were scored blind on the electron microscope, by a colleague, for different types of damage; finally, the electron micrographs were again independently assessed.

Reagents

Sigma Chemical Co., Poole, Dorset: A23187, aprotinin (A1153 from bovine lung), pepstatin A, Ruthenium Red, caffeine. Peptide Institute Inc., Osaka, Japan: chymostatin, leupeptin.

Results

Exposure of intact fibres to A23187

This divalent cation ionophore caused characteristic and progressive damage in the intact cutaneous pectoris of *R. temporaria* over 10–30 min exposure. Fixation was with Karnovsky fixative. Initially, Z-line streaming was detected but, after some 20 min, breakdown of myofibrils occurred and some were contracted independently with Z-lines clearly out of register. After 20–30 min damage was widespread and of two types: (1) dissolution and breakdown of the myofilaments, or (2) a form of hypercontraction with very blurred Z-lines. Exposure of intact fibres to 5 mM or 8 mM-caffeine for 30 min also caused the same characteristic ultrastructural damage.

Preparation of skinned fibres

A total of 240 skinned cutaneous pectoris preparations were used in the present study and it was apparent that there were differences between *R. pipiens* and *R. temporaria* as well as marked variability between preparations (see below). Exposure to high saponin concentrations alone could promote ultrastructural damage and its effects were augmented by treatments that elevated intracellular [Ca]. Thus, the sucrose medium containing 0·5 mM-EGTA (rather than the potassium glutamate) proved more satisfactory and the normal concentrations of saponin used were: *R. pipiens* 37·5 $\mu g\,ml^{-1}$, 25 min; *R. temporaria* 60 $\mu g\,ml^{-1}$, 30 min. Electron micrographs (Fig. 1) of skinned preparations maintained for 90 min revealed a normal ultrastructure with clear myofilaments, sharp Z-lines in register, contracted mitochondria and with the SR not swollen.

Fixation of skinned-fibre preparations

Major changes resulting from damage could be readily detected with transmitted light and ×30 magnification in skinned preparations of the thin cutaneous pectoris muscle. Fixation with glutaraldehyde-based fixatives produced severe and visible damage within 60 s of application and hence subsequently all fixation was with OsO_4.

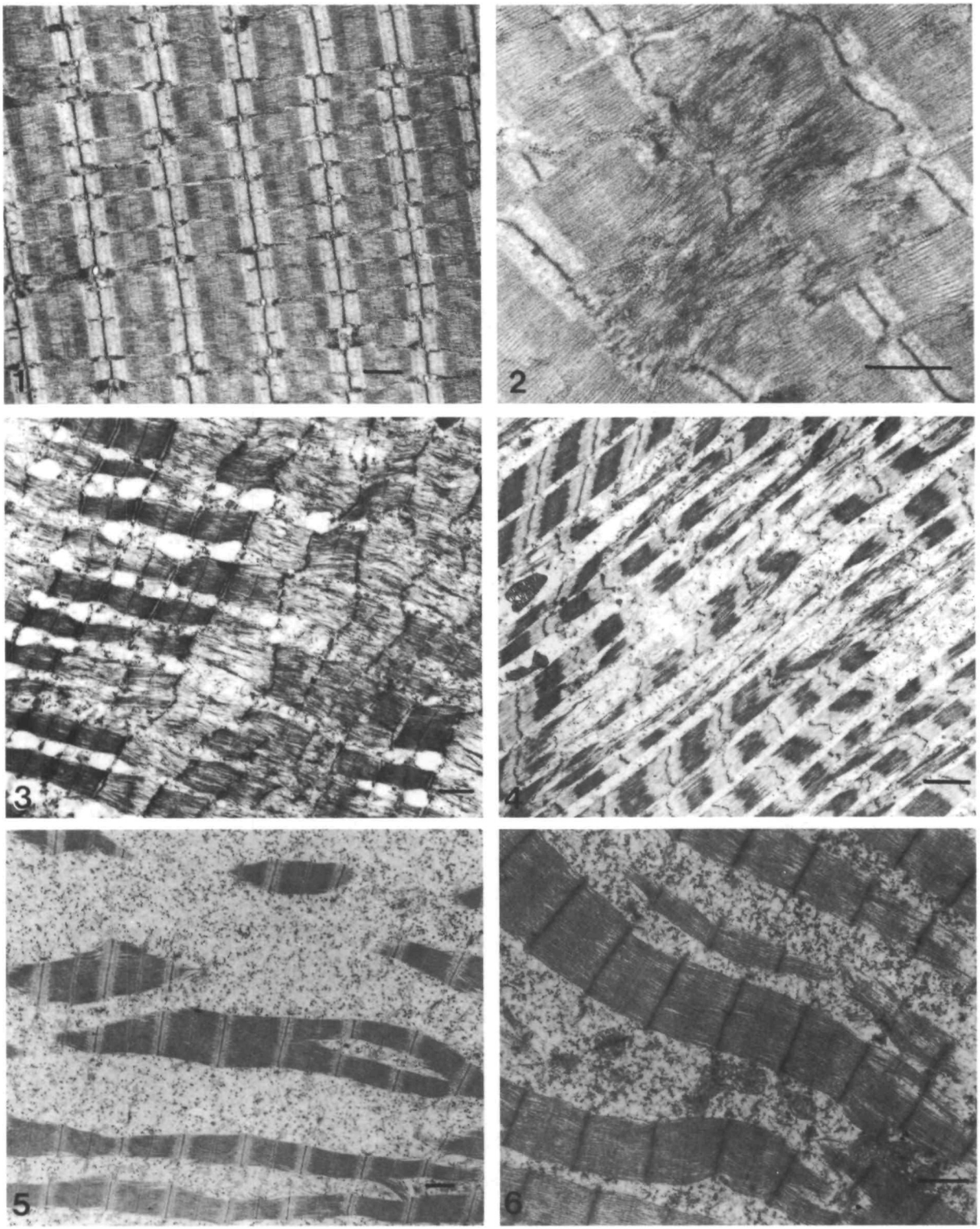
Action of Ca

Exposure of skinned fibres to potassium glutamate solutions in which Ca was buffered at 8×10^{-6} M consistently produced damage, which was severe within 10 min in *R. pipiens* preparations and within 25 min in *R. temporaria*, and was readily detectable at ×30 magnification. Initially, this damage was seen as an area of contraction and abnormal folding of the muscle sheet, which gradually increased in area. Later, the contents in individual cells could be seen concentrated at specific foci and surrounded by clear cytoplasm.

Electron micrographs of Ca-damaged, skinned fibres revealed characteristic patterns of damage and, as with the intact cutaneous pectoris preparations, the results were invariably clear-cut. Whilst areas of undamaged myofibrils were found in all but the severest examples of damage, muscles in which the damage mechanism had been activated were immediately recognizable. The different types of degradative effect are listed in approximate order of severity in Table 1. Although individual sarcomeres appear to respond differently to the damage process (see individual myofibrils in Figs 3 and 14), there was a distinction, as with the results obtained with intact fibres, between sections of the muscle where damage was accompanied by extreme contraction (categories 6 and 7, Table 1), where the myofilament apparatus was clearly damaged but complete destruction did not occur during the 30-min exposure period (Fig. 11), and where hypercontraction did not occur and complete loss of actin and myosin filaments was found (Figs 4, 5, 19). Fig. 14 illustrates

Table 1. *Ultrastructural damage recorded in skinned fibres of amphibian pectoris cutaneous muscles*

Category	Appearance	Figs
1	Small areas with loss of myofilament organization and Z-line damage; frequently no contraction	2, 3
2	Z-line sliding	2, 7
3	Separation of fibrils, sometimes accompanied by SR swelling	4, 9
	Sarcomeres relaxed	4, 5
	Sarcomeres contracted	6, 9
4	Specific loss of actin filaments	7, 8
5	Progressive damage to myofilaments; eventually only Z-lines and cytoskeletal elements remain	17, 18, 19, 21
6	Contraction of the fibril, with heavily blurred Z-line	10, 12, 13, 16
7	Hypercontraction bands with amorphous material; only dark Z-lines distinguishable	11, 12, 14
8	Swollen vesicles	9, 11, 14, 16
9	Mitochondria swollen, damaged, dividing	11, 14, 15, 16
10	Dissolution of cytoplasmic components	5, 6, 13, 14
11	Nuclear damage; margination and clumping of heterochromatin, invagination of the nuclear membrane	



Figs 1–6. Ca-triggered damage in saponin-skinned pectoris cutaneous muscle cells; Bar, 1 μm, except Fig. 4.

Fig. 1. Undamaged area; 8×10^{-6} M-Ca, 25 min; *R. temporaria*.

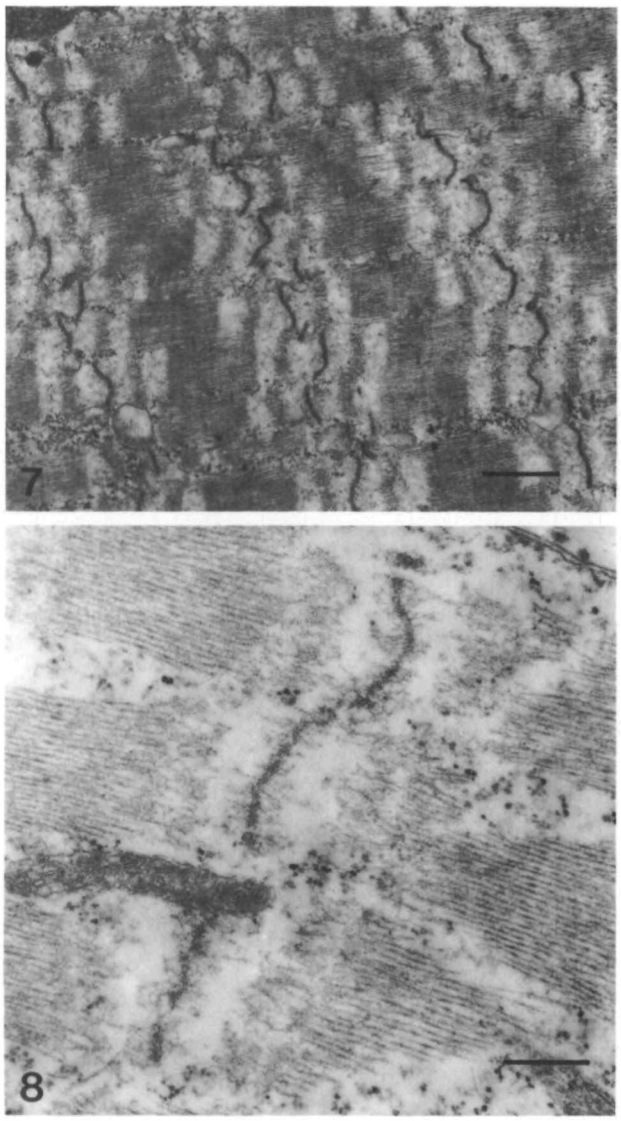
Fig. 2. Early damage with Z-line sliding and disintegration; 5×10^{-7} M-Ca, 25 min; *R. pipiens*.

Fig. 3. Widespread, early damage, swollen vesicles; 8×10^{-6} M-Ca, 40 min; *R. temporaria*.

Fig. 4. Marked separation of relaxed myofibrils with dissolution of parts of the myofilament apparatus; 5×10^{-5} M-Ca, 28 min; *R. temporaria*. Bar, 2 μm.

Fig. 5. Relaxed, broken myofibrils with normal myofilament apparatus but surrounded by the remnants of the other cytoplasmic components; 8×10^{-6} M-Ca plus 14 μg ml^{-1} pepstatin, 25 min; *R. pipiens*.

Fig. 6. As Fig. 5, but with myofibrils contracted; 5×10^{-7} M-Ca, 25 min; *R. pipiens*.



Figs 7, 8. Examples of saponin-skinned pectoris cutaneous fibres where the damage is concentrated on the actin filaments, and is accompanied by marked Z-line sliding and by the extension of the sarcomeres. *R. pipiens*. Fig. 7, 8×10^{-6} M-Ca plus 50 μg ml^{-1} leupeptin, 25 min. Bar, 1 μm. Fig. 8, 5×10^{-4} M-Sr, 25 min.

hypercontraction and complete dissolution in the same fibril. All these different types of damage were found in both species, but substantial differences could be found in different areas of the same muscle sheet; thus different categories of damage (together with apparently undamaged sections) could be found in one preparation and in adjacent sections of the same myofibril. Contraction-type damage (categories 6 and 7) appeared to be associated with the contractions observed with transmitted light. It is evident that the patterns of damage correspond with those caused by A23187 in intact fibres (Statham *et al.* 1976) whilst the hypercontraction bands (category 7; Figs 11, 12, 14), in particular, also compare closely to those found in amphibian cardiac muscle (Rudge & Duncan, 1984*a*). We conclude that the rapid Ca-triggered damage of skinned muscle fibres is identical with that found in intact fibres.

A series of experiments with both species showed that the effect of Ca was both time- and concentration-dependent. With the *R. pipiens* preparations, ultrastructural changes were not detected with [Ca] = 8×10^{-8} M and developed only slowly in some preparations at [Ca] = 10^{-7} M. In most preparations damage was detectable by eye in less than 10 min at 5×10^{-7} M-Ca and in all preparations in less than 5 min at 10^{-6} M-Ca, and in less than 2 min at 8×10^{-6} M. The *R. temporaria* preparation (summer frogs, skinned with 60 μg ml^{-1} saponin), however, was consistently less sensitive and damage was not evident in the electron micrographs after 10 min exposure to 8×10^{-6} M-Ca, but usually began after 18–20 min. Twenty to 25 min were required with 10^{-6} M and usually 30 min at 5×10^{-7} M. It is noteworthy that at low [Ca] ($<10^{-6}$ M) the damage seen was more commonly in categories 1–5 but, nevertheless, contraction-type damage (categories 6 and 7) was also recorded and thus the same damage response was found across the Ca concentration range.

Cytoskeletal elements of the amphibian sarcomere

Some preparations showing category 5 damage (Table 1), where much of the contractile apparatus had been lost, revealed apparent thin, cytoskeletal, longitudinal elements linking the Z-lines, which appeared to be more resistant to the rapid degradative process than the myofilament apparatus. This characteristic effect was found in both intact (Fig. 20) and skinned (Figs 17–19, 21) amphibian muscle cells in which the degradative mechanism had been switched on in a variety of different ways. The appearance of these muscle cells closely resembled those of frog preparations in which the actin and myosin had been extracted with 0·6 M-KI, so revealing the connectin (or titin/nebulin) network (Wang, 1982). This parallel elastic component (Maruyama *et al.* 1985) appears therefore, to be more resistant to the rapid degradative process than are the bulk myofibrillar proteins.

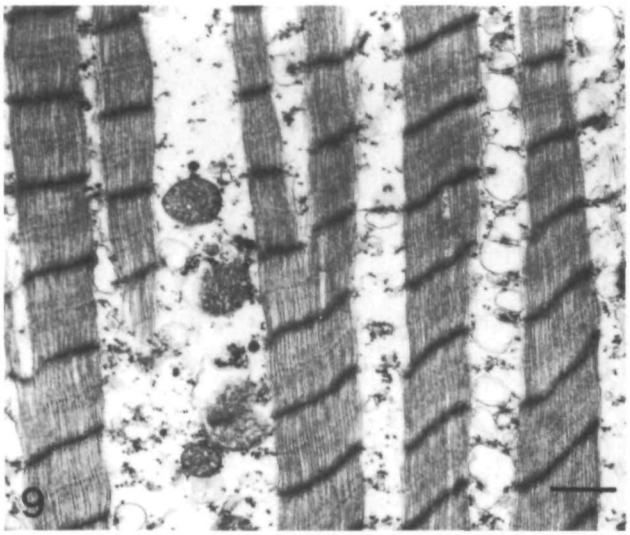
Effect of saponin

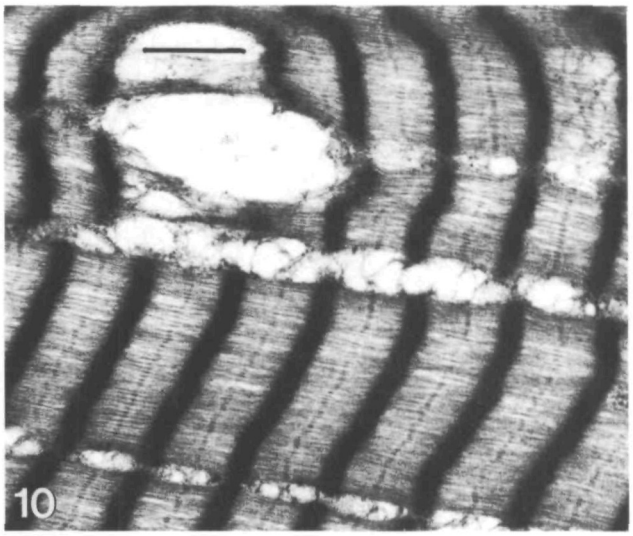
The sensitivity of some preparations to Ca and the variability between different muscles suggested that saponin might directly activate the damage process as well as permeabilize the sarcolemma. *R. temporaria* muscles were skinned for 30 min in a range of saponin concentrations, namely 40, 50, 55, 60, 75, 100, 120 and 150 μg ml^{-1}, and Ca-induced damage was found to develop more rapidly with much more severe effects at

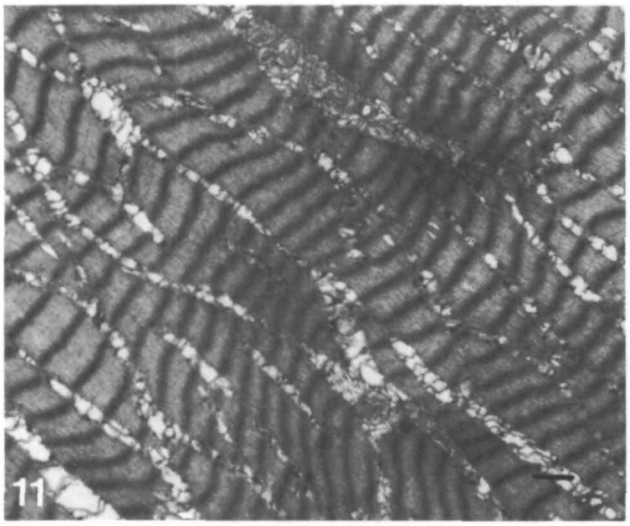
the higher concentrations. At 150 μg ml^{-1}, 8×10^{-6} M-Ca damage could be detected in 90–120 s by transmitted light and, after 9 min, electron micrographs revealed almost complete dissolution of wide areas; a very much more dramatic response than with skinning with 60 μg ml^{-1} saponin in this species. In one series of experiments, 150 μg ml^{-1} saponin directly caused damage in *R. temporaria*.

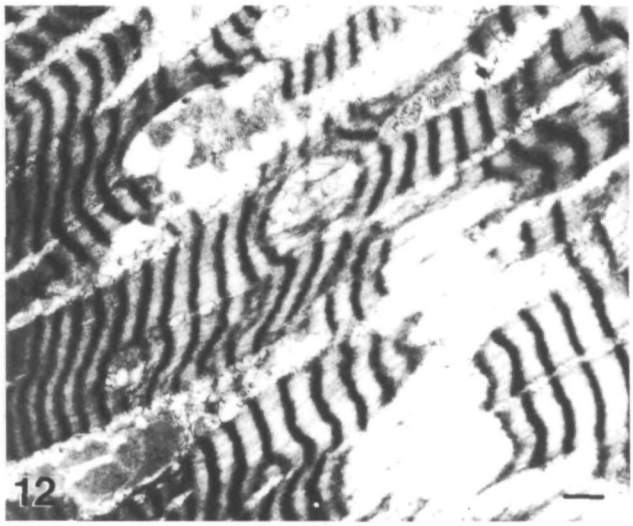
Action of strontium

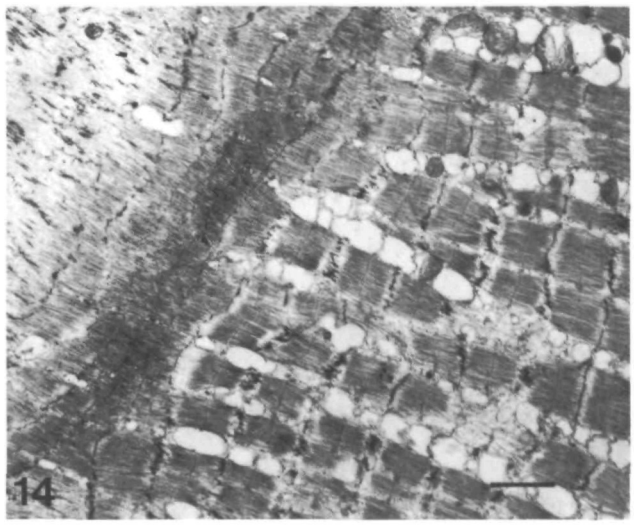
Sr–EGTA buffered solutions also triggered identical patterns of damage in *R. pipiens* skinned preparations











(Fig. 8), although substantially higher concentrations of free Sr were required. At 3×10^{-6} M-Sr (25 min) individual damaged sarcomeres were detected together with some Z-line sliding; at 10^{-5} M-Sr (after 45 min) the SR was additionally swollen in some areas; at 5×10^{-5} M-Sr (25 min) substantial parts of the muscle remained undamaged but characteristic areas of myofilament breakdown were evident (category 1, Table 1). Finally, with 5×10^{-4} M-Sr (25 min), clearly evident contractions and folding were seen under transmitted light and electron micrographs showed a full range of severe damage, including areas of extensive hypercontraction and mitochondrial multiplication (categories 6–9).

Ruthenium Red

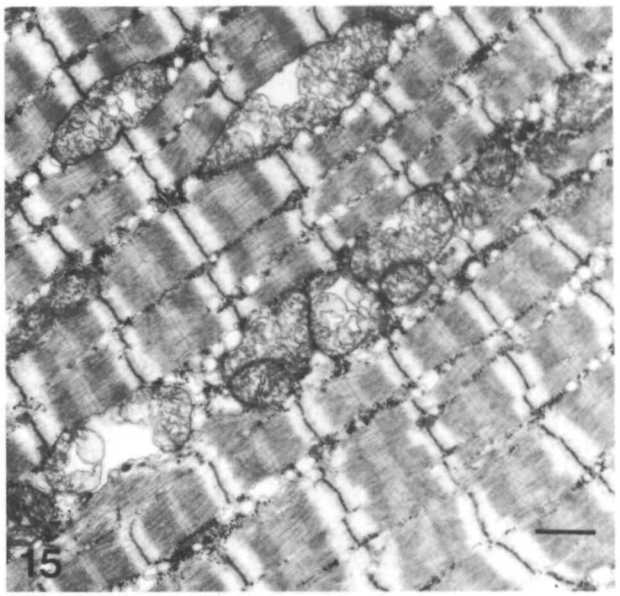
Exposure of the *R. pipiens* skinned muscle to the Ca-ATPase inhibitor Ruthenium Red (2, 10 or 15 μM, 20 min) in the potassium glutamate–EGTA medium, with no added Ca, did not cause the characteristic damage.

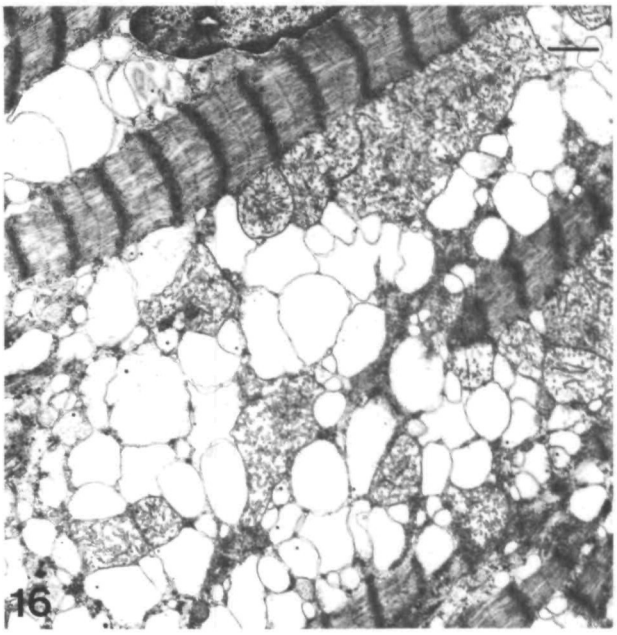
Effect of pH

The skinned preparations were exposed to 8×10^{-6} M-Ca with pH buffered at 7·3, 7·5 or 7·6 (with the dissociation constant of the Ca–EGTA complex recalculated), but no protective effect was noted.

Action of protease inhibitors

The *R. pipiens* skinned preparations were used for a range of experiments in which various protease inhibitors, both singly and in combination, were tested for possible effects in the reduction of damage. Table 2 summarizes the various protocols used and in the first series (experiments 1–6) the preparations were skinned for 30 min with saponin at 40 μg ml^{-1}, whereas in experiments 7 and 8 this procedure was increased to 40 min at 45 μg ml^{-1} so as to increase the severity of skinning. In both series (experiments 1–8) the inhibitor was included in the skinning solution and in the





Figs 15, 16. Ca-triggered changes in the mitochondria in saponin-skinned pectoris cutaneous fibres of *R. temporaria*. Bar, 1 μm.

Fig. 15. Myofibrils largely unaffected and not contracted but mitochondria swollen, damaged and dividing; 8×10^{-6} M-Ca, 40 min.

Fig. 16. Swollen, dividing mitochondria with greatly swollen vesicles, which may represent SR or, more probably in this example, the remnants of mitochondria; contracted myofibrils with blurred Z-lines; 8×10^{-6} M-Ca, 30 min.

Figs 9–14. Examples of extreme, Ca-triggered damage in saponin-skinned pectoris cutaneous fibres. Bar, 1 μm, except Fig. 14.

Fig. 9. Z-line blurring, contracted fibrils, dissolution of cellular contents with separation of fibrils, swollen vesicles (probably SR); 10^{-6} M-Ca, 15 min; *R. temporaria*.

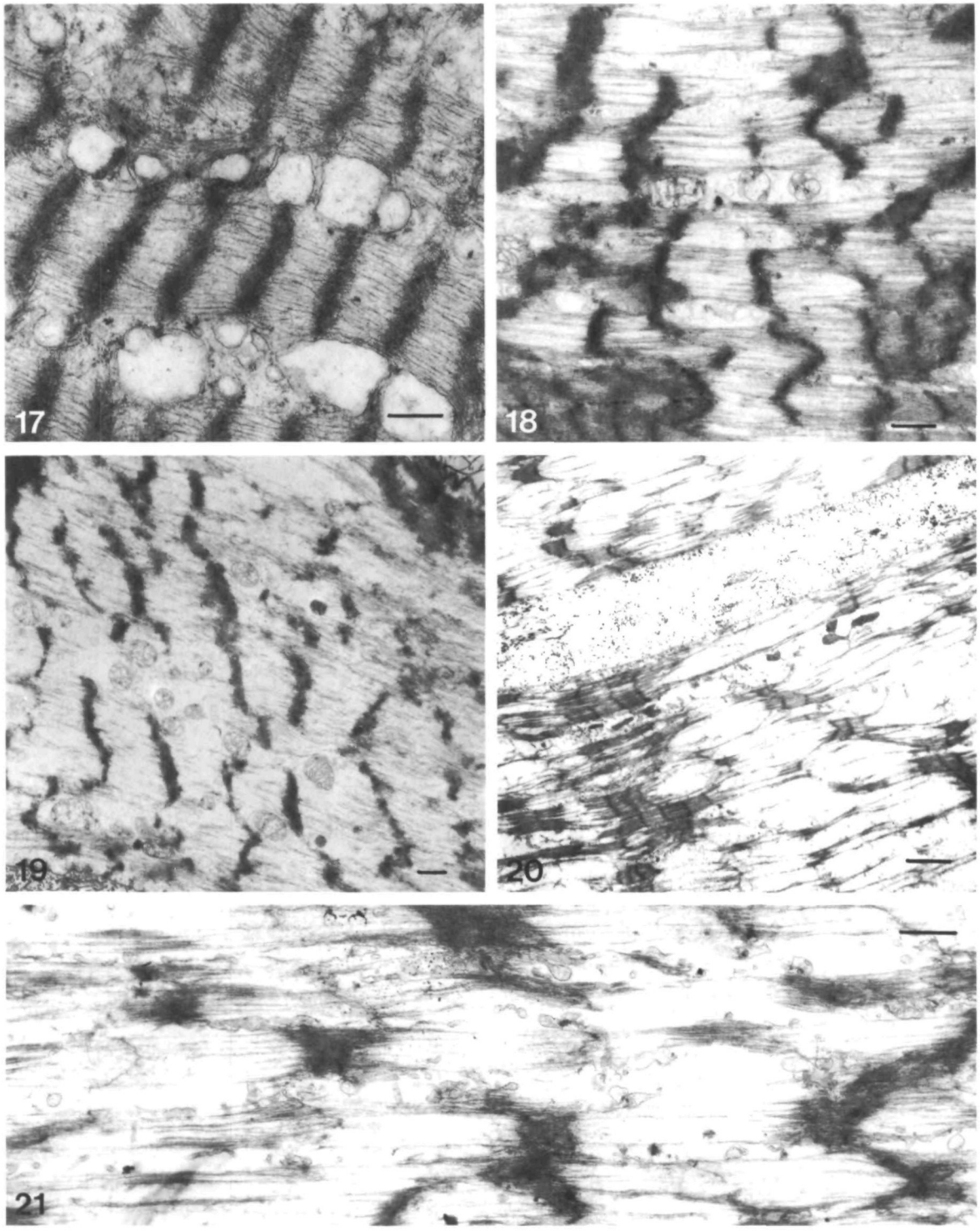
Fig. 10. Very marked Z-line blurring, but otherwise similar to Fig. 9. 5×10^{-5} M-Ca, 28 min, *R. temporaria*.

Fig. 11. Extreme contraction and hypercontraction bands, many swollen vesicles and dividing mitochondria; 8×10^{-6} M-Ca plus 14 μg ml^{-1} pepstatin, 25 min; *R. pipiens*.

Fig. 12. Z-line blurring and hypercontraction with destruction of myofilament apparatus at high [Ca] (5×10^{-5} M), 28 min, *R. temporaria*.

Fig. 13. Z-line blurring and contracted myofibrils with sections in which the myofilaments are destroyed, leaving cytoskeletal elements; swollen mitochondria; 8×10^{-6} M-Ca plus 14 μg ml^{-1} pepstatin, 25 min; *R. pipiens*.

Fig. 14. Central hypercontraction band with progressive dissolution of the myofilaments on one side whilst on the other are markedly swollen vesicles and damaged mitochondria; the myofibrils show early damage in this latter area but the Z-lines show sliding; 8×10^{-6} M-Ca, 30 min; *R. temporaria*. Bar, 2 μm.



Figs 17–21. Different examples of Ca-triggered damage to the myofilament apparatus in which the Z-lines are blurred but largely intact and where they remain held together by longitudinal cytoskeletal elements. Fig. 20 shows intact cells, other figures are of saponin-skinned cells.

Fig. 17. Myofibrils remain in contracted position with Z-lines blurred; 3×10^{-7} M-Ca, 30 min; *R. temporaria*. Bar, 0·5 μm.

Fig. 18. Myofibrils mostly remain in contracted position with areas of hypercontraction; stretched cytoskeletal elements show more clearly than in Fig. 17; 8×10^{-6} M-Ca, 30 min; *R. pipiens*. Bar, 1 μm.

Fig. 19. 8×10^{-6} M-Ca, 30 min; *R. pipiens*. Bar, 1 μm.

Fig. 20. Intact cell, cytoskeletal elements much stretched by small areas of contracted sarcomeres; 10 mM-caffeine, 30 min; *R. temporaria*. Bar, 2 μm.

Fig. 21. Similar effect to Fig. 20 but in a skinned cell; 8×10^{-6} M-Ca, 30 min; *R. temporaria*. Bar, 1 μm.

Table 2. *Protease inhibitors tested that did not prevent rapid ultrastructural damage in skinned cutaneous pectoris preparations of* R. pipiens

Expt no.	Skinning: saponin concn/time ($\mu g\,ml^{-1}$/min)	Inhibitor(s)	Concn ($\mu g\,ml^{-1}$)	Enzymes of degradative pathways inhibited	Mean time for visible damage (min)
1a	40/25	Aprotinin	2	Proteases	2
1b	40/25	Aprotinin	13		2
2a	40/25	Leupeptin	30	CANP; cathepsins B, H and L	2
2b	40/25	Leupeptin	35		5
2c	40/25	Leupeptin	50		6
3	40/25	Chymostatin	40	Chymotrypsin; cathepsins B and D; non-lysosomal	14
4a	40/25	Pepstatin	2·5	Cathepsin D	7
4b	40/25	Pepstatin	14		5
5	40/25	Pepstatin + IAA	$2{\cdot}5+10^{-3}$ M	Cathepsins B, D, L, H and CANP	15
6	40/25	Pepstatin + leupeptin	2·5+50	Cathepsins B, D, L, H + CANP	15
7	45/40	Pepstatin + leupeptin	14+30	Cathepsins B, D, L, H + CANP	5
8	45/40	Pepstatin	14	Cathepsin D	7
9	40/20	pCMB/EGTA	10^{-3} M	Cathepsins B, H, L + CANP	0·5
10	40/25	None		Control	3

After skinning, all preparations (except expt 9) were tested with buffered 8×10^{-6} M-Ca for 25 min before fixation. Expt 9 has 0 Ca and was fixed after 5 min. All preparations showed severe ultrastructural damage, including hypercontraction in expts 1–8. Inhibitors were included during skinning, rinsing and exposure to Ca. For details of the action and concentrations of enzyme inhibitors see Barrett & McDonald (1980), Umezawa (1976). For details of lysosomal and non-lysosomal pathways in hepatocytes see Grinde & Seglen (1980). All experiments were done in triplicate. IAA, iodoacetic acid.

subsequent rinse in the potassium glutamate medium and during the test exposure (25 min) to 8×10^{-6} M-Ca, a concentration that consistently caused damage in control experiments. In experiment 9, the muscles were skinned in the absence of inhibitor and were then transferred to 10^{-3} M-pCMB in 0·5 mM-EGTA (i.e. zero [Ca]) and the preparation was fixed after only 5 min. The inhibitors were chosen to determine whether CANP or individual lysosomal cathepsins were implicated in rapid subcellular damage; combinations of inhibitors were selected to protect against possible lysosomal breakdown and the release of a range of acidic proteases.

In experiments 1–8, transfer to Ca caused rapid, severe and extensive damage, which was usually detectable by transmitted light within 10 min (Table 2). There were differences between preparations or between experiments in the time before changes were clearly visible, but no consistent protection was found in any of the experiments and electron micrographs prepared at the end of the experiment invariably revealed hypercontraction bands in addition to the other types of damage (Figs 5, 7, 11, 13). pCMB, an -SH blocker and hence an inhibitor of cysteine proteases, itself caused visible damage within 30 s in the absence of Ca, and electron micrographs of the preparation after only 5 min revealed widespread damage, both actin loss and disintegration of myofibrils (categories 1, 4 and 5) and also contraction damage (category 6). Thus, this damaged caused with zero [Ca] appears to be identical with the Ca-triggered damage.

Action of trifluoperazine (TFP)

One possibility concerning the action of Ca in triggering subcellular damage is that the ubiquitous calmodulin may be involved at one step in the degradative sequence and hence the anti-calmodulin drug TFP was tested on the saponin-skinned preparation. Three separate series of experiments with different batches of frogs (*R. pipiens* and *R. temporaria*) with appropriate protocols for skinning (*R. temporaria* = 60 $\mu g\,ml^{-1}$ saponin, 30 min; *R. pipiens* = 37·5 $\mu g\,ml^{-1}$ saponin, 30 min) and with pre-exposure to TFP (10^{-5} M or 3×10^{-5} M) for 15 or 20 min were used. Concentrations of TFP were chosen to replicate trials with TFP in intact muscle preparations (Duncan & Rudge, 1984). However, none of these measures provided any protection against the addition of 8×10^{-6} M-Ca in the presence of TFP; both the time-course of the visual damage and the ultrastructural appearance (damage categories

1–8) after 30 min exposure were similar to those seen in the matched controls.

Discussion

Saponin-skinned muscles

The saponin-skinned, cutaneous pectoris preparation (Endo & Iino, 1980) proved to be a satisfactory system for determining the effect of Ca in initiating cellular damage, and in this thin sheet of muscle extreme damage can be detected visually by transmitted light and it has always been subsequently confirmed by electron microscopy. High concentrations of saponin appeared to be able to promote damage directly, suggesting that the system causing damage is membrane-bound and sensitive to membrane disruption. The action of glutaraldehyde-based fixatives in causing visible damage in skinned preparations almost immediately may also be *via* a membrane perturbation, as in its action on the Ca-ATPase of the SR (Thomas & Hidalgo, 1978).

The *R. pipiens* and *R. temporaria* preparations appeared to be genuinely different in their sensitivity to saponin and this observation may reflect a difference in the molecular structure of their muscle membranes. Furthermore, small, but consistent, differences in sensitivity between winter and summer frogs were also found. These poikilotherms have different optimal environmental temperatures and *R. pipiens* had been maintained at 23 °C, whereas *R. temporaria* was kept at 9 °C, so that homeoviscous adaptation may well produce differences in the lipid composition of their membranes.

The role of Ca

The results show unequivocally that intracellular Ca can trigger rapid damage in saponin-skinned amphibian skeletal muscle. The time taken to produce this damage is concentration-dependent and in both *R. temporaria* and *R. pipiens* the effective concentration range is probably about 10^{-7} to 10^{-5} M-Ca, i.e. approximately the same range as the changes in concentration experienced in the cytosol during excitation–contraction–relaxation. As emphasized above, the saponin-skinned muscle may be partially activated; nevertheless the results do show that rapid damage can be triggered at very low cytosolic [Ca]. During contraction in the normal muscle cell the release of Ca will produce local, high levels of $[Ca]_i$, but these will be transient and the Ca^{2+} will be rapidly shuttled between the SR and the troponin C and parvalbumins.

The steady-state level of $[Ca]_i$ in the muscle cell at rest (approximately 10^{-7} M) will be the result of passive influx and active efflux across the sarcolemma and of uptake by the SR (the low affinity mitochondrial Ca uptake is probably not implicated). At normal resting $[Ca]_i$, in the closed system of the muscle cell, net active movement of Ca will be low, [Ca], being close to the lower sensitivity of the pumps for active transport. However, in the skinned muscle cell, immersed in a large volume of Ca–EGTA buffer, even at low free [Ca], active transport will continue as the pumps attempt the impossible task of reducing the free [Ca]. We conclude that it is not the concentration of Ca that is important in triggering myofilament damage, but rather the duration and magnitude of active movement of Ca across key membranes. In muscles where the steady-state level of $[Ca]_i$ is elevated, perhaps quite modestly (e.g. in dystrophic muscle), damage may be triggered by the continued active transport of Ca over a period of many minutes.

Damage can be detected visually after 90 s in skinned preparations at 8×10^{-6} M-Ca and this rapidity is consistent with results obtained with rat hearts in Ca-paradox experiments. Return of extracellular Ca after Ca-free perfusion caused the typical loss of cellular proteins but, significantly, if the hearts were repleted with Ca for only 30 s and then returned to Ca-free perfusion, the resultant protein loss was the same (Hunt & Willis, 1985).

The role of Sr

The action of Sr–EGTA in triggering damage, albeit at higher concentrations (effective range 3×10^{-6} M to 5×10^{-4} M), is in accord with observations on intact preparations, particularly amphibian and mammalian hearts where Sr can substitute for Ca in triggering damage (DeLeiris & Feuvray, 1973; Goshima *et al.* 1978) that was ultrastructurally identical (Rudge, 1983).

Ultrastructural damage in intact and skinned cutaneous pectoris

The damage produced in the skinned cells is summarized in Table 1 and can be classified into two different types of response. First, loss of actin filaments, breaking and disintegration of the myofibrils, and myofilaments separating from the Z-line (categories 1, 3 and 4); this type of damage is characterized by sharp and clearly defined Z-lines. Second, categories 6 and 7 appear to accompany contraction of the myofilament apparatus and concomitantly the Z-line widens and becomes blurred (see Figs 10, 11, 12, 17), particularly in areas of hypercontraction. In areas of hypercontraction, the myofilament apparatus appears to be protected from disassembly and thus a sarcomere may respond with one of the two types of damage. The patterns of damage found in skinned cells closely resemble those found in intact cutaneous pectoris treated with A23187 (Statham *et al.* 1976). Hypercontraction bands and dramatic mitochondrial subdivision

are also characteristically found in damaged amphibian and mammalian cardiac muscle and in mammalian skeletal muscle in which $[Ca]_i$ has been raised (Publicover *et al.* 1978; Duncan & Smith, 1980; Duncan *et al.* 1980; Rudge & Duncan, 1984*a*,*b*).

Thus, we conclude (1) that the characteristic and common patterns of rapid damage (both *in vivo* and *in vitro*) in vertebrate skeletal and cardiac muscle reflect a common underlying sequence of cellular events that culminate in specific ultrastructural degradation of the myofilaments; (2) that the damage caused by A23187 in intact amphibian (Statham *et al.* 1976) and mammalian (Publicover *et al.* 1978) skeletal and cardiac (Rudge & Duncan, 1980, 1984*a*) muscles are indeed triggered by rises in $[Ca]_i$ and in Ca fluxes in the cell.

Ruthenium Red and the regulation of $[Ca]_i$

Ruthenium Red causes rapid and characteristic damage in intact cardiac and skeletal muscles (Duncan *et al.* 1980; Rudge & Duncan, 1980, 1984*a*), but was ineffective in the skinned preparation. We conclude that in the intact cell Ruthenium Red inhibits the Ca-ATPases of the Ca pumps of the sarcolemma, SR or mitochondria and thereby raises $[Ca]_i$ and so, in turn, initiates myofilament damage.

Ultrastructural damage in the absence of Ca

The experiments with pCMB in the presence of EGTA, which cause the same characteristic myofilament damage (presumably *via* an action on key -SH groups), demonstrate that an alteration in $[Ca]_i$ is not the only means whereby the underlying cellular mechanism can be initiated.

Role of calmodulin

Previous *in vitro* experiments with trifluoperazine (TFP) and intact skeletal and cardiac muscles showed that not only did this anti-calmodulin agent fail to protect against the characteristic ultrastructural damage produced by a variety of different means, but that exposure to 10^{-5} M-TFP alone produced identical patterns of damage (Duncan & Rudge, 1984). Clearly, calmodulin could act to modify either the systems regulating $[Ca]_i$ or the Ca-triggered damage process itself. Since TFP does not protect against Ca-induced damage in the skinned cell in the present experiments, we conclude that calmodulin does not have an overriding control of the rapid damage process that proceeds in the presence of TFP. Presumably, TFP *in vitro* inhibits the calmodulin-activation of the Ca-ATPase of the sarcolemma thereby raising $[Ca]_i$.

Mechanisms of cellular damage

Previously, muscle damage has been measured either qualitatively by histological or ultrastructural assessment, or quantitatively by assaying the release of cytoplasmic proteins, a method that reflects only the lack of integrity of the sarcolemma. We do not yet know the sequence of events in cellular damage, but recent studies (Duncan & Jackson, 1987) have shown that at least two independent pathways are involved: (1) PLA_2 (phospholipase A_2) activation and subsequent lipoxygenase activity culminating in sarcolemma damage; and (2) a separate system that produces the characteristic destruction of the myofilament apparatus.

A number of theories are extant concerning the mechanisms underlying cellular damage but the present studies with skinned fibres show that some of these are not implicated in the specific and rapid damage of the myofilaments and these are discussed below.

Intracellular pH. pH_i falls markedly in malignant hyperthermia and in cardiac ischaemia, with irreversible cell injury occurring at pH 6·3 (Gebert *et al.* 1971; Ichihara *et al.* 1979; Schaper & Knoll, 1979). It is proposed that protons are emitted in exchange for Ca uptake by the SR and mitochondria, that acidification also results from ATP breakdown (Grinwald & Nayler, 1981) and that the resulting acidosis injures cells and causes membrane damage (Ganote, 1983). However, 'intracellular' pH is firmly buffered in the skinned cells and no protective effect was found even when the pH was raised to 7·3, 7·5 or 7·6, supporting the results of studies with intact muscle cells showing that rapid damage is triggered even when the mitochondria are uncoupled (Duncan *et al.* 1980).

ATP deletion. During rapid damage, in both skeletal (in malignant hyperthermia) and cardiac (in ischaemia; Ganote, 1983) muscles, the levels of high-energy phosphates fall dramatically. It is proposed that this process directly causes damage and rigor complexes and also permits alterations in membrane permeability (Jennings & Reimer, 1981) or changes in electrolyte levels (Nayler, 1981) to occur.

However, rapid damage in less than 120 s was triggered by Ca in the skinned muscle with 3 mM-ATP and it is unlikely that high-energy phosphate depletion is the direct cause of myofilament damage.

Calcium-activated neutral protease. Since CANP was localized in skeletal and cardiac muscles, considerable work has been directed towards elucidating its possible role in myofibrillar degradation. CANP is also activated by Sr and its properties and role in degradation in muscles have been summarized by Imahori (1982) and Ishiura *et al.* (1982).

However, it is inhibited by leupeptin, iodoacetate, pCMB and EGTA, and none of these agents prevented rapid Ca-activated damage or pCMB-induced damage in skinned muscle preparations (Table 2), confirming previous conclusions from studies with intact skeletal (Duncan *et al.* 1979) and cardiac (Rudge & Duncan, 1984*a*) muscle that CANP is not the major element in rapid cellular damage.

CANP and the cytoskeletal lattice. Titin and nebulin (or connectin) form an elastic network within muscle cells and both are rapidly cleaved by endogenous and exogenously added proteases, especially CANP (Wang, 1982). If the cytoskeletal remnants left after rapid cellular damage (Figs 17–21) are indeed a resistant titin–nebulin network, these observations provide confirmatory evidence that CANP is not involved. It is thus also unlikely that lysosomal proteases (see below) are responsible for rapid myofilament degradation and it seems that this damage, stimulated by pCMB, may be a preferential attack on -SH-linked subunits, since titin and nebulin are believed to be constructed from (non-disulphide) covalently linked subunits (Wang, 1982).

Lysosomal breakdown. Since lysosomal cathepsins B and D degrade myofibrillar proteins (Schwartz & Bird, 1977; Matsumoto *et al.* 1983), a number of workers have suggested that cellular degradation is produced by the release of these enzymes (Kennett & Weglicki, 1978; Wildenthal & Crie, 1980; Katanuma & Noda, 1982), and the fall in pH_i (see above) would permit their activity once free in the cytosol. Lysosomal enzyme activity has been reported to be elevated in various muscular dystrophies (Pearson & Kar, 1979; Noda *et al.* 1981) and in ischaemic heart (Wildenthal, 1978).

Studies with protease inhibitors on intact muscle are bedevilled by uncertainty as to whether they can penetrate the sarcolemma, but none of the inhibitors tested on skinned muscles in this study prevented rapid myofilament damage, nor were any differences from normal, characteristic damage noted, even when the skinning procedure was extended (Expts 7 and 8, Table 2). Cathepsin D is inhibited by pepstatin and chymostatins; cathepsins B, H and L are inhibited by thiol-blocking agents, iodoacetate and leupeptin. The mixtures of inhibitors (Expts 5–7, Table 2) were chosen to protect against the release of mixed lysosomal proteases. It is also unlikely that acidic proteases could achieve complete degradation in 90 s at pH 7·5, and it is evident that lyososmal cathepsins are not involved in rapid myofilament degradation in amphibian skeletal muscle.

To conclude, low levels of Ca trigger rapid and characteristic myofilament damage in skinned skeletal muscle cells. Neither a fall in pH_i, a fall in high-energy phosphate reserves, activation of CANP nor the release of lysosomal enzymes is directly involved, although all may have a peripheral role *in vivo*.

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