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The Action of Caffeine in Promoting Ultrastructural Damage in Frog Skeletal Muscle Fibres

Evidence for the Involvement of the Calcium-Induced Release of Calcium from the Sarcoplasmic Reticulum

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Summary. 1. Caffeine at concentrations above 5 mM was shown to cause rapidly extensive ultrastructural damage to the myofibrils of frog skeletal muscle.

2. The effect was promoted at lower temperatures, whereas the myofibrils were protected by prior exposure to procaine.

3. It is argued that caffeine causes a Ca^{2+}-induced release of Ca^{2+} (the CROC) from the S.R. and that the consequent rise in $[Ca^{2+}]_i$ promotes the ultrastructural damage observed.

4. Myofibril degradation is also produced by treatment of the muscle with the divalent cation ionophore A23187; this effect is not protected by either procaine or Dantrolene sodium.

5. It is suggested that A23187 causes the release of Ca^{2+} from the S.R. by a mechanism that differs from both excitation and the CROC; the resultant rise in $[Ca^{2+}]_i$ again causes myofibril degradation.

6. The ways in which a marked rise in $[Ca^{2+}]_i$ could cause muscle damage and the possible relevance of these findings to the sequence of events in the development of myopathies of human skeletal muscle are discussed.

Key words: Caffeine – Muscle – Muscular dystrophy – Calcium – A23187.

Introduction

We have shown previously that treatment of frog (Statham et al., 1976) or mouse (Publicover et al., 1978) skeletal muscle with the divalent cation ionophore A23187 causes major ultrastructural damage, with dissolution of the myofilaments. The evidence suggests that the ionophore acts primarily at the sarcoplasmic reticulum (S.R.), causing the release of stored Ca^{2+}, and that the consequent rise in $[Ca^{2+}]_i$ promotes (either directly or indirectly) the activity of proteases which are responsible, in turn, for the rapid myofilament degradation.

We have suggested (Publicover et al., 1978; Duncan, 1978) that this action of A23187 may serve to clarify the sequence of events in certain myopathies and in examples of experimentally-induced muscle damage. For example, the suggested sequence in Duchenne muscular dystrophy is as follows: the initial genetic lesion results in a higher net influx of Ca^{2+} at the muscle plasma membrane (perhaps coupled with a reduced Ca^{2+}-uptake at the S.R), resulting in an elevated steady-state position of $[Ca^{2+}]_i$ at rest. The release of Ca^{2+} from the S.R. at excitation therefore causes $[Ca^{2+}]_i$ to rise to an abnormally high level, which may occasionally trigger a further, Ca^{2+}-induced release of Ca^{2+} from the S.R. (the CROC), so that $[Ca^{2+}]_i$ rises further to a concentration that will either release or stimulate the proteases. This positive feedback action of the CROC, involving high levels of $[Ca^{2+}]_i$, is probably of little consequence in normal muscle in vivo, but may well be the mechanism by which an elevated $[Ca^{2+}]_i$ in dystrophic muscle is converted to a dangerously high level that can release or activate the proteases. The CROC could therefore be one of the key steps in the induction of muscle necrosis.

Ca^{2+}-release from the S.R. can, therefore, be achieved either by depolarization or by the CROC (Endo et al., 1970; Ford and Podolsky, 1970, 1972a, b; Taylor and Godt, 1976) and the two processes are, at least partially, independent (see detailed review, Endo, 1977). Dantrolene sodium (DaNa) blocks the depolarized release of Ca^{2+} from the S.R. (Ellis and Carpenter, 1972; Desmedt and Hainaut, 1977) but apparently has little effect on the CROC (Endo, 1977). The action of the CROC, on the other hand, is greatly promoted by caffeine (Carvalho, 1968; Endo, 1977),

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whereas procaine and tetracaine inhibit (Feinstein, 1963; Lüttgau and Oetliker, 1968; Weber and Herz, 1968; Ford and Podolsky, 1972a). This action of caffeine in promoting the CROC explains its familiar effect in releasing intracellular Ca^{2+} in muscle, thereby reversibly potentiating contractions at low concentration and initiating contractions at concentrations above 5mM (Axelsson and Thesleff, 1958; Gebert, 1968; Lüttgau and Oetliker, 1968; Weber and Herz, 1968; Yamaguchi, 1975).

These studies, concerned with elucidating the details of the CROC and with the action of caffeine in its facilitation, have largely been carried out with intact and skinned skeletal muscle fibres of frog. Since, as we have suggested above, the CROC could have a key role in raising $[Ca^{2+}]_i$ in muscle necrosis, we have examined the effect of caffeine and related treatments on the ultrastructure of frog skeletal muscle fibres, thereby complementing our studies with A23187 (Statham et al., 1976). Conway and Sakai (1960) have briefly recorded damage in frog muscle following treatment with caffeine at a concentration of approximately 5 mM but most previous electron micrographic studies with arthropod and frog muscle fibres have shown that the characteristic effect of caffeine treatment is a conspicuous swelling and even disintegration of the S. R. (Huddart and Oates, 1970; Bittar et al., 1974; Uhrik and Zacharova, 1976). We report here that caffeine, like A23187 treatment, rapidly causes myofilament dissolution and degradation of frog skeletal muscle fibres.

Material and Methods

The isolated cutaneous pectoris muscle of the frog *Rana temporaria* was used. Frogs were maintained in the laboratory at 5° C.

The saline in which the preparation was bathed contained NaCl 115 mM, KCl 2.5 mM, Na_2HPO_4 2.15 mM, NaH_2PO_4 0.85 mM at pH 7.1. Normal saline also contained $CaCl_2$ 1.8 mM, but Ca^{2+} was omitted when the muscle was treated with caffeine or A 23187, so as to ensure that any rise in $[Ca^{2+}]_i$ was not associated with Ca^{2+}-influx from the external medium. As far as possible, control and experimental muscles were paired from the same frog.

The stock solution of A 23187 was dissolved in ethanol; the final concentrations for experiments were A 23187 5 μg ml^{-1}; ethanol 5 μl ml^{-1}. Control solutions contained the same concentration of ethanol. Solutions of DaNa were made up at 10 μg ml^{-1} in saline and were vigorously stirred for 1 h; the final solution was then filtered (Statham and Duncan, 1976). All test solutions were prepared immediately before use and maintained at 20° C ± 0.5° C during the experiments, unless otherwise stated.

Muscles were prepared for examination under the electron microscope by replacing the saline with fixative (Karnovsky, 1965) at 21° C for 30 min. Fixation was completed by transfer to a fresh change of the same solution at 21° C for $2^1/_2$ h. Specimens were washed in 0.1 M sodium cacodylate with 2.5 mM $CaCl_2$, pH 7.2, for 30 min (two changes). The tissue was then cut into smaller pieces and postfixed in OsO_4 for 2 h at room temperature. After this, the pieces of muscle were cut into small blocks for subsequent washing in cacodylate buffer (two changes), dehydration through the graded alcohol series and embedding in resin (Spurr, 1969). Sections were cut at 60–90 nm, stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined on an AEI Corinth 500.

All reagents used were AnalaR grade where these were available. DaNa was obtained from Eaton Laboratories, London. Caffeine and procaine hydrochloride were obtained from Sigma Chemical Co., St. Louis. A 23187 was a gift of Lilly Research Centre, Windlesham, Surrey.

Results

Exposure of the pectoris cutaneous muscle to caffeine solutions at concentrations of 0.5 mM to 3 mM at 20° C for periods varying from 5–40 min produced little evidence of ultrastructural damage (Fig. 1) although the S.R. was swollen at 2 mM caffeine in some preparations. Clear evidence of ultrastructural damage was found, however, after exposure to 5 mM caffeine for 45 min. The muscle fibres were frequently contracted and in many parts of the tissue myofilament degradation could be seen which begins with the dissolution of peripheral filaments (Fig. 2), and this stage is followed by the loss of normal ultrastructural organization. Higher concentrations of caffeine (6, 8 or 10 mM) cause the complete destruction of large areas of the muscle (Fig. 3, 8 mM caffeine, 20 min exposure) and electron micrographs reveal only the remnants of the fibrils with a great increase in sarcoplasm. Fig. 4 shows a preparation after exposure to 8 mM caffeine for 30 min with an atypical form of filament dissolution. The effect of 6 mM caffeine (30 min exposure) is shown in Fig. 8. Damaged areas following exposure to these concentrations of caffeine can be detected externally with a low power microscope. The S.R. becomes swollen, whilst the mitochondria undergo configurational changes that are similar to those reported when the muscle is treated with A 23187 (Statham et al., 1976; Fig. 12). As with A 23187, however, areas of undamaged muscle can always be found, even at high caffeine concentration, often adjacent to regions where myofilament dissolution is complete. Muscles were exposed to caffeine both when pinned out and when simply immersed in the test solutions and damage was found with both methods of treatment.

The extensive damage promoted by treatment with these higher concentrations of caffeine is produced quickly and the effect is more rapid than that found with A 23187 (Statham et al., 1976; Figs. 11 and 12). A series of pectoris cutaneous muscles fixed at 8, 10, 20 and 30 min after exposure to 8 mM caffeine all showed extensive areas of damage; the magnitude of the degradation increases with time, but Fig. 5 shows that considerable myofilament dissolution is already complete after only 8 min treatment with caffeine. In general, the major effect of increasing the time of

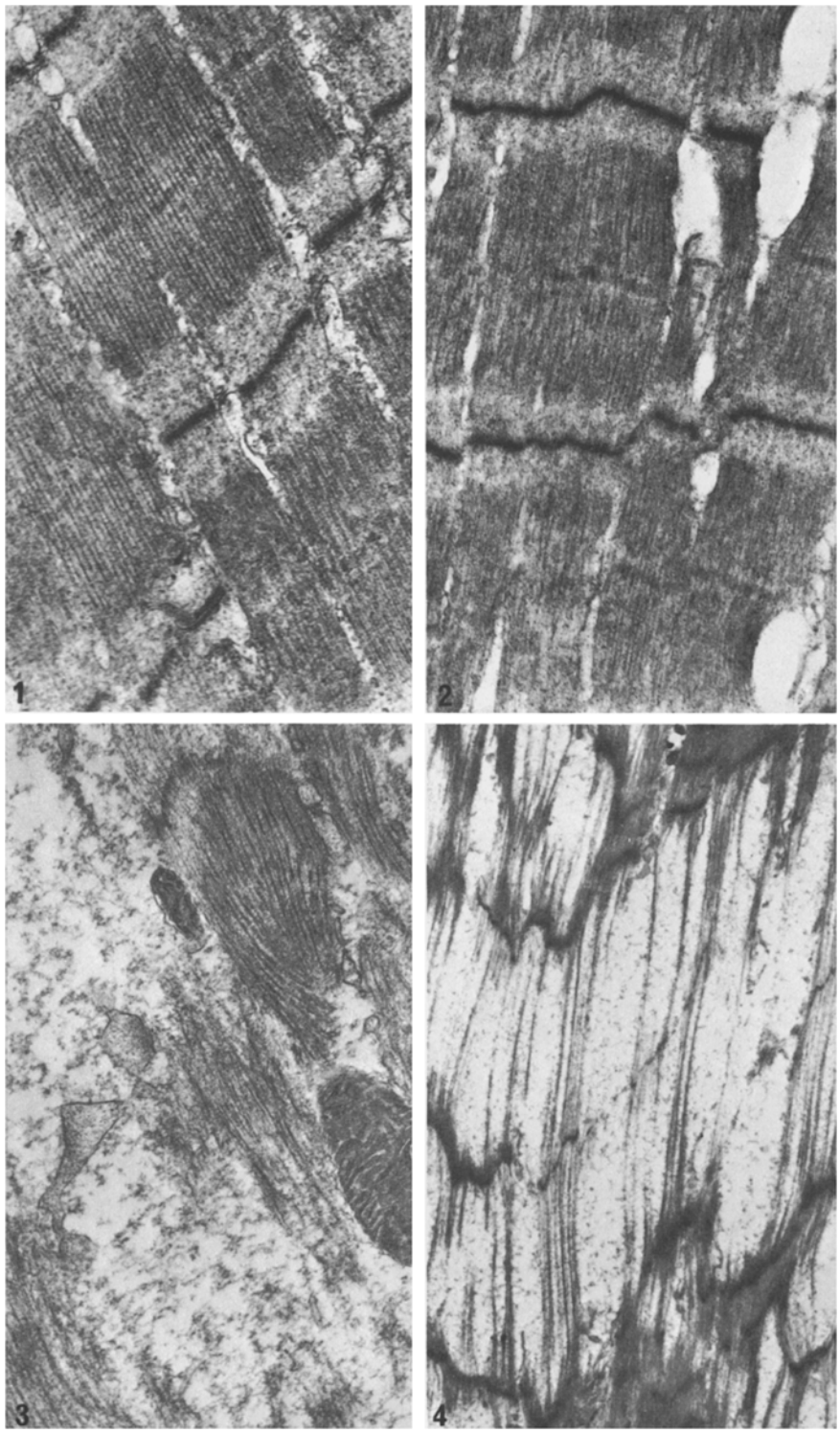


Fig. 1
Muscle exposed to 2 mM caffeine for 45 min at 20° C. × 26,000

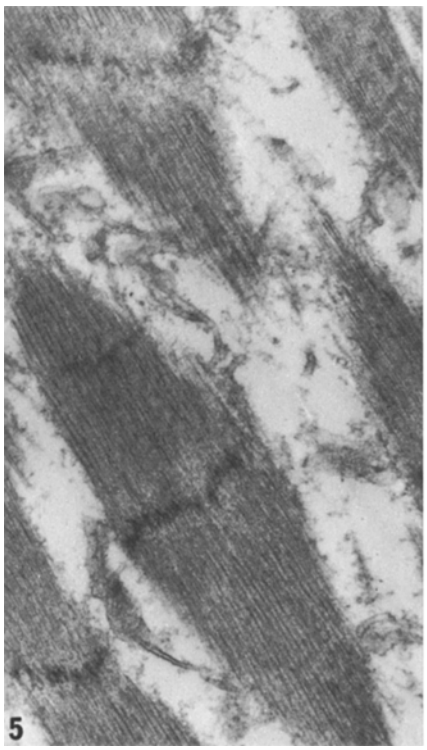
Fig. 2
Muscle exposed to 5 mM caffeine for 45 min at 20° C. Note limited damage in this area, with dissolution of the peripheral myofilaments. × 26,000

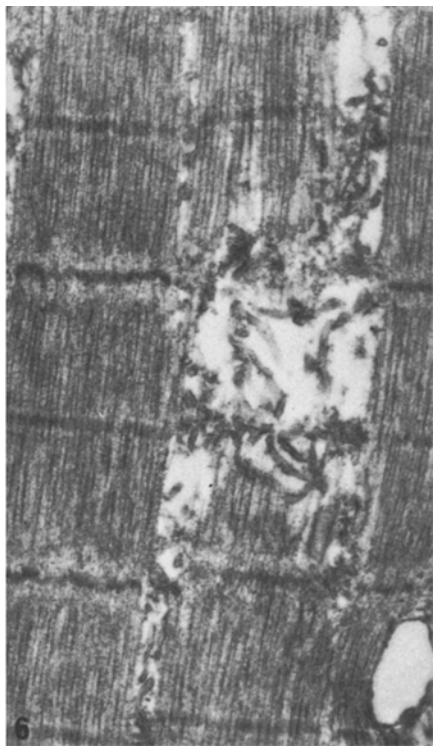
Fig. 3
Effect of exposure to 8 mM caffeine for 20 min at 20° C. Note extensive degradation of myofilaments. × 24,000

Fig. 4
Effect of exposure to 8 mM caffeine for 30 min at 20° C.
This form of myofilament damage was found less commonly. × 6,600

exposure, or of increasing caffeine concentration above 6 mM, was to cause a progressive enlargement of the areas of muscle showing ultrastructural damage.

The effect of temperature on the action of caffeine was explored by exposing muscles to 3 mM caffeine for 40 min at 20° C and at 3° C. This concentration produces little ultrastructural damage at 20° C but peripheral filaments exposed at 3° C occasionally have a greater degree of dissolution (Fig. 6). In general, however, lowering the temperature from 20° C to 3° C





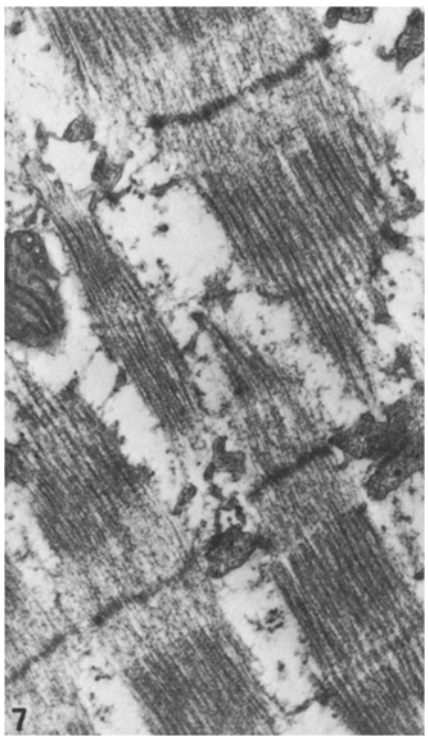


Fig. 5. Muscle exposed to 8 mM caffeine for only 8 min at 20° C. Extensive myofilament degradation even after this short period of exposure. × 26,000

Fig. 6. Effect of 3 mM caffeine for 40 min at 3° C. Note some signs of myofilament damage even at this low concentration of caffeine. × 26,000

Fig. 7. Effect of exposure to 2 mM caffeine for 45 min at 8° C. Extensive myofilament damage at this temperature even at this low concentration of caffeine. × 26,000

does not have a marked effect in promoting degradation of the fibres at low concentrations of caffeine. Much greater damage, however, was found when the muscle was treated at 8° C with 2 mM caffeine, an even lower concentration which normally causes no filament degradation (Fig. 1). Fig. 7 shows that, at this temperature, there is extensive damage of the muscle fibres. Thus, comparison of Figs. 1, 6 and 7 shows that at low concentrations, caffeine is much more effective in producing damage at 8° C than at 3° C, whilst both

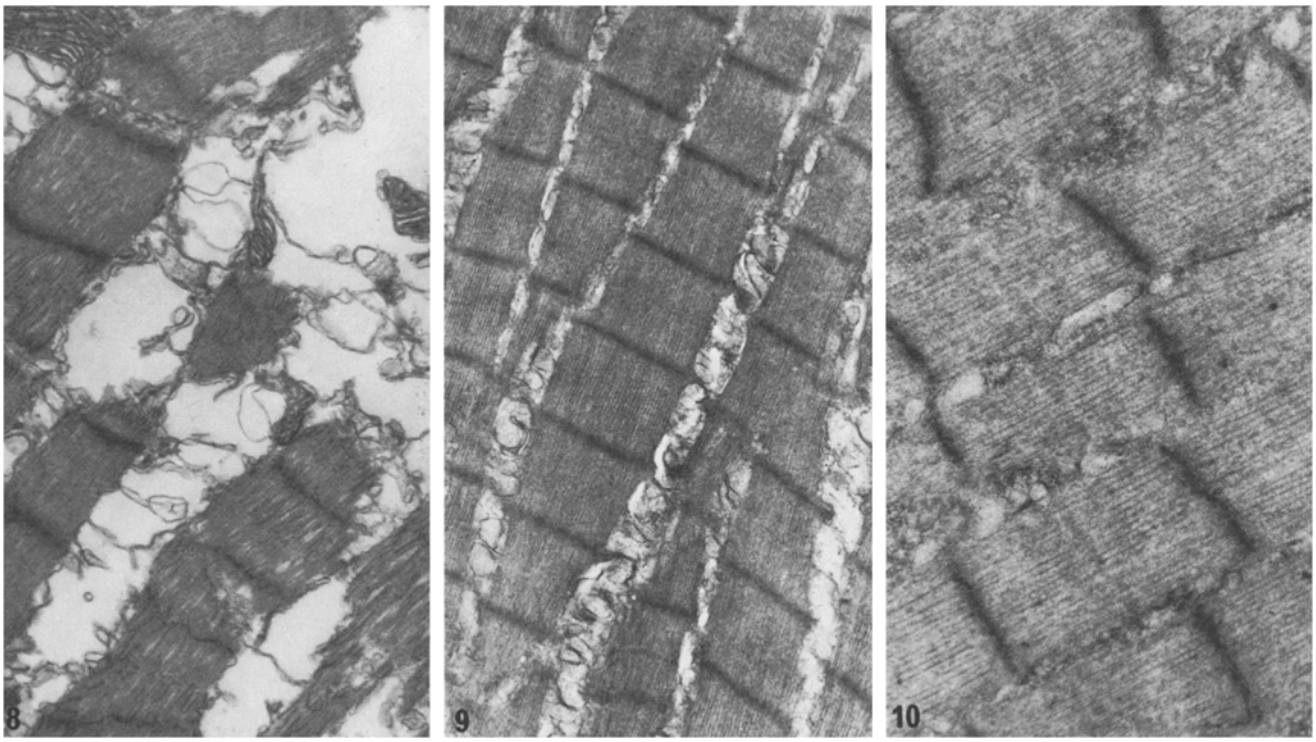


Fig.8–10. Protective effect of procaine. The muscles were pre-exposed to 4 mM procaine (10 min) and then transferred to 4 mM procaine plus 6 mM caffeine (30 min) at 20° C. **Fig.8.** 6 mM caffeine alone; typical damage. × 11,000. **Fig.9.** Pre-exposure to procaine provides substantial protection. × 11,000. **Fig.10.** Pre-exposure to procaine provides almost complete protection. × 26,000

these lower temperatures are able to promote damage that is not seen at 20° C.

The known effects of procaine in protecting the S.R. from the action of caffeine in releasing Ca^{2+} were studied to determine whether this agent was also effective in reducing caffeine-induced damage in frog muscle. Muscles were pre-exposed to Ringers solution with 4 mM procaine for 10 min and were then transferred to Ringers solution with 6 mM caffeine plus 4 mM procaine for 30 min. The results are shown in Figs. 8–10. Muscle exposed to 6 mM caffeine alone showed typical damage (Fig. 8), whilst 4 mM procaine under these conditions gave either substantial (Fig. 9) or almost complete protection (Fig. 10). In Fig. 9 it can be seen that the S.R. is swollen and that the myofilaments are clearly disorganized, but the damage is substantially less than when the muscle is exposed to 6 mM caffeine alone (Fig. 8).

The damage produced in frog muscle by exposure to A23187 has been reported previously (Statham et al., 1976), but experiments with the divalent cation ionophore have been repeated to determine whether procaine or DaNa can protect against ionophore-induced damage. Muscles were exposed to 5 mM procaine for 10 min at 25° C before addition of A23187 (5 μg ml^{-1}) and exposure for a further 20 min. Muscles were exposed to DaNa (10 μg ml^{-1}) for 30 min before addition of A23187 (5 μg ml^{-1}; 25° C) and exposure for a further 20 min. The conditions in these experiments were therefore similar to those with A23187 described previously, except for the inclusion of either procaine or DaNa. Neither procaine (Fig. 11) nor DaNa (Fig. 12) under these conditions served to protect the myofilaments from ionophore-induced damage.

Discussion

There is now substantial evidence for the existence of the CROC in frog skeletal muscle, and the results obtained in the present study are consistent with the hypothesis that the release of Ca^{2+} from the S.R. will rapidly cause myofilament damage and degradation (Statham et al., 1976; Publicover et al., 1978). Thus, Figs. 1–5 reveal a similar pattern of damage to that recorded after treatment with the divalent cation ionophore A23187 (Statham et al., 1976). Myofilament degradation follows rapidly when a CROC is initiated by treatment with concentrations of caffeine above 5 mM. The mitochondria undergo ultrastructural changes during caffeine treatment and it is believed that these are related to Ca^{2+}-uptake by the organelles,

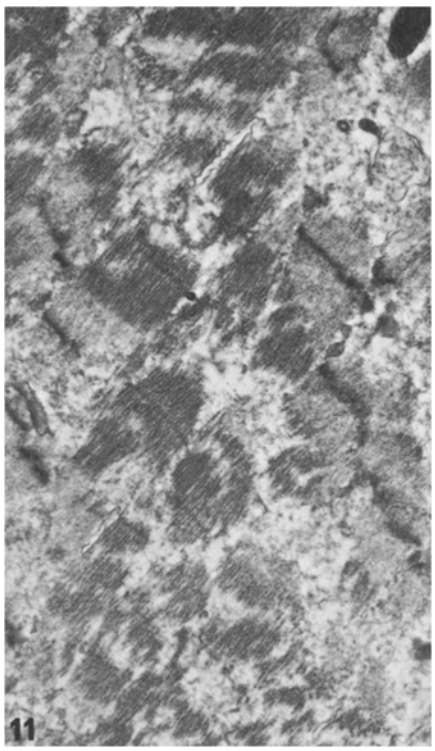


Fig. 11
Failure of procaine to protect against ionophore-induced damage. Muscle exposed to 5 mM procaine (10 min), followed by addition of A23187 (5 μg ml^{-1}; 20 min) at 25° C. ×10,000

Fig. 12
Failure of DaNa to protect against ionophore-induced damage. Muscle exposed to DaNa (10 μg ml^{-1}; 30 min) followed by addition of A23187 (5 μg ml^{-1}; 20 min) at 25° C. Note ultrastructural changes in mitochondrion. ×11,000

as we have discussed previously (Statham et al., 1976; Publicover et al., 1977).

Procaine and tetracaine are known to inhibit the CROC in skinned fibres (Feinstein, 1963; Lüttgau and Oetliker, 1968; Weber and Herz, 1968; Ford and Podolsky, 1972a); these agents nullify the effects of high $[Ca^{2+}]$ and caffeine, but have little effect on depolarization-induced release of Ca^{2+} from the S.R. Procaine (4 mM) also protects against muscle damage caused by 6 mM caffeine (Figs. 9 and 10).

Lowering the temperature apparently labilizes the CROC in frog muscle fibres, and the effect of caffeine in initiating contraction is produced more easily at low temperature (Conway and Sakai, 1960; Lüttgau and Oetliker, 1968; Weber and Herz, 1968; Ogawa, 1970). 3 mM caffeine is a concentration that produces no detectable myofilament degradation at 20° C. However, reducing the temperature to 3° C and exposing the muscle to 3 mM caffeine for 40 min caused only a small increase in muscle damage (Fig. 6). One explanation for this apparent discrepancy may be that not only is the CROC labilized but the proteases responsible for muscle degradation are also inhibited at this temperature and such an hypothesis is supported by the results obtained at 8° C. At this temperature the damage is much more extensive (Fig. 7), even at the lower concentration of 2 mM caffeine (compare Figs. 1 and 7). We conclude that, at 8° C, the action of caffeine in promoting Ca^{2+} release is labilized, whilst the Ca^{2+}- activated proteases are still not completely inhibited.

The experiments also show that A23187 effects the release of Ca^{2+} from the S.R. by a different mechanism from that of caffeine. Neither procaine (an inhibitor of the CROC) nor DaNa (an inhibitor of the depolarization promoted release) at the concentrations tested were able to protect against ionophore-induced damage (Figs. 11 and 12). We therefore conclude that the ionophore A23187 produces directly a major efflux of Ca^{2+} from the S.R. by a process that is different from either depolarization or the CROC.

The results with amphibian (Statham et al., 1976) and mammalian (Publicover et al., 1978) skeletal muscle suggest that the marked rise in $[Ca^{2+}]_i$, produced experimentally by either A23187 or caffeine treatment, is responsible for the rapid myofilament degradation. There are a number of ways in which this effect might be achieved:

1. Ca^{2+}-activated proteases acting at neutral pH have been isolated from rabbit (Reddy et al., 1975) and porcine muscle (Dayton et al., 1976a, b); they are stimulated by Ca^{2+} concentrations in the range 1–10 mM, are free in the myoplasm (Reville et al., 1976) and may have a role in the disassembly of intact myofibrils during the normal metabolic turnover in myofibrillar proteins (Dayton et al., 1976a, b). There are a number of descriptions of other intracellular Ca^{2+}-activated proteases in mammalian muscle which

differ in their substrate specificities and in other detailed properties (Kohn, 1969; Koszalka and Miller, 1960a, b). It is possible that the experimentally-induced rise in $[Ca^{2+}]_i$ acts to stimulate these proteases of the myoplasm directly so causing myofilament degradation.

2. The rapid and extensive damage shown in this study may be the result of the action of acid hydrolases. The evidence suggests that skeletal muscle contains relatively few typical lysosomes and that the lysosomes are part of the sarcotubular system (Seiden, 1973). Thus, the marked rise in $[Ca^{2+}]_i$ may release, by an unidentified mechanism, a mixture of cathepsins and other acid hydrolases known to be present in the lysosomal system (Iodice et al., 1966) and these enzymes effect the dissolution of the myofilaments. Such an hypothesis is supported by studies in which inhibitors of catheptic activity can reduce protein degradation in normal and dystrophic muscle (McGowan et al., 1976; Libby and Goldberg, 1978).

3. The myofilament degradation produced in frog muscle in the present study is particularly rapid (within 8 min) and it is possible that abnormally high levels of $[Ca^{2+}]_i$ activate enzymes that cause the disassembly of the polymers of the myofilaments, rather than their proteolytic digestion.

Frog skeletal muscle is a particularly satisfactory system for the study of this sequence of events in the experimental induction of ultrastructural damage, since the microphysiology of Ca^{2+} uptake and release by the S.R. have been so fully elucidated in skinned fibres. Thus, $[Ca^{2+}]_i$ at rest $\simeq 2\times10^{-7}$ M; the threshold $[Ca^{2+}]_i$ for contraction $\simeq 6-9\times10^{-7}$ M; the physiological loading of the S.R. is 0.25–0.33 of its maximum capacity; in a single twitch 210 nmol Ca^{2+} ml^{-1} are thought to be released, so making the myoplasmic concentration $\simeq 10^{-5}$ M; the CROC starts operating when $[Ca^{2+}]_i \simeq 10^{-4}$ M at physiological levels of $[Mg^{2+}]_i$ (Endo, 1977). The exchangeable Ca^{2+} of living muscle is calculated to be about 0.7 µmol/ml muscle, suggesting that the bulk of Ca^{2+} has to be free in the myoplasm before $[Ca^{2+}]_i$ rises to a level that is sufficient to promote myofilament dissolution.

It could be asked whether the sequence of events in frog muscle constitutes a satisfactory model for degradation in mammalian muscle, since it was suggested in the Introduction that the CROC could well be of importance in promoting necrosis in various myopathies. However, the recent findings of myofilament dissolution in mouse diaphragm caused by application of A23187 (Publicover et al., 1977, 1978) confirms that a rise in $[Ca^{2+}]_i$ may have a key role in the induction of muscle damage and myopathies in mammalian tissues also.

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