Different mechanisms mediate structural changes and intracellular enzyme efflux following damage to skeletal muscle

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Summary

Cellular damage, induced by either A23187 (2×10⁻⁵ M) or 2,4-dinitrophenol (DNP) (10⁻³ M), has been studied in incubated mouse soleus muscle and has been monitored by electron microscopy and measurement of creatine kinase (CK) efflux. CK efflux induced by DNP was dependent on extracellular Ca, whereas the characteristic myofilament breakdown was not. Chlorpromazine (2×10⁻⁴ M; an inhibitor of phospholipase A (PLA₂)) or 5×10⁻⁶ M-nordihydroguaiaretic acid (NDGA) (a lipoxygenase inhibitor) almost completely inhibited CK efflux, following treatment with either A23187 or DNP, but did not affect myofilament damage. It is concluded that there are at least two separate pathways in cellular damage: (1) PLA₂ activation and lipoxygenase activity culminating in sarclemma damage and (2) a system that produces characteristic destruction of the myofilament apparatus.

Key words: muscle damage, phospholipase A₂, lipoxygenase, calcium.

Introduction

Damage to tissues is known to occur in a variety of pathological conditions and a considerable amount of work has been undertaken in order to elucidate the mechanisms by which different stresses induce such damage. It has been assumed that a knowledge of the various processes involved in tissue damage would allow possible therapeutic intervention and hence the maintenance of tissue viability. In particular, the processes involved in damage to cardiac muscle have been extensively studied, with the result that the viability of cardiac tissue either removed for transplant purposes or subjected to major surgery in vivo can be improved by using agents such as calcium antagonists (Nayler et al. 1979) or free radical scavengers (McCord, 1985).

Skeletal muscle has also been studied with a view to finding agents capable of reducing muscle damage in chronic degenerative disorders such as muscular dystrophy (Duncan, 1978; Rodemann et al. 1981; Jones et al. 1983). Unfortunately, the majority of such studies have examined only one indicator of damage. The most popular of these have been protein degradation rates (Rodemann et al. 1981; Baracos et al. 1986), ultrastructural appearance (Publicover et al. 1978) and efflux of intracellular enzymes (Jones et al. 1983; Jackson et al. 1984) or exclusion of non-diffusible compounds, e.g. Trypan Blue (Zuurveld et al. 1985).

A number of compounds have been described that reduce enzyme efflux from skeletal muscle and from which conclusions concerning the mechanism of release of intracellular enzymes have been drawn (Jackson et al. 1983, 1984). In particular, inhibitors of phospholipase A₂ (PLA₂) and of the enzymic oxidation of arachidonic acid via the lipoxygenase pathway (Jackson et al. 1987) have proved to be effective, leading to the suggestion that these pathways are implicated in sarclemma breakdown. The aim of this work was to use chlorpromazine (an inhibitor of phospholipase enzymes) and nordihydroguaiaretic acid, NDGA (an inhibitor of lipoxygenase), to determine whether the characteristic ultrastructural changes that are seen during skeletal muscle damage occur via the same mechanism as the efflux of intracellular enzymes following a damaging stress.
Materials and methods

Female Balb/C mice maintained on a standard laboratory diet were killed by cervical dislocation and the soleus muscles were rapidly dissected and removed. The muscles were 10 mm long and the tendons were ligatured, then attached to glass holders and placed in separate plastic tubes containing 2-5 ml of bicarbonate-buffered Krebs saline. The tubes were mounted in a thermostatically controlled water bath at 37°C (Jones et al. 1983). The incubation medium contained 137 mM-NaCl, 5-0 mM-KCl, 1-0 mM-MgSO4, 1-3 mM-NaH2PO4, 24-0 mM- NaHCO3, 2-0 mM-CaCl2, 10-0 mM-glucose and was gassed with 95% O2, 5% CO2 (pH 7-4). After 30 min incubation the medium surrounding the muscles was exchanged and agents known to damage muscles and induce enzyme efflux were added to the medium. After 30 min the medium was exchanged and this was repeated every 30 min until the end of the experiment. The creatine kinase (CK) activity of the incubation medium was measured by using a linked enzyme assay, the production of NADPH being followed at 340 nm (Jones et al. 1983). When inhibitors of enzyme efflux were used they were added to the incubation medium and the preparations were preincubated for 30 min before the addition of A23187 or 2,4-dinitrophenol (DNP). In media lacking Ca, CaCl2 was simply omitted and EGTA was not included so that [Ca]o was 3x10^-6 to 6x10^-6 M.

Muscles were mounted on a frame under very light tension for fixation in 3% glutaraldehyde at 21°C (15 h) for electron microscopy. Details of subsequent washing, dehydration and sectioning have been given by Duncan et al. (1980) and sections at 60—90 nm were examined on a Zeiss EM10/CR. All agents used were of AnalaR grade or the highest grade commercially available. Calcium ionophore (A23187), DNP, chlorpromazine and nordihydroguaiaretic acid (NDGA) were obtained from Sigma Chemical Company, Poole, Dorset. A23187 was solubilized in absolute ethanol and an equivalent volume of ethanol was included in control preparations.

Results

Effects of calcium ionophore on muscle CK efflux

The CK efflux from a group of muscles treated with the calcium ionophore A23187 (20 μM) are shown in Fig. 1, together with the CK efflux from muscles treated with calcium ionophore in the presence of chlorpromazine (200 μM) or NDGA (5 μM). Treatment of muscles with ionophore induced a large and rapid release of this intracellular enzyme, which was almost completely prevented by the presence of chlorpromazine or NDGA.

Effect of DNP on muscle CK efflux

DNP (10^-3 M) treatment induced a large and rapid efflux of CK from muscles (Fig. 2), which was almost completely eliminated by removal of calcium from the medium bathing the muscles, showing that the increased permeability of the sarcolemma induced by DNP in the soleus preparation is dependent on Ca influx. Similarly, the inhibitors chlorpromazine (200 μM) or NDGA (50 μM), in the presence of extracellular Ca, provided almost complete protection against CK efflux.

Ultrastructural studies of muscle damage

Control muscles fixed either at the start of the experiment or after incubation for 60 min revealed a normal ultrastructure (Fig. 3), confirming that the conditions of incubation were satisfactory. Exposure of soleus muscle to either A23187 or DNP produced the same characteristic pattern of myofilament damage (Figs 4, 5, 7) found in diaphragm muscle exposed to these agents (Publicover et al. 1978; Duncan et al. 1980); namely, (1) Z-line sliding (Figs 4, 5), Z-line blurring or complete Z-line loss (Figs 7, 8); (2) focal myofilament apparatus loss (Fig. 4); (3) disruption of the myofilament apparatus, separating from the Z-line, culminating in disintegration and dissolution of the thick and thin filaments (Figs 5, 6); (4) contraction...
Fig. 2. Effect of removal of extracellular calcium (○), or addition of chlorpromazine (●) or NDGA (■) on the efflux of creatine kinase from muscles treated with DNP, compared with muscles treated with DNP alone (◆). Results are presented as the mean ± S.E.M. of four muscles treated with DNP alone and the mean of two muscles for those treated to reduce enzyme efflux.

(Fig. 3) (particularly associated with Z-line blurring); (5) hypercontraction bands; (6) mitochondrial changes including swelling, division and the development of ‘bars’ within the organelle (see Fig. 8). The difference between the damage triggered by either A23187 or DNP in mouse diaphragm and soleus was that in the latter preparation the damage after 30 min exposure was largely confined to the outer fibres. However, in all of the protocols summarized in Figs 1 and 2, where the inhibitors provided almost complete protection against CK efflux, identical patterns of myofilament damage were found at the end of the experiments. In summary, neither NDGA nor chlorpromazine protected against A23187 (Fig. 8) or DNP-induced ultrastructural damage (Fig. 6). All the different features of myofilament damage and other ultrastructural changes listed above, including swelling of the sarcoplasmic reticulum and mitochondrial division, were found in experiments when chlorpromazine or NDGA was included in the incubation medium.

Furthermore, whereas omission of extracellular Ca protected against DNP-induced CK efflux (Fig. 2), the same concentration of DNP caused severe myofilament damage in both the presence and absence of extracellular Ca (see Figs 4, 5).

Discussion

Evaluation of studies examining the mechanisms of damage to skeletal muscle is hampered by a lack of information on the relationship between the various indices of damage used by different workers. For example, treatment of skeletal muscle with the calcium ionophore (A23187) has been shown to raise intracellular calcium levels, inducing ultrastructural damage (Publicover et al. 1978), intracellular enzyme efflux (Jones et al. 1984), an increase in protein degradation rates (Rodemann et al. 1981) and a loss of Trypan Blue exclusion (Zuurveld et al. 1985), but pretreatment of muscles with the protease inhibitor leupeptin has been reported to reduce the calcium-stimulated increase in protein degradation (Rodemann et al. 1981), but not calcium-induced ultrastructural damage (Duncan et al. 1979) or intracellular enzyme efflux (Jackson et al. 1984). The work described here has examined the relationship between ultrastructural damage and enzyme efflux from skeletal muscle, and demonstrates that certain agents known to inhibit the efflux of intracellular enzymes had little effect on the ultrastructural changes in the same muscle.

Both the calcium ionophore and DNP produced a similar characteristic pattern of rapid myofilament damage in soleus muscle (Figs 3–8) to that found in mouse diaphragm (Publicover et al. 1978; Duncan et al. 1980), with the major difference that in the soleus muscle the damage was largely confined to the outer fibres.

It has been suggested that in many situations an influx of external calcium is an essential part of the process that leads to efflux of intracellular enzymes (Jackson et al. 1984; Jones et al. 1984; Claremont et al. 1984) and calcium-induced ultrastructural damage to muscle has been extensively described (Publicover et al. 1978; Duncan et al. 1980). In 1984 Jones et al. demonstrated that both the intracellular enzyme efflux and ultrastructural damage induced by excessive contractile activity could be inhibited by removal of calcium from the external medium. The results presented here confirm that external calcium is essential for the enzyme efflux that follows DNP treatment (Fig. 2), but they also show that removal of Ca has no effect on the DNP-induced ultrastructural damage. This observation suggests that ultrastructural damage induced by DNP is triggered either solely by intracellular calcium released from the mitochondria or (more probably, Duncan & Rudge, unpublished) by the direct activation of the myofilament damage process by DNP.

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Fig. 3–6. Electron micrographs of mouse soleus muscle fixed after 60 min exposure to the various treatments summarized in the legends to Figs 1 and 2. Bars, 1 μm. Fig. 3, control muscle. Figs 4–6, exposure to $10^{-3}$M-DNP; Fig. 4, with zero extracellular [Ca], notice focal myofilament damage with Z-line sliding; Fig. 5, plus normal extracellular [Ca], notice area of contraction grading into myofilament destruction and Z-line destruction grading into an area of dissolution of myofilaments; Fig. 6, plus normal extracellular [Ca] plus 50 μM-NDGA, notice sarcomeres with Z-line loss and swollen mitochondria grading into an area of complete cellular dissolution.

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The results presented here confirm the fact that both NDGA and chlorpromazine have a substantial protective effect against intracellular enzyme efflux, but are ineffective against the ultrastructural changes. Chlorpromazine is an agent that has many properties, but which is an extremely efficient inhibitor of PLA₂, while NDGA is a potent antioxidant and lipoxygenase inhibitor, and their lack of effect against the specific ultrastructural damage to the myofilament apparatus suggests that activation of PLA₂ and the oxidation of the released arachidonic acid are not involved in the process of ultrastructural damage in a similar manner to that proposed for the mechanism of enzyme efflux (Jackson et al. 1984, 1985, 1987). We conclude that there are likely to be at least two separate mechanisms that can operate during rapid subcellular damage in mammalian skeletal muscle. First, a rise in [Ca]ᵢ activates PLA₂ and the consequent oxidation of arachidonic acid culminates in sarcolemma damage and the release of CK. Second, a process can be switched on by rises in [Ca]ᵢ, which initially can cause focal myofilament damage in the centre of the cell (Fig. 4). However, results to be presented separately suggest that this system is not obligatorily dependent on Ca, but can also be triggered directly by other agents (Duncan, unpublished data).

This finding of at least two different mechanisms that mediate enzyme efflux and ultrastructural damage following treatment with a damaging agent receives support from clinical observations during therapeutic intervention in chronic myopathies. In Duchenne muscular dystrophy it has been reported that treatment with corticosteroids reduces the plasma creatine kinase activity of the patients without significant effect on the course of the disease (Drachman et al. 1974), while in patients with polymyositis treated with steroids there may be a significant discrepancy between the plasma creatine kinase activity and the clinical course of the disease (Edwards et al. 1979). Steroids are known to act to inhibit phospholipase enzymes (Blackwell & Flower, 1983) and so may be analogous in their action to the substances (e.g. chlorpromazine) used in this study.

Despite our findings of apparently disparate mechanisms underlying the two processes of muscle damage, both were activated by either A23187 or DNP and it is probable that the two mechanisms are linked in various ways. In particular, it is likely that changes in sarcolemmal permeability that are sufficient to allow the leakage of large intracellular enzymes will lead also to ultrastructural damage to the muscle via an associated influx of extracellular calcium. However, the data

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presented here indicate that the reverse situation, i.e. ultrastructural damage without intracellular enzyme efflux, can also exist in skeletal muscle in vitro.

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References


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