Dissociation between Metabolite Levels and Force Fatigue in the Early Stages of Stimulation-induced Transformation of Mammalian Skeletal Muscle

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Abstract

Rabbit tibialis anterior (TA) muscles were arranged to perform shortening contractions at a frequency of 10 Hz. Frozen milligram quantities of tissue were removed during the course of the contractions by a new sampling technique. This made it possible to monitor simultaneous changes in metabolite levels, force and mass action potentials within a single muscle.

Marked decreases in levels of PCr and ATP were observed after only 2 minutes of stimulation. After a further 40 minutes of stimulation these levels had recovered almost to their initial values. While metabolites were undergoing this phasic change, force and electromyographic potentials were declining progressively. Muscles that had been made resistant to fatigue by chronic stimulation showed a similar response when tested under more severe conditions. Hypothetical mechanisms underlying the recovery of metabolite levels under these conditions are discussed. The phenomenon provides some clues to the general nature of the intracellular events responsible for the initiation of stimulation-induced transformation.

Key words: metabolites, force, fatigue, chronic stimulation.

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Indirect electrical stimulation can be used to effect a substantial increase in the contractile activity of a skeletal muscle. In the long term, a mammalian fast-twitch skeletal muscle responds to such conditions by developing slow-contracting, highly fatigue-resistant properties. This transformation is brought about by a sequence of profound changes in the expression of proteins involved in the major molecular systems of the muscle: contraction, calcium transport and storage, and the generation of ATP (reviewed in 37, 41). The availability of suitable probes has facilitated studies of changes in the levels of the corresponding muscle-type-specific mRNAs. Such experiments have contributed to a growing body of evidence that many of the protein changes induced by chronic stimulation are the result of regulatory events taking palce at a pre-translational level [5, 6, 7, 26, 27, 28, 34, 42, 46]. However, the intracellular signals responsible for initiating these changes remain unknown.

Examination of the time course of the response of rabbit fast-twitch muscle to stimulation provides an approximate indication as to when these signals might be expect to appear. The earliest changes in proteins are observed about 2 days after the onset of stimulation and include changes in calcium transport ATPase [18] and in some oxidative enzymes, such as hexokinase and 3-oxoacid CoA transferase [10, 20, 35, 36]. Changes in mRNA - even those corresponding to changes in myosin heavy chain isoforms, which occur at a much later stage of transformation [6, 7, 27] - are also detectable after 4 days of stimulation. Candidate signalling mechanisms must therefore be sought among events which occur earlier than this.

The possibility has already been considered that changes in the concentrations of substrates and products of energy metabolism could provide molecular cues, related to functional demand, that initiate more permanent changes. For example, there is evidence that oxidative phosphorylation is activated by elevated ADP levels [9] and a similar mechanism could be responsible for the dramatic increase that takes place in mitochondrial volume fraction during chronic stimulation [14, 38]. These, and other reasons, prompted an earlier study of changes in metabolite levels during stimulation for periods up to 8 weeks [21]. That work contained a surprising observation: at the earliest measured time point, 30 hours after the onset of stimulation, there were no more than minor changes in ATP, phosphocreatine and most of the other metabolites studied. The well-documented results of acute experimet in which fast muscles had been subjected to exercise or stimulation would have led one to expect a rapid and early decline in PCr [9, 12, 13, 23, 25, 33] and, with prolonged intense stimulation, a decline in ATP levels as well [13, 22, 24, 31, 32, 33, 40]. Henriksson et al. [21] therefore suggested that large changes in these components could have occurred at an earlier stage, but that fatigue, possibly at the level of the contractile apparatus, had intermittently relieved the muscle of the energy demand and so provided opportunities for metabolic recovery.

We therefore resolved to re-examine this response in further detail, concentrating on the period between 0 and 24 hours of stimulation. Since this period has been the focus of acute studies of the mechanisms responsible for muscle fatigue, and since these have yielded conflicting results with regard to a possible relationship between force fatigue and the levels of energy metabolites, we elected to include in our study measurements of both force and electromyographic activity.

A preliminary account of these results has been communicated previously [30].

Methods

Chronic stimulation procedure

Adult New Zealand White rabbits of either sex, weighing between 2.5 and 4.5 Kg, were prepared for aseptic surgery with a pre-anaesthetic medication of diazepam (Valium^{TM} Roche Products Ltd., 5 mg Kg^{-1}) and atropine sulphate (Bimeda, 3 mg Kg^{-1}) delivered subcutaneously, followed after 20 minutes by an intramuscular injection of Hypnorm^{TM} (Janssen Pharmaceuticals: fluanisone, 10 mg ml^{-1} and fentanyl citrate, 0.315 mg ml^{-1}; 0.3 ml kg^{-1}).

Miniature electrical stimulators were implanted under strictly aseptic conditions. The electrodes were arranged to stimulate the tibialis anterior (TA) muscle via the common peroneal nerve. Details of this procedure have been described previously [20]. In all cases stimulation was delivered to the left TA muscle continuously at a frequency of 10 Hz, for 24 hours per day. The contralateral muscle served as a control.

Acute stimulation procedure

Animals were anaesthetized by intravenous injection of urethane (Sigma Chemical Co., 500 mg Kg^{-1}) followed immediately by pentobarbitone sodium (Sagatal^{TM}, approximately 30 mg Kg^{-1}, to effect). Anaesthesia was maintained by supplementary intravenous injection of Sagatal ^{TM}.

Physiological measurements

For all physiological measurements, animals were placed on a myograph table in a supine position with their hind limbs clamped rigidly at the knee and ankle joints. The distal portions of both TA muscles were freed and attached via tendon hooks to force transducers (Statham Universal Transducer Cell, type UC.3, fitted with 2lb Load Cells, type UL 4-2). Each force transducer was connected to a Fenlow ZA2 strain-gauge amplifier. In order to simulate shortening, rather than isometric conditions, a compliance of 2.85 mm N^{-1} was introduced by incorporating a spring in series with the transducer. Contractions were evoked by pulses of amplitude 6 V and duration 0.02 ms delivered via electrodes placed beneath the common peroneal nerve. To measure mass action potentials, multistranded stainless steel wire electrodes (AS634, Cooner Wire Co., California) were sutured to the epimysium of proximal and distal portions of the muscle. The signals were amplified by Devices 3160 difference pre-amplifiers, set at a fixed gain of 100.

The amplified signals were monitored on oscilloscopes, and recorded simultaneously on a chart recorder (Gould Electronics Ltd., Warrington).

Instruments were calibrated as described by Brown et al. [4].

Sample collection by freeze-clamping

Samples were obtained from whole TA muscles by freeze-clamping the distal section with brass tongs cooled to the temperature of liquid nitrogen.

Tissue was then wrapped in cooled aluminium foil and stored at -77°C in hermetically sealed bags, pending analysis.

Sample collection by freeze-nibbling

In order to measure metabolic responses while the muscle was being stimulated, we developed a method which we have called 'freeze-nibbling'.

This consisted of removing small tissue samples from the superficial layer of the exposed muscle by clamping with forceps that had been cooled in liquid nitrogen; a small cut underneath the forceps freed the frozen tissue with minimal bleeding or other disturbance to the muscle. In this way samples could be taken at different stages of stimulation, in parallel with the measurement of muscle force and electromyographic potentials. Tissue biopsies were stored at -77°C, pending analysis.

Metabolite analysis

Metabolites were extracted from the muscle samples with perchloric acid at -10°C as described previously [29]. As the biopsied mass was only about 3 mg, a microhomogenisation system [17] was used to extract metabolites from the tissue. Metabolite levels were determined by HPLC on a Shimadzu system. The separation method, based on that of Bedford and Chiong [3], provided for the measurement of adenosine monophosphate (AMP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), inosine monophosphate (IMP), nicotinamide adenine dinucleotide (NAD), phosphocreatine (PCr) and creatine (Cr), in a single chromatographic analysis of duration 55 minutes. Metabolite levels were internally standardised by reference to NAD [39].

Experimental protocols

Control fast-twitch muscle

TA muscles were prepared for the measurement of force and electromyographic potentials as described above. Stimulation patterns of either (a) 10 Hz continuously, or (b) 40 Hz, for 330 ms every 1000 ms [8] were delivered to the muscle via the common peroneal nerve. Measurements of mass action potential and force during shortening contractions were made over a period of up to 2 hours. In the early experiments samples were collected at a predetermined interval by freeze-clamping. In later studies, biopsies were removed at regular intervals by freeze-nibbling.

Fatigue-resistant fast-twitch muscle

Fast-twitch muscle was conditioned so that it acquired more fatigue-resistant characteristics by stimulating it at 10 Hz continuously for 8 weeks. Muscles were subjected to 3 fatigue tests in turn. The stimulation patterns were: (a) 40 Hz, for 330 ms every 1000 ms [8]; (b) 40 Hz, for 330 ms every 500 ms; (c) 80 Hz, for 330 ms every 500 ms. Muscles were not allowed to rest between successive fatigue tests. Force produced during shortening contractions was recorded together with electromyographic potentials throughout the tests, and tissue biopsies were removed at regular intervals by the freeze-nibbling technique.

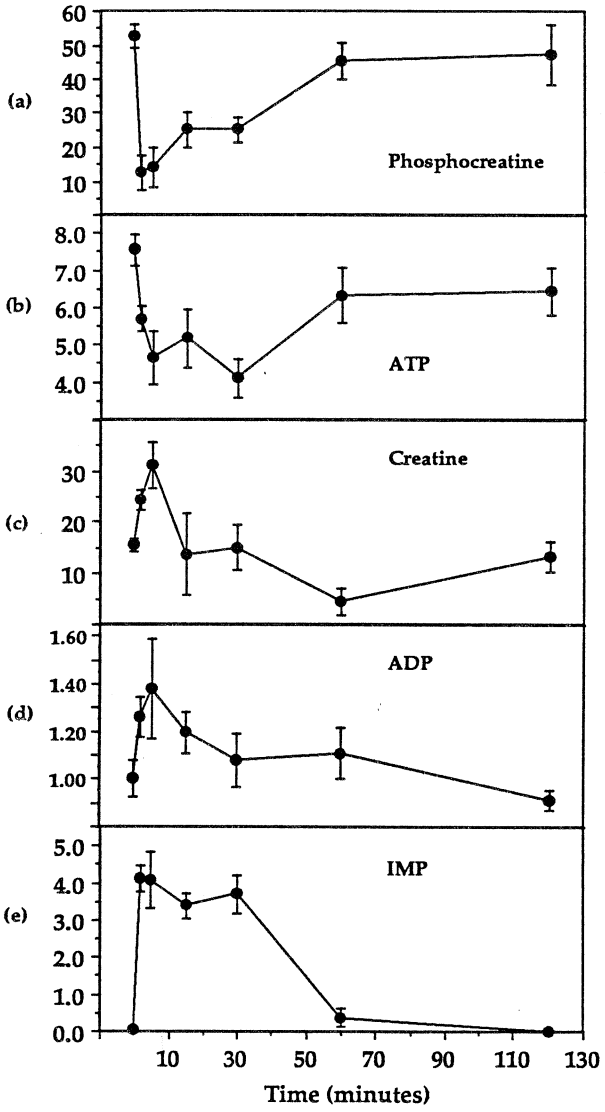

Figure 1 Changes in metabolite levels in TA muscle in response to stimulation at 10 Hz, monitored over a period of 120 minutes. **(a)** *Phosphocreatine;* **(b)** *ATP;* **(c)** *Creatine;* **(d)** *ADP;* **(e)** *IMP. Units; mole/mole NAD . n=14 at t=0; n=5-7 at each time point.*

Direct stimulation

In control TA muscles the force of contraction was reduced to approximately 10% by acute stimulation of the common peroneal nerve at 40 Hz, for 330 ms every 1000 ms. The stimulating voltage was then increased to 80 V and a prolonged 40 Hz tetanic contraction elicited by a pair of large-area surface electrodes placed on opposite sides of the muscle.

Results

TA muscles subjected to continuous stimulation at 10 Hz

Changes in metabolite levels in TA muscles stimulated at a constant frequency of 10 Hz are shown in Figure 1. Each time

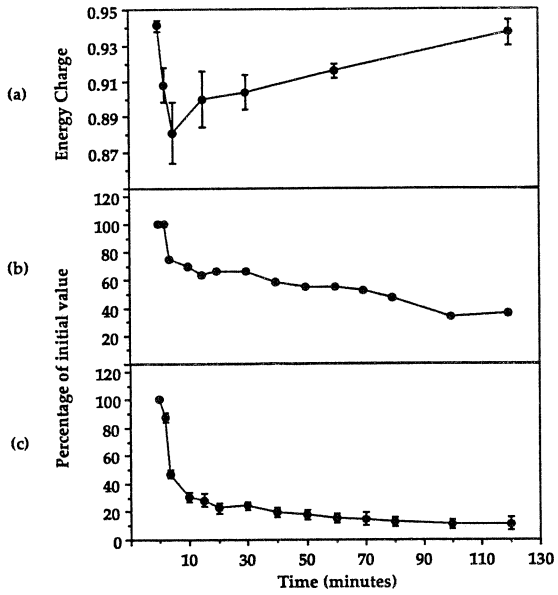

Figure 2 (a) Changes in energy charge, defined here as ([ATP] + [ADP]/2 / [ATP] + [ADP]), in response to stimulation at 10 Hz, monitored over a period of 120 minutes. (b) Corresponding force produced in shortening contraction. Values expressed as a percentage of the initial amplitude of contraction. (c) Corresponding mass electromyographic potentials. Values expressed as a percentage of the iniutial amplitude, measured from peak to peak of the depolarization and repolarization phases.

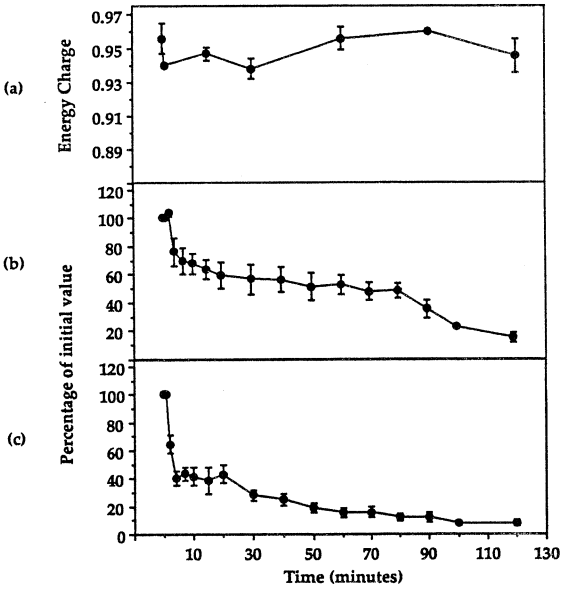

Figure 3 Responses of TA muscle to stimulation at a frequency of 40 Hz, for 330 ms every 1000ms, over 120 minutes. **(a)** *Energy charge;* **(b)** *force produced in shortening contractions;* **(c)** *electromyographic potentials. (b) and (c) values expressed as percentage initial measurements. Mean of 3 experiments.*

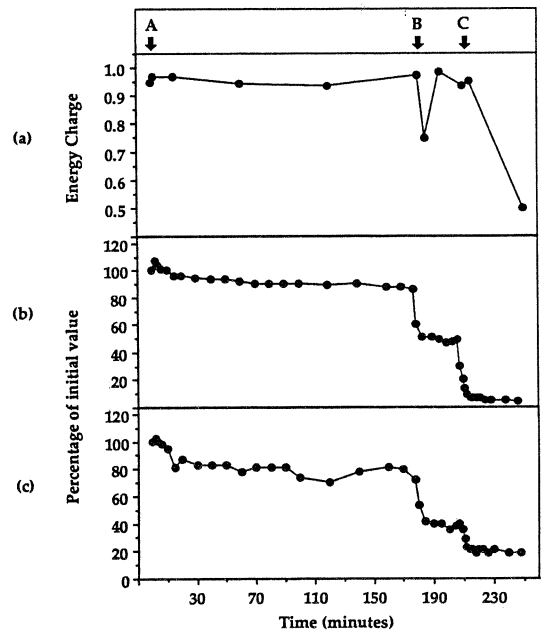

Figure 4 Responses of conditioned TA muscle to 3 stimulation patterns: (A) 40 Hz for 330 ms every 1000 ms; (B) 40 Hz for 330 ms every 500 ms; (C) 80 Hz for 330 ms every 500 ms. (a) Energy charge; (b) force in shortening contractions; (c) electromyographic potentials. In (b) and (c), measurements are expressed as a percentage of the initial values.

point represents the mean (± S.E.M.) of several experiments (14 at t = 0, and 5-7 at all other time points).

After 2 minutes of stimulation there were significant decreases in the levels of both PCr (76%) and ATP (55%) (Figures 1a and 1b). Conversely, there were increases in the content of Cr (50%), ADP (38%) and IMP (100-fold) within 5 minutes of the onset of stimulation (Figures 1c, d, e). After 15 minutes of stimulation, however, all of the metabolites had recovered to levels closer to those of control, unstimulated muscles. This process continued over the next 45 minutes, by which time the values attained did not differ significantly from those of control muscles (Figure 1). Despite continuing stimulation, the metabolites showed no significant departures from control levels from this point up to 24 hours (data not shown).

The adenylate energy charge [1] was used as a method of assessing the amount of stored energy available to the cell. This value is normally obtained from the equation:

$$\frac{[ATP] + [ADP]/2}{[ATP] + [ADP] + [AMP]}$$

The analytical HPLC method is capable of detecting AMP levels as low as 300 picomoles per sample. Nevertheless this detection limit was not low enough to register the AMP present either in unstimulated muscles or in stimulated muscles at any point during the time course. We therefore excluded from the above equation the small, but indeterminate, contribution from AMP, and computed the energy charge from the ATP and ADP contents alone.

Figure 2a shows the changes in energy charge resulting from stimulation. The resting level in the cells was 0.941±0.003.

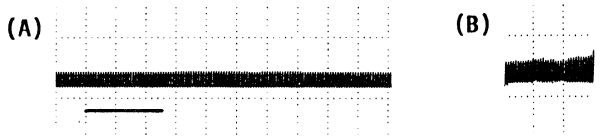

Figure 5 Response of fatigued TA muscle to direct stimulation (original unretouched trace). (a) Force produced in shortening contractions, elicited at 40 Hz for 330ms every 1000 ms, at the end of 150 minutes. Force has fatigued to 8% of initial value. (b)Effect of immediate application of direct stimulation at 40 Hz. Bar represents 30 s.

After stimulation for 5 minutes the value was 0.881±0.017, a reduction of 6.4% ($P < 0.0001$, unpaired Student's t-test). With continued stimulation the energy charge again increased, recovering to levels that were not significantly different from control values after 120 minutes. Since energy charge encompassed changes in the levels of both ADP and ATP, it served as a useful index of metabolic response when comparison was made with physiological and electrophysiological changes in these and subsequent experiments.

In response to stimulation at 10 Hz, force and peak-to-peak electromyographic potentials decreased rapidly over the first 4 minutes to 75% and 46%, respectively, of their initial levels. They declined more slowly over the next 115 minutes, force reaching 27% and electromyographic potentials 10% of initial values (Figures 2b and 2c).

TA muscles subjected to a standard fatigue test

To facilitate comparison with the response of the conditioned muscle (see below), further experiments were conducted in which control TA muscles were subjected to a standard fatigue test, consisting of 40Hz for 330 ms every 1000 ms [8]. The results, shown in Figure 3, were obtained with the freeze-nibbling biopsy technique and represent the mean of 3 experiments.

Energy charge showed a small phasic response to stimulation which did not achieve significance (Figure 3a). Force and electromyographic potentials decreased rapidly over the first 4 minutes to 76% and 65% of their initial levels, and declined less steeply over the subsequent 115 minutes to 15% in the case of force and 2% in the case of electromyographic potentials (Figures 3b and 3c).

Conditioned TA muscle

Well-documented changes in blood flow, metabolism and myosin isoforms render a rabbit TA muscle highly resistant to fatigue after prolonged stimulation at 10 Hz [4, 37, 41]. In the present study a muscle that had been stimulated in this way for 8 weeks was subjected to the standard fatigue test (40 Hz for 330 ms every 1000 ms). Analysis of muscle biopsies obtained by freeze-nibbling provided no evidence of significant changes in energy charge (Figure 4a). After 178 minutes of stimulation,

the force had declined to 86%, and electromyographic potentials to 72%, of control values (Figures 4b and 4c).

At this stage the functional demand on the muscle was escalated by changing the stimulation pattern to 40 Hz for 330 ms every 500 ms. Metabolites, force and electromyographic potentials then showed a marked response. Within 2 minutes, the energy charge had decreased from 0.946 to 0.748 (Figure 4a). From this point, however, the muscle began to recover metabolically, and the energy charge regained pre-stimulation levels after only 15 minutes. The initial decline in the energy charge coincided with significant decreases in force and electromyographic activity; thus after only 2 minutes of the new stimulation régime, force had decreased to 51% and electromyographic potentials to 41% of their initial values (Figures 4b, c). These levels were then maintained.

A further escalation of demand was effected by changing the stimulation pattern to 80 Hz for 330 ms every 500 ms. As with the previous stimulation pattern, the muscle responded with a reduction in energy charge. After 30 minutes of stimulation with this régime, energy charge had declined to 0.5, a value which corresponds to zero ATP. Re-examination of the chromatogram confirmed that ATP was indeed at an undetectable level at this point. The force and electromyographic signals, which had stabilised at about 49% and 39% of their respective initial levels, decreased rapidly when the stimulation régime was changed; by the end of the test, force had declined to 4%, and electromyographic potentials to 19%, of their amplitudes at the beginning of the experiment.

Direct stimulation

In this experiment, direct stimulation of a fatigued muscle was used with a view to determining how much of the decline in force could be attributed to neuromuscular block. A control TA muscle was stimulated indirectly with the standard test pattern (40 Hz for 330 ms every 1000 ms) for 150 minutes, by which time the force had declined to 8% of its initial value (Figure 5a). The intensity was then increased to 80 V and stimulation applied directly to the muscle at a frequency of 40 Hz. As Figure 5b shows, the force did not appreciably increase in amplitude.

Discussion

The initial effects of subjecting skeletal muscle to an increase in energy demand are known to include changes in the levels of PCr and ATP [9, 12, 13, 22, 23, 25, 33] and secondary changes involving the purine nucleotide cycle [15, 31, 32, 33, 40]. The response of the purine cycle is indicative of metabolic stress [19]; by activating glycogenolysis [15] and regulating the relative concentrations of adenine nucleotides, it helps to preserve the energy state of the system [40]. Nevertheless, when the demand is prolonged, these mechanisms are unable to prevent a fall in the levels of both PCr and ATP.

Many authors have sought to interpret such a decline in high-energy phosphates as a primary cause of force fatigue in skeletal muscle. Chance and his colleagues [9] suggested that isometric force declined with PCr and Dawson et al. [13] reported that the reduction in force was correlated with the rate of ATP utilization. There have, however, been reports of metabolic recovery in the presence of force fatigue. Meyer & Terjung [32], Hultmann & Spriet [24] and Takata et al. [43] all observed a phasic change in metabolites which took place within a period of less than 60 minutes. These authors measured metabolites by analysis of biopsy samples or by nuclear magnetic resonance spectroscopy (NMR).

The former technique, which was used in the early stages of the present study, has the disadvantage that a large number of separate experiments is needed in order to build up a detailed time course of events. NMR overcomes this objection, but it yields measurements of relative, rather than absolute levels, it has limited temporal resolution, and the intolerance of the equipment to the presence of ferromagnetic materials can create technical problems in providing for simultaneous measurement of force. The 'freeze-nibbling' technique developed in the course of the present experiments affords some of the advantages of each of these methods. It is capable of providing absolute measurements of metabolite levels at as many as 15 points in the time course of a single experiment, and samples can be removed within seconds of each other if required. Force and electromyographic potentials can be monitored throughout the procedure, and in control experiments we sought, and were unable to find, any evidence of perturbations in these measurements caused by the removal of biopsies. The technique is open to the general objection that force and electrical responses reflect bulk properties of the muscle, whereas sampling by the freeze-nibble approach is necessarily limited to the superficial layers of the muscle. Our experience nevertheless indicates a high level of agreement between the results of analysing freeze-nibbled samples on the one hand, and large, more representative, samples obtained by freeze-clamping on the other. Such agreement would clearly be even greater in muscles, such as conditioned muscles, in which there was less regional variation in composition. The freeze-nibbling technique therefore offers a tool for increasing our understanding of a possible involvement of metabolite depletion in the aetiology of force fatigue.

Continuous simulation at 10 Hz represented a major metabolic challenge to the rabbit TA muscles used in these experiments. The inability of the muscle to meet the associated energy demand was clearly reflected in the precipitous initial fall in the levels of high energy phosphates (Figures 1a and b). These results are in close agreement with the reports cited previously, which described a reduction in ATP and PCr and an increase in Cr, ADP and IMP taking place over a similar period of stimulation. At this rate of decline, the short-term energy stores of the muscle should have been totally depleted within 15 minutes. Contrary to this expectation, however, measurements after 15 minutes of stimulation showed evidence of a recovery in the energy charge of the muscle. This process continued until, after 2 hours, high-energy phosphate metabolites and compounds involved in the purine nucleotide pathway had regained completely their levels prior to the onset of stimulation.

At no point did AMP rise to an extent that was measurable by HPLC. This was probably due to the prompt removal of this compound by deamination to IMP within the purine nucleotide cycle, in agreement with the findings of Norman et al. [33]. IMP showed the rise which is the hallmark of metabolic fatigue, but declined again to undetectable levels as ATP increased.

A standard fatigue test, consisting of bursts of stimuli at 40 Hz lasting 330 ms and repeated every 1000 ms [8], elicited a similar response, although the change in energy charge was less marked.

In both sets of experiments, the phasic metabolic responses to stimulation were accompanied by a decline, rapid at first and

then becoming more gradual, in both force production and the amplitude of electromyographic potentials. Force fatigue was more severe for the standard fatigue test than for continuous stimulation at 10 Hz. In either case, stimulation for 2 hours produced a condition in which the metabolites had returned to control levels but the muscle was exhibiting profound force fatigue. Although explanations of force fatigue in terms of a decline in the availability of high-energy metabolites were not inconsistent with the effects we saw over the first 15 minutes of stimulation, they were completely incompatible with the dissociation between metabolic status and force fatigue which appeared during longer periods of stimulation.

Further experiments were performed on muscles which had been transformed by chronic electrical stimulation at a frequency of 10 Hz for 8 weeks. Such muscles are highly resistant to fatigue as a result of changes in blood flow, metabolism and myosin isoforms [4, 20. 37, 41]. For this reason there were problems at first in devising tests that would tax the muscle sufficiently to produce force fatigue. One of these experiments, illustrated in Figure 4, consisted of 3 fatigue patterns of increasing severity, delivered in sequence without intervening opportunities for recovery.

Although the muscle showed prolonged resistance to force and metabolic fatigue under the conditions of the standard fatigue test, the two more demanding patterns both produced a well-defined response. When the first of these was applied, the amplitudes of the force and electromyographic potentials decreased rapidly to a new lower level, which appeared to be well maintained. The associated changes in metabolite levels were similar to the response seen earlier in unconditioned muscles: a rapid decline in energy charge with a subsequent recovery. In fact, the energy charge recovered to control values within 30 minutes, more quickly than in unconditioned muscles stimulated at 10 Hz. When the final stimulation pattern was applied, the energy charge fell to 0.5, the level at which ATP is zero. It is not possible to say whether it recovered subsequently, because the time course had not been anticipated, and no samples had been taken at later time points.

Our observations on non-conditioned muscles confirm earlier findings that force fatigue can persist in spite of recovery of high-energy phosphates to normal levels [24, 32, 43]. Such muscles are composed predominantly of Type 2 fibres and it has been suggested that metabolic recovery is possible because of failure of these fibres in the early stages of stimulation [32]. In the present experiments, however, a muscle that had been completely conditioned at 10 Hz for 8 weeks was equally capable of mounting a phasic response in metabolite levels. Since the muscle in question had been transformed, and was therefore homogeneously composed of Type 1 fibres, this behaviour cannot be unique to Type 2 fibres. Rather it appears that the fall and subsequent recovery of metabolite levels is a feature of all muscle fibres that have been taxed with an energy demand beyond their aerobic capacity. The greater resistance to fatigue which is a feature of conditioned muscle simply means that the response can only be demonstrated at a metabolically more challenging level.

Recently Green et al. [16] also reported a phasic response in metabolites in rabbit TA muscles subjected to stimulation at 10 Hz. Although they sampled at only 3 time points in the period covered by the present experiments, results from the part of the time course which is common to the two studies are in good agreement.

The transient nature of the metabolic response accounts for the previous observations of Henriksson et al. [21], who failed to detect any departure from normal in the levels of metabolites 30 hours after the onset of stimulation, and for findings reported previously by this group [30], in which metabolite levels were found to be normal in a muscle with severe force fatigue induced by stimulation for 6 hours. Henriksson et al. [21], suspecting that the muscle may have shown a reduction in metabolites at an earlier stage, suggested that it may have been relieved of the energy demands by some form of inactivation. The inclusion of continuous monitoring of force and electromyographic potential in the present study enables us to say a little more about the nature of this mechanism.

Acute muscle damage can be eliminated as a factor, because substantial increases in force and electromyographic potentials could be recorded from the fatigued muscle after only 2 minutes of rest (data not shown). The decline in electromyographic potentials that accompanied force fatigue suggested that some degree of neuromuscular block was present. However, since force was not even partially restored by direct stimulation (Figure 5) we conclude that failure of the muscle fibres to contract could not be due to neuromuscular block alone. Our data therefore supports the conclusions reached by previous observers: that contractile fatigue associated with metabolic recovery of the muscle is due to a progressive failure of activation [21, 24, 32, 43]. We think it likely that the block occurs at the level of excitation-contraction coupling. The net effect of this response is to restore the energy charge and so to preserve the viability of the cell pending either the removal of the conditions responsible for the excessive demand or an expansion in the ATP-generating capacity of the cell. The latter response is, of course a well-known part of the stimulation-induced transformation of mammalian fast skeletal muscle, but it is relevant to observe that it also takes place in avian muscle, in the apparent presence of a profound and prolonged block of contractile activity [2].

These phenomena have some important consequences. In recent years a vigorous research field has developed around the potential clinical use of fatigue-resistant skeletal muscle grafts. Examples of these applications include various ways of augmenting the function of a failing heart by means of a *latissimus dorsi* graft [11] and surgical construction of a neosphincter from the *gracilis* muscle [44]. Fatigue resistance in these grafts must be induced by conditioning the muscle, and interest has been expressed in the possibility of monitoring this process in patients non-invasively by ^{31}P-NMR spectroscopy. Our results show that such measurements must be interpreted with extreme caution, for normal metabolite levels can mean either that the muscle has not been challenged unduly by the test pattern or that it has been challenged so severely that excitation-contraction coupling has been blocked. Where a reduction in the energy charge forms part of the response, it appears to be limited to the initial stages. Any test must therefore record the full time course of the response, with a temporal resolution that may be beyond current NMR capabilities.

As pointed out in the Introduction, part of the reason for seeking a detailed picture of early events in the response of skeletal muscle to chronic stimulation is to establish what signalling mechanisms might be responsible for initiating the subsequent changes in gene expression. If the products of energy metabolism were among the triggers, they could provide a direct link between phenotype and functional demand.

This attractive possibility now appears to be more remote. While it is conceivable that the transient changes in metabolites observed in these experiments could act as intracellular signals, they would need to be linked to longer-lasting events if they were to be effective on an appropriate time scale. On the whole it now seems more likely that the signalling pathway for transformation is independent of the levels of high-energy phosphates and other metabolites, and that it is related instead to events associated with the depolarization of the muscle membrane. Such events would continue to exert their influence even after the uncoupling of excitation-contraction, and by orchestrating the development of aerobic pathways better adapted to the increased energy demand could create the conditions for a gradual return of force.

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