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CHANGES IN CROSSBRIDGE AND NON-CROSSBRIDGE ENERGETICS DURING MODERATE FATIGUE OF FROG MUSCLE FIBRES

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SUMMARY

1. The effect of sarcomere length (SL) during a fatiguing series of isometric tetani of frog muscle fibres was investigated. Tetani at 2·3 μm SL were more fatiguing than tetani at 3·2 μm SL, in that force declined twice as much and relaxation became much slower.

2. In a second set of experiments the force and heat production were measured during a series of fatiguing tetani. Heat was separated into two components: (a) crossbridge heat which is dependent on filament overlap and interaction, and (b) non-crossbridge heat which is independent of filament overlap and due to Ca^{2+} turnover.

3. In a series of fifty tetani, force, crossbridge heat and non-crossbridge heat each declined by 25–30 % of its initial value.

4. The 25 % reduction in non-crossbridge heat occurred completely during the first few tetani of the fatiguing series while force declined by less than 3 %. This may be due to a reduction in Ca^{2+} binding to parvalbumin and to Ca^{2+} remaining bound during the remainder of the fatigue series.

5. After the first few tetani of the fatigue series the non-crossbridge heat hardly changed as force declined by a further 25 % of its initial value. Continuing reduction of force with constant Ca^{2+} turnover indicates a reduction in the Ca^{2+} sensitivity of the filaments, and/or a reduction in the average force per attached crossbridge.

6. At the start of the fatiguing series, as force declines by about 7·5 % there is a much larger decline of crossbridge heat (17 %). The reason for this is unknown. Later in the series, force declined more rapidly than heat. This is probably due to a progressive accumulation of inorganic phosphate which acts by depressing force more than it depresses ATP breakdown.

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INTRODUCTION

Fatigue, the progressive loss of the ability to produce active force, is a complex process in which many factors have a role (see review by Westerblad, Lee, Lännergren & Allen, 1991). In intact muscle fibres, fatigue can be caused by fully activated crossbridges failing to produce their normal isometric force (Edman & Lou, 1990). Further evidence that the crossbridges are changed by fatigue is the decrease in the maximum velocity of shortening (Edman & Mattiazzi, 1981). Fatigue can also be caused by changes in the Ca^{2+} reactions, so that fewer than the normal number of crossbridges produce force. The reactions of Ca^{2+} include: release from the sarcoplasmic reticulum, binding to troponin and parvalbumin, thin filament 'dis-inhibition', release of Ca^{2+} from binding sites, ATP-powered active transport of Ca^{2+} into the sarcoplasmic reticulum. The turnover of Ca^{2+} through these reactions is coupled to ATP breakdown, as is the crossbridge cycle. Heat is produced in proportion to the amount of ATP that is split and therefore heat production can be used to monitor these processes. The heat can be partitioned into a crossbridge component and a Ca^{2+} turnover component on the basis of its dependence on filament overlap. This partitioning of energy output gives information about the amount of Ca^{2+} turnover, which is not available from other complementary methods used to investigate activation, which measure the level of free Ca^{2+}, not Ca^{2+} flux.

The rationale of the experiments was that if fatigue includes changes in crossbridge reactions, such as the rate or extent of ATP breakdown or other steps in the cycle, these would be detected as a change in the crossbridge heat. If the fatigue produced here involves a reduction in Ca^{2+} turnover, this would be detected as a reduction in the non-crossbridge heat. It should be noted that a reduction in the sensitivity of the thin filaments to Ca^{2+}, which does not change Ca^{2+} turnover but can contribute to fatigue, would not be detected in this way.

We investigated moderate fatigue, about a 25 % reduction of force, produced by repeated tetani of intact frog muscle fibres. The results show that there is a reduction in heat from Ca^{2+} turnover at the start of a fatiguing series when there is little loss of force. However, this component of the heat remains relatively constant during the time when most of the force loss occurs; this supports evidence from other experiments (Westerblad *et al.* 1991) that fatigue involves changes in the co-operative and non-linear relation between activation and force. During most of the fatiguing series, the crossbridge heat changes in a way that is probably due to the accumulation of inorganic phosphate. However, early changes in crossbridge heat indicate that there is some additional factor at this time.

METHODS

Frogs, *Rana temporaria*, were killed by decapitation followed by destruction of the brain and spinal cord. Single or small bundles of up to twenty fibres were dissected from the lateral tibialis anterior. The bathing solution contained (mmol l^{-1}): NaCl, 98; KCl, 2·0; $CaCl_2$ 1·8; Hepes, 20; and the pH was adjusted to 7·2 with NaOH. During experiments, the temperature was maintained at 5·6–5·7 °C. The tendons were held in platinum foil T-shaped clips.

The relation between stimulus strength and twitch force was determined to find the supramaximal stimulus. For tetanic stimulation, a pulse frequency of 10–15 Hz was sufficient to produce a fused tetanus.

At the end of each experiment the fibre length was measured under a dissecting microscope. Then tendons and adhering solution were removed and the fibres allowed to dry in air. Dry weight was measured using an electronic balance (Cahn Instruments, USA; model 26). Force and heat were normalized to take account of differences due to the sizes of the fibre preparations as follows. Dry weight divided by fibre length was used as an estimate of cross-sectional area, and force was divided by this estimate. Heat was divided by dry weight.

Experiment 1: *effect of filament overlap during fatique on force*

In this experiment, force but not heat was measured. Fibres were held horizontally in a water-cooled, Perspex bath; pre-cooled solution flowed slowly (about 2 ml min^{-1}) through the bath. One end of the preparation was fixed to semiconductor strain gauge (Sensonor, model AE801) and the other to a hook attached to a micrometer which allowed fibre length to be adjusted. Sarcomere length was measured by laser diffraction and set to either 2·3 or 3·2 μm. Stimuli were given via platinum electrodes parallel to the fibres. The fatiguing series consisted of fifty 1 s tetani given every 15 s. Control of stimulation and tension recording were performed using a laboratory microcomputer (Tandon, PAC286+) using a multi-function interface board (Scientific Solutions, LabMaster, Solon, OH, USA) and running custom-written programs.

Experiment 2: *crossbridge heat and non-crossbridge heat during fatigue*

In this experiment, both force and heat were measured. The fibre preparation was mounted between a motor (Ling Dynamic Systems, Series 100, Royston, UK), for altering fibre length, and a tension transducer (Cambridge Technology, model 401, Watertown, MA, USA). The fibres were in contact with a thermopile to record temperature. Signals were recorded on a digital oscilloscope.

The thermopile was made by vacuum deposition of antimony and bismuth on a mica substrate as described by Mulieri, Luhr, Trefry & Alpert (1977). Each thermocouple produced 83·2 $\mu V\ °C^{-1}$ temperature difference.

The thermopile was mounted in a metal block and enclosed in a thermostated, thick-walled chamber. The air space in the chamber was relatively small (about 10 ml). A few drops of bathing solution were put on the thermopile surface before mounting the fibres and most of this solution was removed just before the chamber was closed. Evaporation of water from the surface of the thermopile in the closed chamber was found to produce a detectable absorption of heat. This was prevented, however, by placing a layer of filter paper soaked in bathing solution diluted by 20 % in the groove in the lid directly above the thermopile surface.

The number of thermocouples used for recording was varied between twelve and twenty (3–5 mm of thermopile). The region of thermopile used for recording was selected so as to span the central portion of the fibre preparation. As described later, there was very little change in sarcomere length in this region of the fibre preparation after the initial rapid rise of force during a tetanus.

Temperature records were converted to heat and corrected for heat loss using characteristics determined for each fibre preparation at each of the fibre lengths used. These characteristics were determined by passing a known current through the whole thermopile; this produces a known quantity of heat due to the Peltier effect. The time course of heat loss following a period of Peltier heating was adequately described by a single exponential function. Typically the time constant of this process was between 2 and 4 s.

The values that are reported here are the heat produced by the time of the last stimulus. They have not been corrected for the small amount of stimulus heat, which was estimated to be less than 5·5 % of the heat produced by an unfatigued preparation at the length optimum for force. This estimate was based on measurements made on three fibre bundles that had either become inexcitable (no force response to stimulation) in the course of the experiment, or were given a long train of strong stimuli to make them inexcitable. Heat measured during application of usual strength stimuli was taken as stimulus heat. Records were not corrected for lag in the recording system.

Creep during tetani at long sarcomere length

At long sarcomere lengths the force records showed 'creep', the relatively slow increase in force after the initial rapid rise (see Huxley, 1980, pp. 56–59). Creep is due to shortening of some sarcomeres which stretch others in series with them (Huxley & Peachey, 1961; Julian, Sollins & Moss, 1978). The shortening sarcomeres produce an increasing amount of force because their filament overlap is increasing, and the lengthening sarcomeres produce more than the isometric force because crossbridges resist stretching with a force which is greater than the force they produce when their length is constant.

To exclude creep force, we measured force at the intersection of extrapolations of the early rapid rise and later creeping force produced in tetani at long sarcomere length (Gordon, Huxley & Julian 1966*a*).

Control experiments were done to see whether creep was accompanied by sarcomere movements in the region of the fibre where heat was to be recorded. In experiments on four fibre preparations, the laser diffraction patterns were recorded on film during tetani after setting the sarcomere length to 3·0 μm. In one of the fibre preparations a shortening of 1 % was detected, and in the other three, no movement was detected. The 1 % change corresponds to the limit of change that could be reliably detected. We concluded that during force creep in our preparations, a small proportion of the sarcomeres at the ends of the fibres shortened, where heat was not being recorded. The rest of the sarcomeres were stretched, but each sarcomere moved by an undetectably small amount, which would not significantly affect our records of heat production.

Filament overlap

It was not possible to measure sarcomere lengths in the fibres on the thermopile because of physical constraints of the thermopile and chamber. Therefore, filament overlap during isometric tetani at different fibre lengths was calculated from the measured value of the active force in the unfatigued state, corrected for any creep of force. The following assumptions were made: filament overlap was 100 % at the fibre length giving maximum force, and filament overlap decreases in direct proportion to active force for isometric tetani at longer lengths.

Stimulation protocols

Heat and force were recorded during 0·5 s tetani at 3 min intervals at a range of sarcomere lengths to establish their dependence on filament overlap in the unfatigued state.

Records were then made on each fibre preparation during the fatiguing protocol, which consisted of fifty tetani of duration 0·5 or 1·0 s every 10 s (duty cycle 0·05 or 0·10). Two different degrees of filament overlap were used during the fatiguing series. For most tetani filament overlap was optimal for active force development (100 % overlap). However, at regular intervals between tetani, the motor stretched the fibre to reduce filament overlap, and then after one tetanus the motor returned the fibre preparation to its original length. Filament overlap was reduced to 31·8 $\pm$ 2·2 % ($\pm$ S.E.M.; $n = 7$ fibre preparations) for the 5th, 10th, 20th, 30th, 40th and 50th tetani in the series. As will be described later, we used these results to partition the energy into the component due to crossbridge reactions and the component due to non-crossbridge reactions.

Statistical analysis

One-way analysis of variance was used to determine the significance of slopes of the relations between filament overlap and heat or force. The 0·95 level of confidence was used to determine statistical significance. Values are means $\pm$ S.E.M. throughout the text.

RESULTS

Experiment 1: effects of filament overlap during fatigue on force

The force produced by a small bundle of muscle fibres in the first and last isometric tetani (1 s duration) in a series of fifty (1 s tetanus every 10 s) are shown

in Fig. 1. The recordings in Fig. 1*A* correspond to tetani performed with resting sarcomere length 2·3 μm throughout the fatiguing protocol. In the 50th tetanus, peak force was reduced relative to that in the first contraction and relaxation was markedly slower. In Fig. 1*B*, recordings from the same preparation are shown.

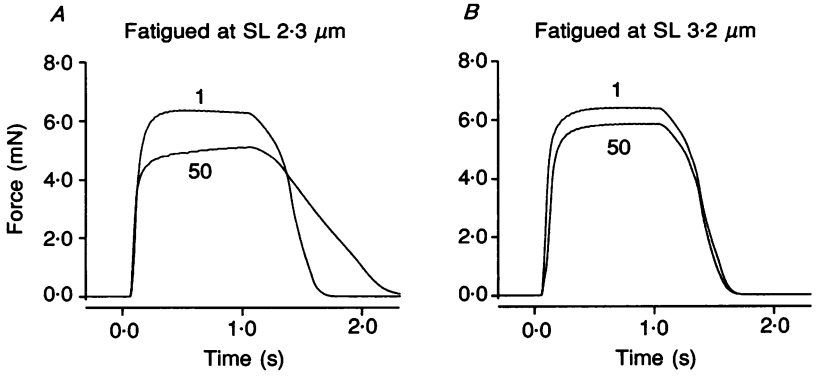

Fig. 1. Force records from Expt 1. Force produced by the same fibre bundle in the 1st and 50th tetani in two series of isometric tetani. The number near each record indicates the tetanus number in the series. The start of the first stimulus pulse was at time 0. *A*, resting sarcomere length was 2·3 μm for all tetani. *B*, records from 1st and 50th tetani where the resting sarcomere length was 2·3 μm. For all the intervening tetani of the series (2nd to 49th) the sarcomere length was set to 3·2 μm before stimulation.

However, in this case only the first and last tetani of the series (those shown) were performed with sarcomere length 2·3 μm; for contractions 2–49 the fibre was at a sarcomere length of 3·2 μm. The total tension–time integral during the series of tetani at long sarcomere length was only 40·2 ± 8·1 % (mean ± S.E.M., $n = 5$ fibre preparations) of that produced during the series at the shorter sarcomere length. When the fibre was fatigued at the long sarcomere length, the decline in force was smaller and there was little slowing of relaxation compared with the results for fatigue at 2·3 μm. Experiments on five preparations showed that force declined by only half as much when fatigued at long sarcomere length (12·0 ± 3·1 % for 3·2 μm sarcomere length, and 24·6 ± 1·0 % for 2·3 μm). Changes in the time course of relaxation were determined from the time taken for force to fall from 98 to 2 % of maximum force. The effect of fatigue on relaxation was also much smaller at the long sarcomere length; relaxation time was increased by 10·0 ± 8·1 % at 3·2 μm, and 37·4 ± 7·8 % when the fibre was fatigued at 2·3 μm.

Experiment 2: crossbridge heat and non-crossbridge heat during fatigue

The average maximum force developed by the seven fibre preparations used for the myothermal experiments was 0·73 ± 0·08 N m (g dry weight)$^{-1}$. The average heat produced during the 0·5 s tetanus was 141·7 ± 26·6 mJ (g dry wt)$^{-1}$, comparable to previous reports (Curtin, Howarth & Woledge, 1983; Curtin, Rall & Woledge, 1986; Elzinga, Stienen & Wilson, 1989).

Non-crossbridge heat

Before recording heat production during fatiguing tetani, the fraction of the heat that was unrelated to crossbridge interaction (non-crossbridge heat) was determined from the relation between heat and filament overlap. Figure 4 (●)

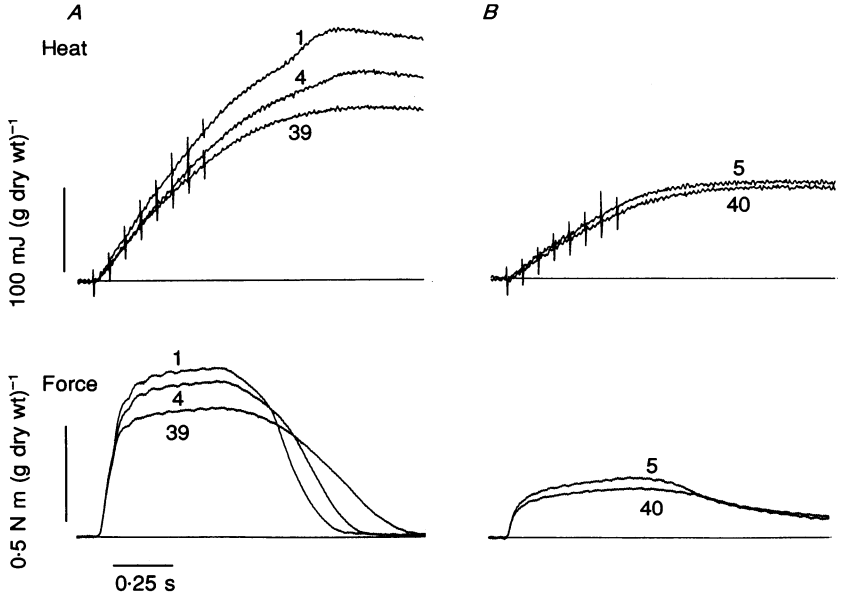

Fig. 2. Records of heat and force from Expt 2, fifty 0·5 s tetani at 10 s intervals. Superimposed force records and heat records from a fibre preparation. The number near each record indicates the tetanus number in the series. *A*, tetani with fibre length set to give maximum active force (100 % filament overlap). *B*, tetani with 32·7 % filament overlap. Resting fibre length at 100 % filament overlap = 8·4 mm; dry weight = 0·194 mg; cross-sectional area = 0·115 mm^2, calculated assuming 5 mg dry weight is equivalent to 1 mm^3 wet fibres.

shows this relation for one fibre preparation; as previously described the relationship is linear. The non-crossbridge heat was estimated by extrapolating the relation to zero filament overlap as shown in the figure. The results for all seven fibres preparations showed that in the unfatigued state, the non-crossbridge heat was 26·5 ± 1·5 % (± S.E.M., $n = 7$) of the heat. Control experiments were done on three fibre preparations that confirmed that the relation between heat and filament overlap remained linear in the fatigued state.

Force and heat production during fatigue

Fatigue was produced by a series of fifty tetani of 0·5 or 1 s duration. Ten observations, using seven fibre bundles, were made. Three preparations performed a series of fifty 0·5 s tetani and also a series of fifty 1 s tetani.

Figure 2 shows records of the force and heat produced by one preparation at various times during a series of tetani. The time course of changes in force and heat

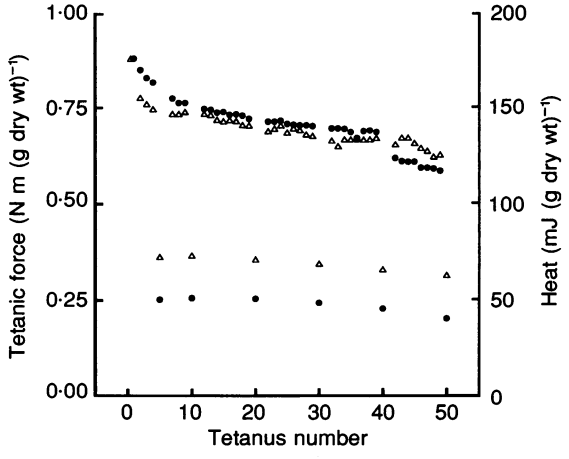

Fig. 3. The tetanic force (●) and heat production (△), measured at the time of the last stimulus, in tetani during a series of fifty 0·5 s tetani at 10 s intervals. The upper two sets of points are for 100 % filament overlap. The lower sets are for tetani at 32·7 % overlap (5th, 10th, 20th, 30th, 40th and 50th tetani in the series). Values for the tetanus immediately following tetani at 32·7 % overlap are not shown because the fibres were shortening.

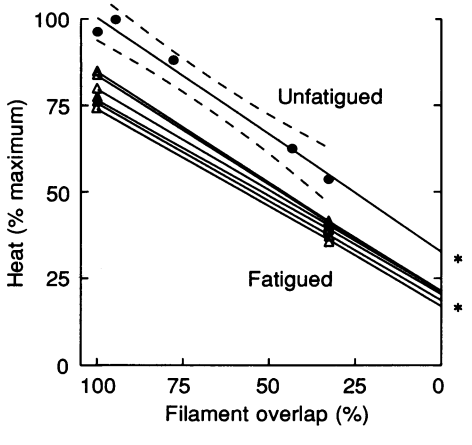

Fig. 4. Heat produced by one fibre bundle plotted as a function of filament overlap. The extent of filament overlap was estimated from the active force, corrected for force creep (see text). ●, unfatigued: heat produced in 0·5 s isometric tetani at 3 min intervals. The length of the preparation was changed between tetani to vary the filament overlap. The regression line is the continuous line and the dashed lines show the 95 % confidence bands. △, fatigued: heat produced during a fatigue series with 100 % filament overlap and with 32·7 % filament overlap. Each line joins values for consecutive tetani; the uppermost line joins values from the 4th tetanus (100 % overlap) and the 5th tetanus (32·7 % overlap). Extrapolation of these lines to 0 % filament overlap was used to estimate non-crossbridge heat in the unfatigued state and during the fatigued series (*).

for the same preparation are detailed in Fig. 3. The force and heat are shown for tetani at two different degrees of filament overlap during the fatigue protocol; the upper values are for 100 % overlap and the lower values are for 32·7 % overlap (tetanus numbers 5, 10, 20, 30, 40 and 50).

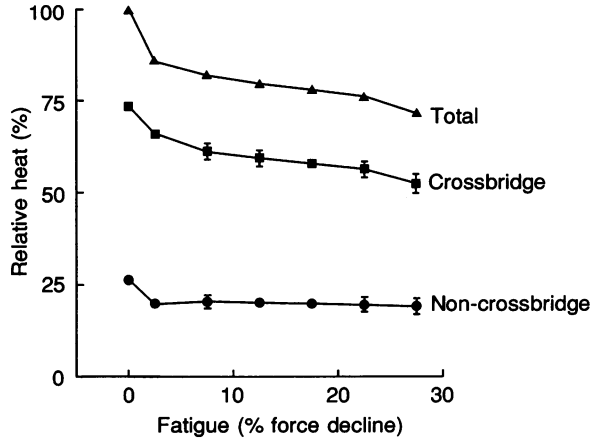

Fig. 5. Mean (± S.E.M.) total heat, crossbridge heat, and non-crossbridge heat as a function of force decline during a series of fifty tetani. Results for 10 experiments on 7 fibre preparations. All heat values are expressed relative to the total initial heat in the 1st tetanus of the fatigue series. To get average values of heat as a function of force decline, data were 'binned' according to the magnitude of force decline. Bin width was 5 %. Each mean value of non-crossbridge heat is the average of between 6 and 15 data points. Mean values of total heat are from 10 to 22 data points. The number of points for non-crossbridge heat is smaller because estimates were only made 6 times during the series of fifty tetani. Crossbridge heat was the difference between the mean values of non-crossbridge heat and total heat. Standard errors for crossbridge heat were obtained by taking the square root of the sum of the squares of the errors of the total heat and non-crossbridge heat.

Both force and heat production decreased during the series of tetani. However, results for 100 % overlap show that the decrease in force occurred more steadily throughout the fifty contractions than the decrease in heat production. In ten experiments on seven fibre preparations, force decreased by 6·1 ± 1·2 % in the first four tetani and a further 20·9 ± 3·7 % in the remaining forty-five tetani. In contrast, the amount of heat produced decreased as much in the first four tetani (15·8 ± 0·3 %) as it did in the remainder of the fatigue series (15·0 ± 4·2 %). In tetani with reduced overlap, force and heat changed only a small, but significant, amount during the contraction protocol. Force decreased 3·0 ± 1·3 % and heat production decreased 6·7 ± 1·9 % from tetanus 5 to tetanus 50.

Changes in crossbridge and non-crossbridge heat during fatigue

The main aim of this study was to determine relative changes in crossbridge and non-crossbridge heat during a fatiguing series of tetani. As described above, the filament overlap was changed at intervals during the fatigue protocol so that

successive tetani were performed with either 100 % or about 30 % overlap. To determine how non-crossbridge heat changed during fatigue, the heat produced in tetani during the fatigue protocol was expressed as a function of filament overlap. In Fig. 4 (△), the heat values for the two tetani of a pair with different filament

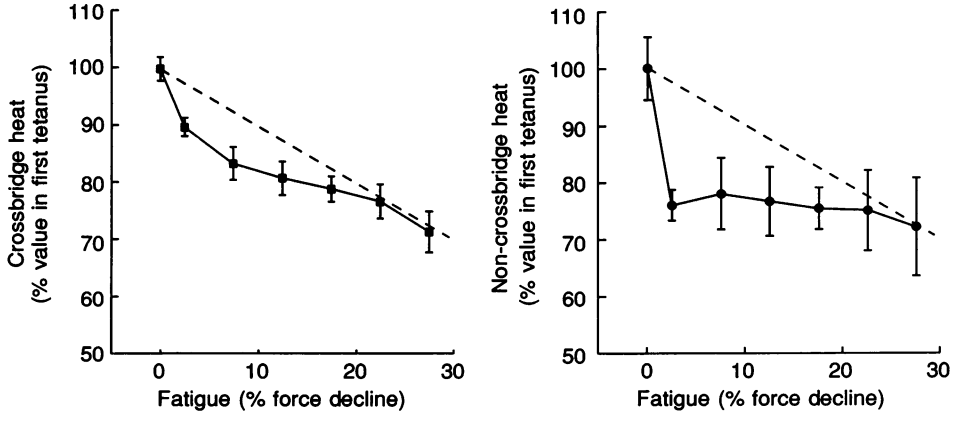

Fig. 6. Mean values (± S.E.M.) of crossbridge heat (*A*), non-crossbridge heat (*B*) as a function of force decline in a fatiguing series of fifty tetani. Results for 10 fatigue series on 7 fibre preparations; 3 preparations performed 2 fatigue series with different tetanus durations. The dashed line is the line of identity of percentage decline of force and heat.

overlap were joined by a line which, when extrapolated to 0 % filament overlap, provided an estimate of the non-crossbridge heat. This method provided six estimates of non-crossbridge heat during each fatiguing series of fifty tetani. The example shown in Fig. 4 is typical in showing that most of the decrease in non-crossbridge heat took place early in the series of tetani, between the first tetanus (unfatigued) and the first estimate of non-crossbridge heat (i.e. between tetanus 1 and pair of tetani 4 and 5).

The partitioning of heat production during fatigue is summarized in Fig. 5, which shows the mean results for the ten experiments on seven fibre preparations. The values of total heat, crossbridge, and non-crossbridge heat are shown expressed as a fraction of the total heat in the first tetanus of the fatiguing series. Crossbridge heat was calculated as the difference between the mean total heat and mean non-crossbridge heat.

At the end of the fatiguing series, force was 73·1 ± 9·5 % of that in the first tetanus of the series and the total heat was 69·2 ± 8·8 %. Both the crossbridge heat and the non-crossbridge heat decreased significantly as force decreased (Fig. 6*A* and *B*, respectively). However, their dependence on force was different. The decrease in non-crossbridge heat was entirely associated with the first 2·5 % decrease in force. Thereafter, although force declined a further 25 %, the non-crossbridge heat remained constant. Crossbridge heat declined continuously during the fatiguing series. At the start of the series, it declined more rapidly than force, but later on it declined less rapidly than force.

DISCUSSION

Experiment 1: effects of filament overlap during fatigue on force

If the fatigue were caused by Ca^{2+} release failing when it is repeated, the prediction would be that a series of tetani with reduced filament overlap would be as fatiguing as a series at full filament overlap. The results show that this is not the case. When filament overlap is small (about 30 % of its maximum values), the decline of force is only half that produced by the same pattern of stimulation at full filament overlap. The slowing of relaxation is even less than half. We concluded that the loss of force investigated here is not simply due to reduced Ca^{2+} cycling when activation is repeated. Comparing the results for the two degrees of filament overlap, the difference in fatigue (50 %) was similar to the difference (60 %) in total tension–time integral during the series of tetani that produced the fatigue. If we take tension–time integral as an indication of metabolic cost, the results suggest that the higher metabolic requirements of tetani at full overlap seem the most likely explanation for the greater fatigue at full overlap.

Experiment 2: crossbridge heat and non-crossbridge heat during fatigue

The second series of experiments was done because the purely mechanical evidence from Expt 1 does not indicate which mechanism(s) is more affected by tetani at full filament overlap than by tetani at reduced filament overlap: it could be that *crossbridge reactions* change so that each attached bridge produces less force than a normal bridge, and/or the *non-crossbridge reactions* which include all the steps of excitation–contraction coupling, reactions governing Ca^{2+} availability and binding to the thin filaments.

The measurements of heat production are relevant to the question of mechanism because this method can give separate measures of the chemical processes of crossbridge interaction and of the non-crossbridge reactions.

Justifications for assumptions

The separation of heat production in a tetanus into its crossbridge and non-crossbridge components rests on two assumptions. (1) The heat produced by crossbridge activity is proprotional to the number of active crossbridge sites; that is, the sites are independent and equivalent ATPases. As it is well established that the sites are independent as force generators (Gordon, Huxley & Julian, 1966*b*), this assumption seems reasonable. (2) It is assumed that non-crossbridge heat, which is considered to be due largely to Ca^{2+} reactions, is independent of muscle length, over the range of lengths used here (100–31·8 % filament overlap).

The fact that heat production declined linearly with filament overlap (Fig. 4, ●) supports the validity of these assumptions. The linear relation between heat and force is most simply explained by the decline in the number of crossbridge ATPase sites with decreasing filament overlap, while Ca^{2+} turnover remains unchanged, but does exclude the possibility of a linear decline in Ca^{2+} turnover with length.

Here we must consider the results of Blinks, Rudel & Taylor (1978) which showed that the free Ca^{2+} during a twitch or a tetanus was lower at reduced filament overlap; the size of the effect and filament overlap for onset of the effect varied

among fibres. In relating this finding to the results reported here, it is relevant that the free Ca^{2+} is not a measure of the energetic cost of activation. The amount of Ca^{2+} turnover in the cell, mainly uptake by the sarcoplasmic reticulum (SR), determines the cost of activation. Most of the Ca^{2+} that is released from the SR becomes bound, and only a small amount remains free. Consequently a 5 % reduction in Ca^{2+} release would, on the basis of plausible assumptions, result in a 50 % reduction in the free Ca^{2+} (Homsher & Kean, 1978, pp. 103–104 for details). Thus, the results of Blinks *et al.* (1978) showing a large change in free Ca^{2+} with length are compatible with there being only a small change in the cost of activation, negligible in the context of the results reported here.

There are, in addition, several other lines of experimental evidence that Ca^{2+} turnover is not reduced at long sarcomere lengths (for review see Rall, 1982). These include the finding that the twitch-to-tetanus force ratio is independent of sarcomere length (Smith, 1972) and the finding that the threshold K^+ required for production of K^+ contracture force is independent of length (Homsher, Mommaerts, Ricchiuti & Wallner, 1972). Rall (1980) has shown that when force production is blocked by D_2O, the heat production, which must be mostly non-crossbridge heat, is hardly affected by sarcomere length. In addition, temperature dependence of heat production at full and reduced filament overlap shows that force-dependent heat is strongly influenced by temperature, whereas activation heat is not (Rall, 1979). This result can easily be explained if Ca^{2+} cycling is independent of filament overlap in the range tested; if it varies with filament overlap, a quite contrived mechanism would be required to account for Rall's results.

Interpretation of changes in non-crossbridge heat

The results in Fig. 6*B* show that the non-crossbridge heat decreases by about 25 % and force declines by less than 3 % in the first few tetani of the fatiguing series. Then the heat production remains relatively constant for the remainder of the series, while force declines considerably. Clearly, non-crossbridge heat and force do not decline in parallel. This extends our conclusion from Expt 1 that repeated stimulation does not simply reduce Ca^{2+} availability.

The non-crossbridge heat is due to Ca^{2+} movement from the sarcoplasmic reticulum to its binding sites on troponin and parvalbumin, consequent changes in the thin filament, active transport of Ca^{2+} back into the SR, and reactions directly coupled to these processes. The ATP-driven active transport of Ca^{2+} is probably quantitatively the most important source of heat.

Which of these reactions might be responsible for the decline in non-crossbridge heat early in the fatiguing series? Non-crossbridge heat may be reduced because both Ca^{2+} release and reuptake by the SR decrease early in the fatiguing series. However, force does not decline much at this time, so there would have to be some compensating mechanism. An increase in Ca^{2+} sensitivity of the thin filament would in principle have this effect, but the evidence about Ca^{2+} sensitivity during fatigue all favours a decrease rather than an increase (Allen, Lee & Westerblad, 1989; Godt & Nosek, 1989; Lee, Westerblad & Allen, 1991; reviewed by Westerblad *et al.* 1991).

If the reduction in heat in the first four tetani is due to a reduction in Ca^{2+} turnover (release and uptake by the SR), how else can the maintenance of force be explained? Some of the Ca^{2+} binds and is later released from parvalbumin and therefore does not lead to force development. A reduction in this part of the Ca^{2+} turnover would explain the finding. Such an effect is expected because the release of Ca^{2+} from parvalbumin is slow (Hou, Johnson & Rall, 1991) and thus once it has bound to parvalbumin, turnover is not completed in the time between the tetani in the fatiguing series.

In spite of reduced Ca^{2+} release from the SR, Ca^{2+} availability for binding to troponin would not necessarily be reduced and may even increase, which is consistent with the force remaining almost constant. Measurements have indeed shown that in *Xenopus* fibres at 20 °C the aequorin Ca^{2+} signal does increase early in a fatiguing series (Allen *et al.* 1989).

Interpretation of change in crossbridge heat

Figure 6*A* shows that early in the series, the percentage decline in crossbridge heat is faster than the percentage decline in force, whereas later the force declines faster than heat. This non-linear relation between heat and force argues, like the results of Expt 1, against the loss of force being due to a failure to activate a progressively larger proportion of the crossbridges, as might occur if Ca^{2+} release from the SR were reduced or the propagation of the action potential into the T-tubule progressively failed. These events would lead to a proportional decline of force and ATPase.

Could changes in intracellular pH during the fatiguing series produce the non-linear relation between force and heat shown in Fig. 6*A*? A change in pH *alone* could not be responsible because when intracellular pH is varied in the range 6·0–7·3 in unfatigued fibres, force and heat change in the same proportion (Curtin, Kometani & Woledge, 1988). However, it is likely that any change in intracellular pH that does occur will *contribute* to the changes in force that occur during moderate fatigue.

Although the crossbridge heat declines faster than the force early in the series, later in the series it declines at a lower rate than the force does; this latter behaviour would be expected if there were accumulation of inorganic phosphate (P_i). During a fatiguing series of tetani, inorganic phosphate probably does accumulate because the time constant for its removal by oxidative phosphorylation (Kawano, Tanokura & Yamada, 1988) is much longer than the 10 s intervals between tetani used here. It is well established from experiments on skinned fibres that an increase in P_i level depresses both force and myofibrillar ATPase and that the effect on ATPase is less than that on force (Bowater & Sleep, 1988). If the same relation holds in intact frog fibres, the decrease in ATP splitting and thus heat production, would be less than the decrease in force.

The fact that the behaviour of the crossbridge heat during the first few contractions in the series is different from what we expect if P_i is the main factor causing fatigue, suggests that the crossbridges are influenced during this time by some additional factor which has not yet been recognized, and which causes heat and ATP turnover to decline more than force does.

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