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FORCE RELAXATION, LABILE HEAT AND PARVALBUMIN CONTENT OF SKELETAL MUSCLE FIBRES OF *XENOPUS LAEVIS*

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SUMMARY

1. Measurements were made of stable (h_b) and labile (h_a) maintenance heat rate, slowing of relaxation as a function of tetanus duration, and parvalbumin (PA) content in intact single muscle fibres of types 1 and 2 from *Xenopus laevis*. The majority of experiments were performed at 20 °C. In addition, total and myofibrillar ATPase activity was measured in skinned *Xenopus* fibres, also of types 1 and 2; these studies were performed at 4 °C.

2. In agreement with a previous study h_b was significantly higher in type 1 (175 ± 13 mW (g wet wt)$^{-1}$; $n = 8$) than in type 2 fibres (88 ± 9 mW (g wet wt)$^{-1}$; $n = 7$). The value of h_a was 236 ± 22 and 117 ± 16 mW (g wet wt)$^{-1}$, respectively (means ± S.E.M.). h_a decayed with a time constant of $0{\cdot}27 \pm 0{\cdot}02$ ($n = 8$) and $0{\cdot}33 \pm 0{\cdot}02$ s ($n = 7$).

3. The early relaxation rate of tetanic force, extrapolated to the onset of stimulation ($y_o + y_b$; where y_o is 'extra' rate of relaxation and y_b steady rate) was $85{\cdot}6 \pm 4{\cdot}2$ s^{-1} for type 1 fibres ($n = 8$) and $62{\cdot}7 \pm 7{\cdot}3$ s^{-1} for type 2 fibres ($n = 7$). Relaxation rate at the end of a 1·8 s tetanus (y_b) was $29{\cdot}4 \pm 1{\cdot}6$ and $33{\cdot}3 \pm 1{\cdot}5$ s^{-1}, respectively; thus, there was more slowing with tetanus duration in type 1 fibres. The time constant for slowing of relaxation with tetanus duration was similar to that for decay of h_a.

4. Parvalbumin concentration, [PA], was $0{\cdot}45 \pm 0{\cdot}04$ mM in type 1 ($n = 7$) and $0{\cdot}22 \pm 0{\cdot}04$ mM ($n = 7$) in type 2 fibres.

5. For individual fibres positive correlations were found between the 'extra' rate of relaxation (y_o), labile heat (h_a) and [PA]. Significantly more labile heat was liberated than can be accounted for by the enthalpy change of Ca^{2+} binding to PA.

6. For five fibres (type 1) studied both at 20 and 10 °C, the magnitude of slowing of relaxation, expressed as $y_o/(y_o + y_b)$, was $0{\cdot}58 \pm 0{\cdot}03$ at 20 °C and $0{\cdot}65 \pm 0{\cdot}03$ at 10 °C.

7. Both slowing of relaxation and labile heat were depressed in the second of two closely spaced tetani in type 1 fibres. Repriming of both effects followed similar, biphasic time courses and required more than 10 min for completion at 20 °C.

8. Maximum Ca^{2+}-activated myofibrillar ATPase of chemically skinned fibres at 4 °C was $3{\cdot}42 \pm 0{\cdot}21$ and $1{\cdot}77 \pm 0{\cdot}14$ μmol s^{-1} (g dry wt)$^{-1}$ for type 1 ($n = 9$) and

type 2 ($n = 13$) fibres, respectively. Sarcoplasmic reticulum ATPase was $1{\cdot}47 \pm 0{\cdot}15$ and $0{\cdot}95 \pm 0{\cdot}10$ μmol s^{-1} (g dry wt)$^{-1}$, respectively.

9. The results suggest that at 20 °C, PA may promote relaxation early in a tetanus and that its interaction with Ca^{2+} is responsible for part of labile heat production; a substantial fraction, about 70%, of labile heat, however, must be due to some other process. Similarly, slowing of relaxation with tetanus duration can only be partly explained by Ca^{2+} saturation of PA.

INTRODUCTION

In frog muscle at optimal filament overlap and under isometric conditions, heat production during a tetanic contraction usually occurs at a high initial rate and then declines exponentially to a lower value. This observation led Aubert (1956) to distinguish two components of isometric heat: a rapidly decaying component, which he called labile heat and a steady component, which was named stable maintenance heat. From studies where heat production has been correlated with chemical change it has become apparent that stable maintenance heat is most probably due to hydrolysis of phosphocreatine (PCr) which continuously replenishes the ATP hydrolysed by the myofibrillar and sarcoplasmic ATPases (Homsher & Kean, 1978; Curtin & Woledge, 1979). The origin of the labile heat is known with less certainty, but one current theory (Peckham & Woledge, 1986) suggests that it represents calcium binding to parvalbumin (PA), a soluble, low molecular weight protein known to be present in some muscles, especially those with fast contractile properties. However, Berquin & Lebacq (1992) have recently shown that labile heat is produced in mouse soleus muscles, which contain negligible amounts of parvalbumin. Also, an earlier report (Homsher, Lacktis, Yamada & Zohman, 1987) suggested that not all labile heat is due to Ca^{2+}–PA interaction.

Apart from a decline in the rate of heat production the speed of relaxation usually changes with tetanus duration, being high for a short tetanus and decreasing as the tetanus duration increases. Since parvalbumin has a limited calcium binding capacity it has been natural to assume that the slowing of relaxation is due to a gradual calcium saturation of parvalbumin, i.e. a decrease in binding capacity. Attempts have been made to test the idea that a high initial rate of relaxation and labile heat production are both due to calcium binding to PA. Peckham & Woledge (1986) studied slowing of relaxation and labile heat production in two kinds of whole muscles, sartorius and the extensor digitorum longus IV of the frog, and found that both processes were less conspicuous in the latter, more slowly contracting muscle, which probably contains less PA. The PA content was not measured in their study. In a more recent investigation, Hou, Johnson & Rall (1991) studied slowing of relaxation in single frog fibres for which the PA concentration was determined and also measured Ca^{2+} on and off rates from purified PA. Their conclusion was that both slowing of relaxation and repriming, i.e. return to original relaxation rate, could be explained by calcium interaction with PA. No heat measurements were performed in their study.

The aim of the present investigation was to obtain combined data for changes of relaxation, heat production and PA content of individual fibres to allow a more comprehensive evaluation of the parvalbumin hypothesis. We chose to work on

Xenopus, an amphibian in which fibres of different types can be singled out during dissection (Lännergren & Smith, 1966), and selected two kinds of fibres, types 1 and 2. The PA content of these two types has recently been determined by Simonides & van Hardeveld (1989) and was found to differ by a factor of about 3. Further, we have previously measured some mechanical parameters and stable maintenance heat rate in different fibre types in *Xenopus*, including types 1 and 2 (Elzinga, Lännergren & Stienen, 1987).

The parvalbumin hypothesis assumes that the rate of fall of Ca_i^{2+} determines relaxation rate. At an early stage of a tetanus calcium-free PA as well as the calcium pumps of the sarcoplasmic reticulum (SR) combine to lower Ca_i^{2+} when stimulation is stopped. Once PA is calcium saturated the activity of the SR is the major determinant of the rate of fall of Ca_i^{2+}. We therefore thought it to be of interest to study also relaxation late in tetanic contractions in the two fibre types and relate it to the SR ATPase activity. The results of the study show that slowing of relaxation and labile heat production is more marked in type 1 fibres. In both fibre types more labile heat is produced than can be accounted for by binding of calcium to PA. The nature of additional heat producing processes is discussed in relation to the relatively high initial relaxation rate of type 1 fibres.

Brief accounts of some of the results have already been given (Elzinga & Lännergren, 1988; Elzinga, Lännergren & Simonides, 1989).

METHODS

Fibre preparation and mounting on the thermopile

Single fibres were dissected from the iliofibularis muscle under dark-field illumination from adult, female *Xenopus laevis* (clawed frogs). The animals were kept in tap water at room temperature and fed with meal worms every second day. The frogs were killed by rapid decapitation, followed by pithing. Large diameter, transparent fibres (type 1) were selected from the outer layers of the muscle and more granular ones (type 2) from the inner regions. The type of fibre was determined from its position in the muscle and its microscopic appearance according to the criteria developed by Lännergren & Smith (1966).

After dissection, the largest and smallest diameter was measured at three different places by rotating the fibre and using an ocular scale. The values were used to calculate the cross-sectional area which was assumed to have an elliptical shape. A small platinum hook was tied to each trimmed-down tendon with fine nylon thread. The fibre was then mounted on the thermopile in a small pool of Ringer solution between two platinum flags, each provided with a hole to accommodate the platinum hooks. One of the flags extended from the beam of a force transducer (AE 801, SensoNor, Horten, Norway), the other flag was fixed to a platinum wire extending from a micrometer screw which could be used to adjust fibre length to just above slack.

The thermopiles used were made by evaporation of bismuth and antimony in vacuum, as described by Mulieri, Luhr, Trefry & Alpert (1977). The length of the piles was 15·5 mm with sixty-two junctions; electrical connections were made to every second warm junction. The sensitivities of the piles used were similar to those used previously (Elzinga *et al.* 1987). The temperature change during contraction was measured over at least 75% of the fibre length.

Stimulation and protocol

Fibres mounted on the thermopile were made to contract by end-to-end stimulation with 0·4 ms monophasic pulses at 1·2–1·3 times threshold, delivered via the platinum flags. Fibres mounted in a separate recording chamber (see below) were stimulated via flanking platinum plate electrodes using biphasic pulses of 0·6 ms total duration.

After the fibre had been mounted on the thermopile the frame holding the pile was inserted into a plexiglass cylinder containing Ringer solution. The cylinder was submerged vertically in a tank containing temperature-controlled water at 20·0 °C. When the Ringer solution had attained the

same temperature as the water in the tank, the cylinder was drained so that only a thin film of solution adhered to the fibre. In the majority of experiments fibres were given interrupted tetani (cf. Abbott, 1951) with a total duration of 1·8 s. The tetani consisted of recurring 120 ms periods of 70 Hz stimulation and 60 ms periods without stimulation. These tetani resulted in contractions (Fig. 1) in which the relaxation rate could be measured during the relaxation intervals between the 120 ms trains. To study repriming, pairs of interrupted tetani were given with intervals varying between 5 s and 30 min.

Experiments were also performed in a separate recording chamber with the fibre suspended in a perfusion channel with continuous flow of temperature-controlled Ringer solution. Parameters for interrupted tetani at 20 °C were the same as for fibres on the thermopile, at 10 °C 160 ms 'on' periods (50 Hz) and 150 ms 'off' periods were used. In some experiments at 20 °C continuous 70 Hz tetani of various durations (0·12–1·6 s) were given. Repriming was measured in a few fibres at 20 °C by giving a long tetanus (1·0–1·6 s), followed by a 120 ms test tetanus after various times (cf. Hou *et al.* 1991).

Analysis of force records

The rate of relaxation was measured as the reciprocal of the time for force to decay by 5% from its maximum value after the last stimulus. The reason for using this interval rather than e.g. 95–90% of maximum tension was that in some interrupted tetanic contractions tension did not drop as low as to 90% of the maximum. Changes in relaxation rate (y) were analysed by fitting data obtained at various times with the equation

$$y = y_0 e^{-t/\tau_r} + y_b, \quad (1)$$

where y_0 is the initial 'extra' rate of relaxation at $t = 0$ s, τ_r is the time constant for the effect and y_b is the final rate of relaxation.

Analysis of temperature records

At the end of each experiment on the thermopile, Peltier heating was done to obtain values for heat loss correction and calibration. Heat loss was corrected for by deconvolution of the temperature record using a system function obtained by fitting the temperature decay at the end of Peltier heating. The deconvoluted heat records (H) were analysed by fitting to the following equation, derived from Aubert (1956),

$$H = h_a \tau_h (1 - e^{-t/\tau_h}) + h_b t, \quad (2)$$

where h_a is labile heat rate, τ_h is its decay time constant, h_b is stable heat rate. The total amount of labile heat (H_a) is $h_a \tau_h$. The following equation was used for analysing the decay of heat rate with stimulation time:

$$\dot{H} = h_a e^{-t/\tau_h} + h_b. \quad (3)$$

Parvalbumin analysis

At the end of the experiment the fibre was taken off the thermopile, dried and weighed on a Cahn 29 electrobalance. The dried fibre was then stored at −80 °C for later determination of the parvalbumin content. Parvalbumin was quantified as described by Simonides & van Hardeveld (1989). Briefly, the fibre was homogenized in 87·5 mM Tris buffer with 10% glycerol and 2% sodium dodecyl sulphate (SDS) and the homogenate directly loaded on SDS polyacrylamide gels (0·75 mm thick, 6·5% stacking and 15% separating gel) run in a conventional minigel apparatus (Bio-Rad, Richmond, CA, USA). Frog and rabbit parvalbumins (Sigma, USA) run on the same gel were used for calibration. Gels were scanned with a laser densitometer. For conversion of mg protein (dry wt)$^{-1}$ to mM an average molecular weight for PA of 13000 and a fibre volume–dry wt ratio of 3·7 was assumed.

ATPase activity measurements

The ATPase activity of the skinned fibres was measured by means of a coupled enzyme system, in which resynthesis of ATP is coupled to the breakdown of NADH which is followed photometrically (Glyn & Sleep, 1985; Stienen, Roosemalen, Wilson & Elzinga, 1990). Single type 1 and 2 fibres were dissected in relaxing solution, mounted in the experimental set-up by means of aluminium T-clips, and skinned by saponin (50 μg ml^{-1}) for 30 min. This saponin treatment disrupts the sarcolemma but leaves the SR functionally intact (Stienen *et al.* 1990). The bath used for the ATPase assay (volume 30 μl) had quartz windows to allow transmission of near-UV light for the measurement of NADH absorbance. This bath contained activating solution and was

continuously stirred by motor-driven vibration of a diaphragm at the base. The other baths (volume 80 μl) contained relaxing and pre-activating solution. The fibre could be transferred manually between baths. The composition of the relaxing, pre-activating, and activating solutions was (mM) 7·35, 6·96, 6·82 $MgCl_2$; 5·58, 5·58, 5·66 Na_2ATP; 20, 0·5, 0 EGTA; 0, 19·5, 0 HDTA (1,6-diaminohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid); 0, 0, 20 CaEGTA; and 88·6, 89·4, 89·3 potassium propionate to adjust final ionic strength to 200 mM. In addition all solutions contained: 100 mM Tes (*N*-tris (hydroxymethyl)methyl-2-aminoethanesulphonic acid; pH adjusted to 7·1 with KOH), 5 mM phosphoenolpyruvate, 0·9 mM NADH, 4 mg ml^{-1} pyruvate kinase, 0·24 mg ml^{-1} lactate dehydrogenase, 0·2 mM P^1,P^5-di(adenosine-5′)pentaphosphate and 10 μM oligomycin and 5 mM sodium azide. The free Mg^{2+} and MgATP concentrations were 1 and 5 mM, respectively. The temperature of the solutions was kept at 4 ± 1 °C. The ATPase activity was measured from the slope of the NADH absorbance signal which was corrected for the change in absorbance when the fibre was not in the measuring chamber.

The ATPase difference method

The ATPase activity of the SR was derived from the difference in the maximally calcium activated ATPase activity of the saponin skinned preparations in the presence of 5 mM caffeine, before and after Triton X-100 treatment. Caffeine prevents Ca^{2+} accumulation inside the SR and ensures that the SR ATPase activity is at its maximum value or close to maximal (G. J. M. Stienen, R. Zaremba & G. Elzinga, unpublished observations). Triton X-100 treatment (0·5%, 30 min) disrupts SR membranes, and after washing out the remaining fragments, SR activity is lost (cf. Kurebayashi & Ogawa, 1991). By subtraction of the (myofibrillar) ATPase activity measured after Triton treatment from the total ATPase activity measured initially, the fully Ca^{2+}-activated SR ATPase activity can be obtained. Some of the experiments were carried out in the presence of 10 μM oligomycin and 5 mM sodium azide. The results were similar to those in which these blockers of mitochondrial activity were omitted, indicating that mitochondrial activity does not interfere with the ATPase measurements.

RESULTS

Changes in relaxation and labile heat rate

The change in relaxation rate with tetanus duration was studied both in fibres on the thermopile and in fibres suspended in a chamber with slowly flowing Ringer solution. Figure 1 shows records obtained in the latter set-up. The top part of the figure shows examples of interrupted tetani of a type 1 fibre (*A*) and a type 2 fibre (*B*). It can be clearly seen that the relaxation rate is higher in the type 1 fibre during the first stimulus pause and also that relaxation slows down more markedly in this fibre for successive stimulus pauses so that the relaxation rate becomes similar to that of the type 2 fibre at the end of the long tetanus. Values for initial relaxation rate ($1/t_{5\%}$) for the two fibres are plotted in the lower part of the figure.

A large number of experiments were performed with fibres on the thermopile which allowed us to record simultaneously changes in mechanical performance and in heat production. Figure 2 shows two examples, recorded from a type 1 and a type 2 fibre, respectively. The mechanical records (upper part) are similar to those in Fig. 1 and show again the marked difference in changes in relaxation rate between the two fibres during an interrupted tetanus. The heat records (lower part) were obtained at different amplification which was necessitated by the marked difference in heat rate for the two fibres. The temperature rise of the type 2 fibre is very nearly linear whereas the slope of the trace for the type 1 fibre is higher during the initial part, which reflects a substantial component of labile heat.

Combined force and heat records were obtained from eight type 1 fibres and seven type 2 fibres. Figure 3*A* and *B* summarizes how relaxation rate and heat production

change with stimulation time for the two fibre types. The figure shows the striking similarity in the time course of decay of relaxation rate (*A*) and labile heat (*B*). Figure 3*B* also illustrates the approximately twofold larger heat parameters (h_a and h_b) in type 1 *vs.* type 2 fibres.

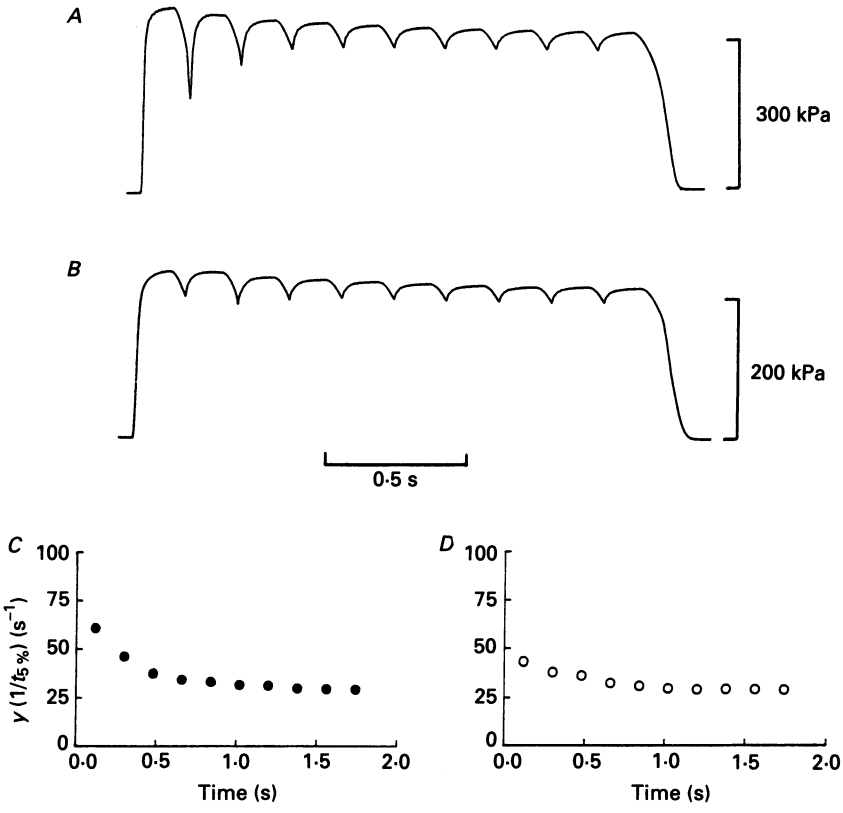

Fig. 1. Tension records of an interrupted tetanus in a type 1 (*A*) and a type 2 fibre (*B*). *C* and *D*, relaxation rate of the same fibres, measured as the reciprocal of the time taken for force to fall by 5% at the end of each stimulation period, and plotted against tetanus duration.

Parvalbumin analysis

Parvalbumin analysis by gel electrophoresis was successfully carried out in seven of the type 1 fibres and in all of the type 2 fibres. The parvalbumin content was significantly higher in type 1 than in type 2.

Mechanical data, heat data and parvalbumin content for the two fibre types are summarized in Table 1.

Interrelations of different parameters in individual fibres

It is clear from Table 1 that the mean values for some parameters for the two fibre types are significantly different. In order to see more clearly if there is a functional connection between slowing of relaxation and labile heat and also between parvalbumin content and relaxation and heat parameters data for the single different fibres were plotted. These plots are shown in Fig. 4*A–C*. Statistical analysis

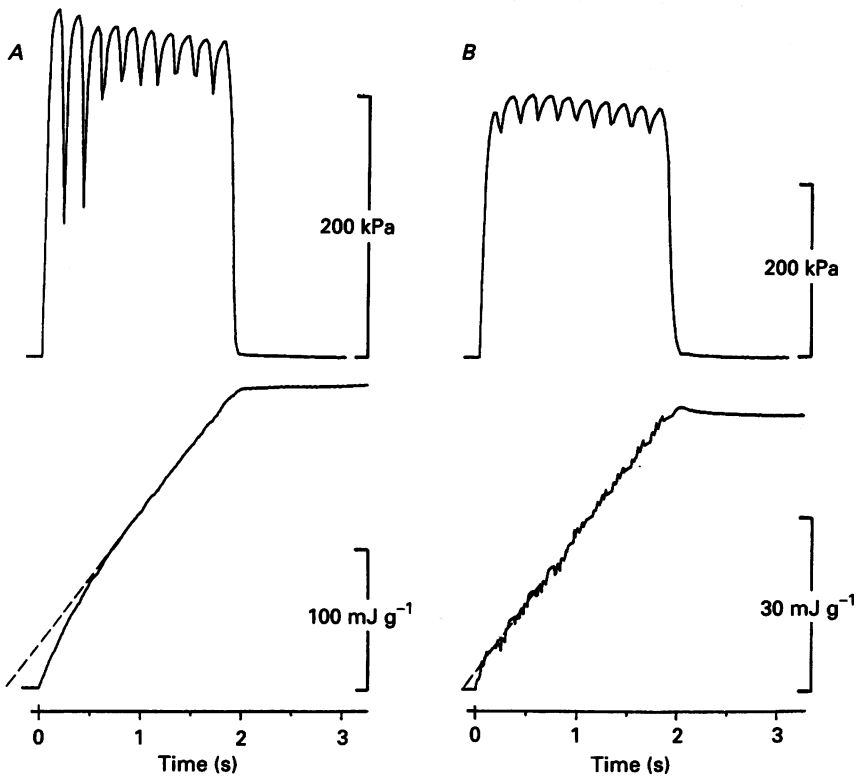

Fig. 2. Simultaneous records of force and heat production in the rested state of a type 1 fibre (*A*) and a type 2 fibre (*B*). The interrupted lines are extrapolations from the late, final parts of the records; the slope gives the stable heat rate.

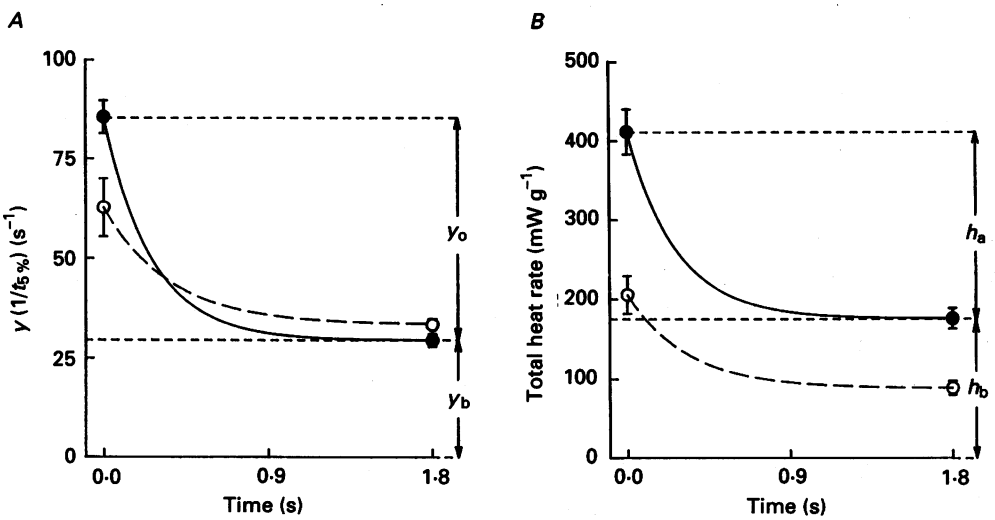

Fig. 3. *A*, summary graph of the slowing of relaxation with tetanus duration in eight type 1 fibres (●) and seven type 2 fibres (○). The mean values for y_o ('extra' relaxation rate), y_b (final relaxation rate) and τ_{rel} have been taken from Table 1. *B*, similar graph of the decay of heat production rate for the same fibres as in *A*. Values for h_a (labile heat rate), h_b (stable heat rate) and τ_{ha} from Table 1.

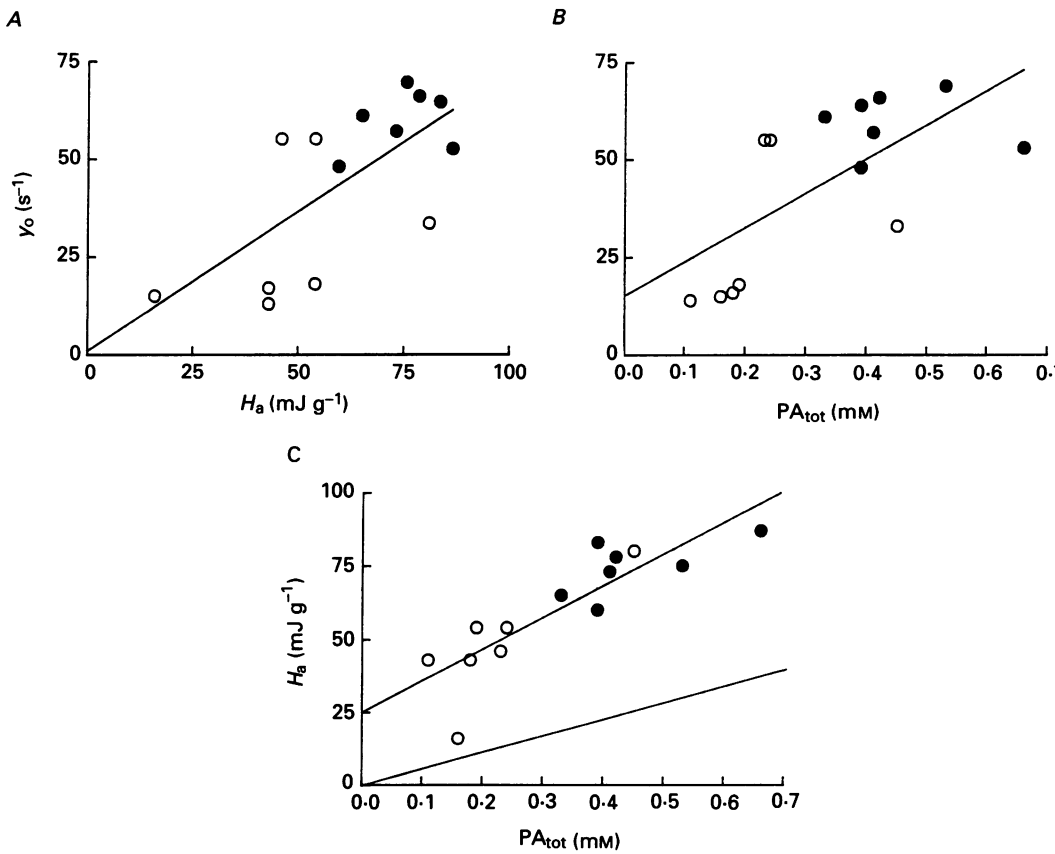

Fig. 4. *A*, plot of 'extra' relaxation rate (y_o) against labile heat (H_a) for the same fibres as in Fig. 3, except one type 1 fibre which was lost during PA analysis. *B*, plot of y_o against total PA concentration. *C*, plot of H_a against total PA concentration PA_{tot}. The lower straight line represents 56 kJ mol^{-1}, the molar enthalpy of Mg^{2+}–Ca^{2+} exchange.

TABLE 1. Mechanical data, heat data and parvalbumin content for the two fibre types

Quantity	Type 1 Mean ± S.E.M.	Type 2 Mean ± S.E.M.
Mechanical		
y_b (s^{-1})	29·4 ± 1·6	33·3 ± 1·5
y_o (s^{-1})	56·3 ± 4·2	29·4 ± 7·0*
τ_{rel} (s)	0·26 ± 0·04	0·36 ± 0·06
Heat		
h_b (mW g^{-1})	175 ± 13	88 ± 9*
h_a (mW g^{-1})	236 ± 22	117 ± 16*
τ_{ha} (s)	0·27 ± 0·02	0·33 ± 0·02
H_a (mJ g^{-1})	74·5 ± 3·7	48·3 ± 7·3*
Parvalbumin (mM)	0·45 ± 0·04	0·22 ± 0·04

The meaning of the different parameters is explained in Methods and also in Fig. 3*A* and *B*.
* Denotes significant difference between fibre types (Student's *t* test, $P < 0·01$).

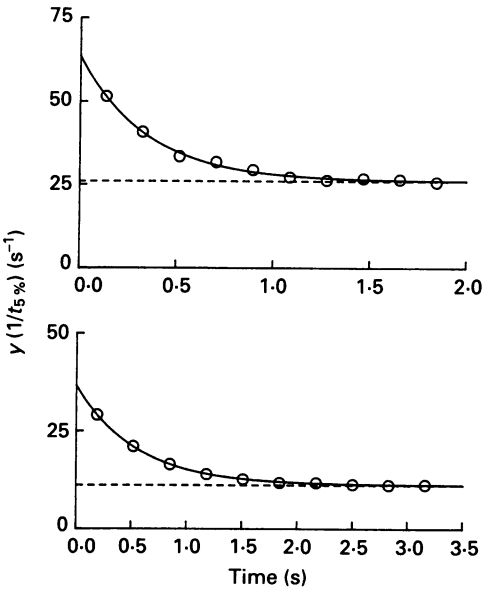

Fig. 5. The effect of temperature on time course and extent of slowing of relaxation in a type 1 fibre. Relaxation rate at various times during an interrupted tetanus is plotted against tetanus duration. The values in the upper graph were obtained at 20 °C, those in the lower at 10 °C. Lines drawn in full are exponentials according to eqn (1) in Methods. The interrupted line represents the final relaxation rate (y_b).

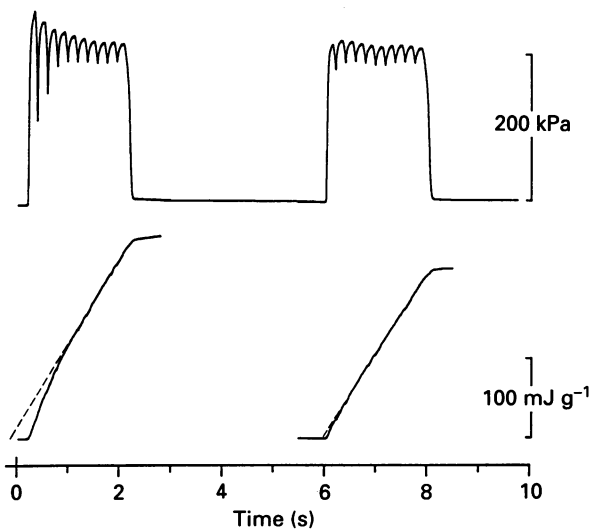

Fig. 6. Effects of a preceding tetanus. Force and heat records from a type 1 fibre. Interrupted lines drawn as in Fig. 2.

shows that there is a reasonably good correlation ($r = 0{\cdot}69$) between slowing of relaxation and labile heat (H_a) and also between y_o and parvalbumin content ($r = 0{\cdot}67$). In Fig. 4*C* the lower line corresponds to the molar enthalpy change of Mg^{2+}–Ca^{2+} exchange on parvalbumin (56 kJ per mol of PA; Tanokura & Yamada,

1987). It can be seen that significantly more labile heat is produced than can be accounted for by Ca^{2+}–PA interaction. For example, for a PA concentration of 0·4 mM the enthalpy change due to Ca^{2+}–PA interaction would be 23 mJ g^{-1} whereas the measured value (regression line, $r = 0{\cdot}85$) is 67 mJ g^{-1}, i.e. 2·9-fold higher.

Effects of temperature on time course and magnitude of slowing of relaxation

All the experiments described so far were performed at 20 °C. An additional series was performed both at 20 and 10 °C (on freely suspended type 1 fibres with perfusion) in order to allow a comparison with results on frog (*Rana temporaria*) fibres (Hou & Rall, 1987). Changes in relaxation rate were determined from interrupted tetani. Figure 5 shows one example. It can be seen that the exponential component of the early relaxation rate became slower at 10 °C and that its relative magnitude increased. Values for $y_o/(y_o+y_b)$ were $0{\cdot}58 \pm 0{\cdot}03$ at 20 °C and $0{\cdot}65 \pm 0{\cdot}03$ at 10 °C (means ± S.E.M.; $n = 5$).

Repriming of relaxation rate and labile heat

Peckham & Woledge (1986) have shown, for frog sartorius muscles, that if interrupted tetani are repeated at short intervals both slowing of relaxation and labile heat rate are considerably diminished in the second tetanus. They studied how the extent of repriming, i.e. the return to the rested values, of these two parameters depends on the interval between contractions. They found that repriming occurred in two phases, an initial, rapid phase with restoration to about 75% of the rested value within 1 min and a slow phase, requiring up to 30 min. Hou *et al.* (1991), on the other hand, found nearly complete repriming of relaxation rate within 30 s, also at low temperature. We performed similar experiments at 20 °C on type 1 fibres, which show the biggest change in relaxation rate with tetanus duration. Figure 6 shows an example. As can be seen, when the interval between tetani was short, 3·5 s in this case, both slowing of relaxation and labile heat rate of the second tetanus were greatly reduced.

Figure 7*A* shows mean values from eight fibres for repriming of h_a, the labile heat rate and Fig. 7*B* shows repriming of the maximum relaxation rate. Both parameters recovered in a biphasic manner with a rapid early phase followed by a much slower phase. At 1 min h_a and y_o had recovered to, on average, 77 and 62%, respectively, of their fully rested values.

In order to see if the conditions used for studying repriming are important, two experiments were performed on fibres freely suspended in a recording chamber with perfusion. A 1 s continuous tetanus was used for conditioning, followed by a 120 ms tetanus after various repriming times as a test (Fig. 7*C*). The result was similar to that obtained on the thermopile: recovery of y_o at 1 min repriming time was only 49 and 57% of the fully rested value. Thus, there appears to be a slow phase of repriming, both at 0 and 20 °C.

The final relaxation rate y_b

The difference in relaxation rate between type 1 and type 2 fibres decreases with duration of stimulation and at the end of a long tetanus relaxation rate is very nearly the same. This is surprising since type 2 fibres are in general slower than type 1 fibres:

tension rise in a tetanus is slower, maximum shortening velocity is lower, and stable maintenance heat rate (h_b) is lower (Elzinga *et al.* 1987); for the present set of fibres the value for h_b was only 50% of that of type 1 fibres. The value of h_b is considered to reflect two processes: cross-bridge cycling and calcium pumping by the SR, of

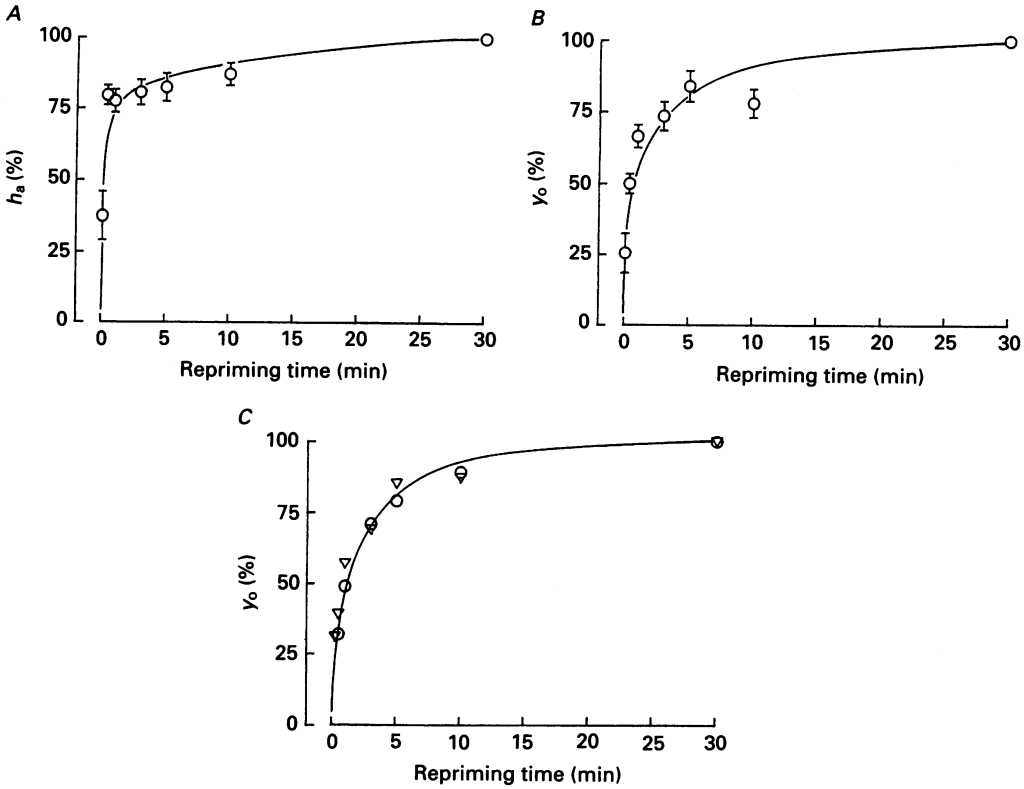

Fig. 7. The effect of varying the interval between two successive tetani. *A*, time course of repriming of labile heat rate. Data from eight type 1 fibres. *B*, repriming of 'extra' relaxation rate. Same fibres as in *A*. *C*, repriming of 'extra' relaxation rate studied with a long conditioning tetanus (1 s), followed by a 120 ms test tetanus. Data from two type 1 fibres (different symbols). Curves drawn by eye.

which cross-bridge cycling is the dominant process (Curtin & Woledge, 1979; Homsher, Kean, Wallner & Sarian-Garbian, 1979). Thus cross-bridge kinetics do not seem to be of major importance for determining relaxation rate under the present conditions.

The other possibility, that y_b is determined by the fall in cytoplasmic calcium, then becomes the major alternative. From an energetic point of view, SR ATPase activity, underlying calcium pumping, is thought to be reflected by activation heat (Homsher & Kean, 1978). Activation heat can be measured by recording heat and force production in preparations stretched to increasing lengths so that filament overlap decreases. Its value is then measured from the intercept on the heat axis when

force production is nil (Homsher, Mommaerts, Ricchiuti & Wallner, 1972). We attempted such experiments on *Xenopus* fibres but were unsuccessful because sarcomere inhomogeneities developed with stretch, resulting in internal movements and pronounced 'creep' on tension records. We then decided to try to measure SR ATPase activity by another method.

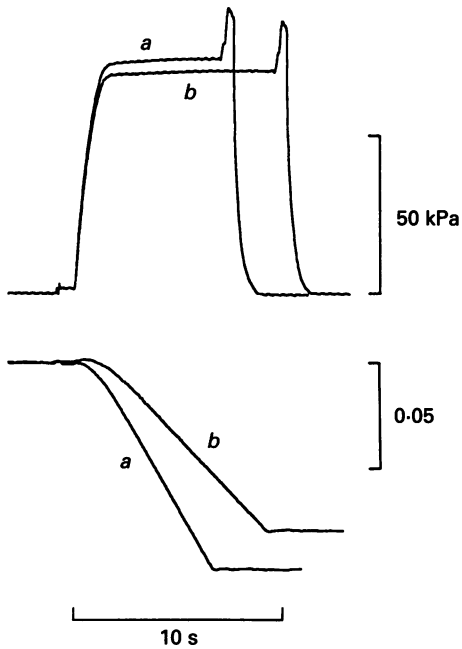

Fig. 8. Measurements of ATPase rate in a skinned type 1 fibre. Upper records show force, lower records NADH absorbance. Traces marked *a* were obtained after skinning in saponin, traces marked *b* were recorded after destroying the SR with Triton X-100 (0·5%, 30 min). The fibre was activated by changing from pCa = 9·0 to pCa = 4·5.

Using skinned fibres, fully activated in the presence of caffeine, it is possible to determine ATPase rate from the ATP consumption, either of the fibre as a whole or, after destroying the internal membrane system, of the myofibrils only. By subtracting the latter from the former an estimate of SR ATPase activity can be obtained.

Figure 8 shows original recordings from an experiment of this kind on a saponin skinned type 1 fibre. Two sets of traces are shown, obtained before and after Triton X-100 treatment. It can be seen that after Triton treatment ATPase activity is reduced whereas force development is very little affected. In general, both force development and ATPase activity deteriorated somewhat with repeated activations; fibres in which the isometric force before and after Triton treatment differed by more than 15% were discarded. The average reduction of force and ATPase activity after Triton treatment was 5 ± 1 and 8 ± 2%, respectively. This factor has been neglected. Several controls were carried out to verify that other membrane-bound ATPases did not interfere with the measurements to a significant extent. These controls were:

measurement of the ATPase activity, after Triton treatment (i) at a free Ca^{2+} concentration just below the threshold for force generation and (ii) in the presence of quercetin (0·24 mM), ouabain (1 μM) and cyclopiazonic acid (5 μM).

The results for myofibrillar (AM) and SR ATPase activity for the two fibre types are summarized in Fig. 9. Total ATPase activity (AM+SR) for the two fibre types

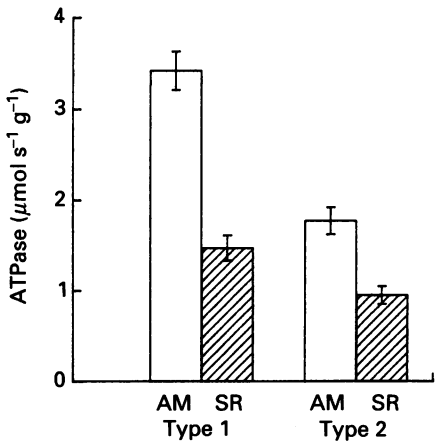

Fig. 9. Summary of results from skinned fibres. Total ATPase activity was first measured, then myofibrillar ATPase (AM), from which SR ATPase could be calculated. Results from nine type 1 fibres and thirteen type 2 fibres.

was 4·89±0·27 ($n = 9$) and 2·72±0·20 ($n = 13$) μmol s^{-1} g^{-1}, respectively, thus 1·8 times higher in type 1 than in type 2 fibres; this is in good agreement with the ratio of the stable maintenance heat rate values for the two fibre types found previously (Elzinga *et al.* 1987) and in this study (Table 1) although the temperature is different. This indicates that the temperature coefficient (Q_{10}) values for h_b and ATPase activity are similar. SR ATPase activity was more similar for the two types (1·47±0·15 and 0·95±0·10 μmol s^{-1} g^{-1}); statistical analysis, however, showed a significant difference (Student's t test, $P < 0·01$).

DISCUSSION

We have shown here that some mechanical and energetical parameters vary considerably for a collection of *Xenopus* fibres, selected to encompass two fibre varieties, types 1 and 2. The use of this broad group enabled us to find fibres which differed markedly in slowing of relaxation during a long tetanus, in labile heat production and in parvalbumin content. In contrast to previous studies we have been able to perform mechanical and heat measurements as well as biochemical analysis on individual fibres; furthermore we have measured myofibrillar and SR ATPase activity of skinned fibres of the two types. Our study has mainly been carried out at 20 °C whereas most of the other work in this field has been done at 0–4 °C. One reason for this is that we had already available various data for *Xenopus* fibres of different

types at this temperature such as force–velocity values, rate of tension redevelopment and stable maintenance heat rate (Elzinga *et al.* 1987). Another reason is that fibres from *Xenopus* cannot be activated at temperatures below ≈ 5 °C (Hill, 1965; J. Lännergren, unpublished observations).

Comparison with previous results

The general trend was similar to that observed by Peckham & Woledge (1986), namely that fibres of a faster type had a higher initial relaxation rate and also slowed down more markedly during a long tetanus; they also produced more labile heat. We also found that y_b, the 'steady' relaxation rate, was slightly higher in type 2 than in type 1 fibres. A corresponding result, i.e. for extensor digitorum longus IV (EDL) *vs.* sartorius muscles, can be extracted from Table 1 of Peckham & Woledge: y_b was 2·96 s^{-1} for EDL (which in general is a 'slower' muscle) and 2·59 s^{-1} for sartorius.

For labile heat we found a mean value of 74·5 mJ g^{-1} for type 1 fibres and 48·3 mJ g^{-1} for type 2 fibres. This is about 4 times more than Peckham & Woledge's values at 0 °C, which would give a Q_{10} value of about 2. There is an uncertainty about this calculation, however, since, in general, heat values for single fibres tend to be larger than those for whole muscles (Curtin, Howarth, Rall, Wilson & Woledge, 1986). The ratio of labile heat rate–stable heat rate, which was 1·35 and 1·33 for types 1 and 2 respectively, was higher than in Peckham & Woledge's case (1·09 and 0·58). It should be noted, however, that our type 2 fibres appear to be slower than their ELD muscles.

The mean value for the total concentration of parvalbumins was $0{\cdot}45 \pm 0{\cdot}04$ for type 1 fibres and $0{\cdot}22 \pm 0{\cdot}04$ for type 2 fibres. This agrees well with values reported by Simonides & van Hardeveld (0·43 and 0·16 mM) but the type 1 value is lower than that given by Hou *et al.* (1991) for frog fibres (presumably comparable to type 1; $0{\cdot}76 \pm 0{\cdot}04$ mM).

Relaxation rate, labile heat and parvalbumin content

For fibres stimulated after a 30 min rest period positive correlations were found between y_o, the 'extra' rate of relaxation, and H_a, the magnitude of the labile heat, between y_o and PA content, and between labile heat and PA content (Fig. 4*A*, *B* and *C*). These results suggest that a causal relationship exists between these variables. There was also a close similarity between the time constants for the decay of labile heat rate and for slowing of relaxation (Fig. 3; Table 1). Further, the repriming of y_o and h_a followed similar time courses (Fig. 7). The simplest explanation of this whole set of results would be that labile heat production is due to calcium binding to parvalbumin and also that the 'extra' rate of relaxation is due to this process. On this scheme labile heat rate and y_o diminish during a long contraction because parvalbumin becomes gradually saturated with calcium. During the repriming period calcium dissociates from parvalbumin and is again replaced by magnesium.

This simple picture is confounded by some findings. One is the quite fundamental observation that the amount of labile heat produced is significantly greater than can be accounted for by calcium binding to parvalbumin (Fig. 4*C*). The interaction of PA with metal ions has been studied in some detail and the binding of calcium to PA in

exchange for magnesium is an exothermic process liberating close to 56 kJ per mol of PA (Tanokura & Yamada, 1987). Although there is some uncertainty about the molar enthalpy value the deviation is too large to explain all of the discrepancy.

Another complication arises from the time course of repriming. The repriming curves for y_0 and labile heat were both non-exponential with a slow final phase and the time for full repriming was more than 10 min rather than 30 s as obtained by Hou *et al.* (1991). The method of studying repriming was found not to influence the result: also with continuous tetani the repriming of y_0 was only 50–60 % at 30 s. Our results closely resemble those of Peckham & Woledge for repriming of y_0 and h_a (at 0 °C) and those of Homsher *et al.* (1987) and Kitano (1988) for repriming of h_a (frog sartorius at 0 °C), but deviate from those of Hou *et al.* (1991) where repriming was monoexponential with a rate constant of 0·14 s (0 °C). A slow phase of repriming would agree with the late removal of Ca^{2+} from PA being governed by the rate of Ca^{2+} by the SR, as suggested by the results of Klein, Kovacs, Simon & Schneider (1991). However, their slow rate constant (1/13·5 s) is still too fast to explain the slow final phase of repriming; thus, for this some other mechanism which is not related to Ca^{2+} handling appears to be responsible.

The finding that more labile heat was produced than could be accounted for by the binding of calcium to parvalbumin suggests that there is yet another heat-liberating process which contributes to labile heat. There are reports in the literature which support this notion. Berquin & Lebacq (1992) have found an appreciable amount of labile heat in mouse soleus muscles (about 64 % of stable heat for a 3 s tetanus) although these (slow) muscles are devoid of parvalbumin. This labile heat is substantially reduced in a second tetanus given a few seconds after a preceding one; the nature of the underlying process is not clear. Another point of interest is the time course of PCr splitting during tetanic contraction of frog muscles. This has been studied by several authors and the results have been summarized by Homsher & Kean (1978). The collected studies show that there is an initial, high rate of PCr splitting both in *Rana temporaria* and *Rana pipiens* muscles which then settles down to a lower value. A similar result was obtained by Homsher *et al.* (1987). Thus it is conceivable that part of the labile heat which we define by fitting records to eqn (2) derives from a higher ATPase activity early during a contraction. Whether this hypothetical extra ATPase activity would be of myofibrillar or SR origin cannot be clearly decided although some observations may link it with SR processes.

One such observation is that activation heat, i.e. heat not related to cross-bridge activity and assumed to correlate with SR calcium pumping, is markedly reduced for some tens of milliseconds following a single twitch at 23 °C and then recovers within 100–200 ms (Homsher & Kean, 1978). This was interpreted to mean that calcium release normally decreases with consecutive twitches. Also, the records of calcium transient of Blinks, Rüdel & Taylor (1978) show a continuous decline in their amplitude. Whether there is a similar decrease during a long tetanus has not been studied in detail.

Another argument in favour of changes of ATPase activity occurring early during a tetanus derives from a comparison of the magnitude of slowing of relaxation of the present fibres with that observed for tibialis anterior (TA) fibres of *Rana temporaria* by Hou & Rall (1987). The extent of slowing can be expressed as $y_o/(y_o+y_b)$ and this

ratio was found by Hou & Rall to be 0·63 at 0 °C, 0·33 at 10 °C and 0·37 at 20 °C. For *Xenopus* type 1 fibres, which in many respects resemble their TA fibres, we found considerably higher values (Figs 3*A* and 5): 0·65 at 10 °C and 0·58–0·65 at 20 °C. *Xenopus* type 1 fibres display this more pronounced slowing despite a lower PA content, 0·45 mM as compared to 0·73 mM for TA fibres. If the slowing of TA fibres is wholly accounted for by calcium saturation of parvalbumin, as claimed by Hou *et al.* (1991) then some additional mechanism has to be invoked to explain the more extensive slowing in *Xenopus* fibres at 10–20 °C. One possible change taking place early in a tetanus might be diminished calcium pumping by the SR, but we do not know of any direct evidence for such an effect.

The final relaxation rate (y_b)

In contrast to the difference in the magnitude of slowing at 10 °C between *Rana temporaria* and *Xenopus* fibres, the temperature dependence of the final relaxation rate, y_b, seems to be similar. From the experiments at 10 and 20 °C a Q_{10} of 2·4 for y_b was obtained, which is the same as that reported by Hou & Rall (1987) for *Rana temporaria* fibres for this temperature interval.

It is not yet clear what process is the major determinant of relaxation rate in the 'adapted state', i.e. when calcium buffering by parvalbumin presumably is complete. Two alternatives are usually considered: (i) cross-bridge detachment rate and (ii) the decay rate of calcium occupancy of troponin C, the latter being determined by the fall in Ca_i^{2+} caused by calcium recapture by the SR.

From the present data we have already argued (p. 133) that, at least in the present temperature range, cross-bridge detachment rate would be of lesser importance. The argument is somewhat indirect in that it assumes that detachment rate is proportional to cross-bridge turnover rate, which may not necessarily be true. However, consideration of Q_{10} values points in the same direction. Cross-bridge turnover rate has a Q_{10} of about 4, whereas relaxation rate has a Q_{10} of about 2·5 (Rall & Woledge, 1990; present results). Further evidence for changes in Ca_i^{2+} being the major determinant of relaxation rate comes from recent experiments, also on *Xenopus* fibres, with a 'caged' calcium chelator (Lännergren & Arner, 1992). Single toe muscle fibres (type 1) were incubated with a membrane permeable form of diazo-2, which upon photolysis liberates the powerful calcium chelator 1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) intracellularly. Flashes given at the beginning of the linear phase of relaxation increased relaxation rate 1·8-fold at 20 °C; thus lowering of Ca_i^{2+} has a marked effect on the speed of relaxation.

The results of the ATPase measurements on skinned type 1 and type 2 fibres are in reasonable agreement with the view that calcium handling is the major determinant of relaxation rate. Figure 9 shows that SR ATPase activity was nearly in the same range for type 1 and type 2 fibres with a large scatter for each type, whereas myofibrillar (AM) ATPase activity was clearly higher for type 1 fibres. Statistical analysis (Student's t test, $P < 0·01$) showed that the mean value for SR ATPase was significantly higher for type 1 than for type 2 fibres. It appears unsafe to correlate this directly with y_b, the final relaxation rate, since the ATPase measurements were performed on a different group of fibres and performed at 4 °C whereas the living fibres were studied at 20 °C, but the results suggest that there are

no large functional differences in design or number of Ca^{2+} pumps between the fibre types.

Conclusion

The main results of this study suggest that for *Xenopus* fibres at 20 °C, slowing of relaxation during a long tetanus and subsequent recovery of relaxation rate are not solely determined by Ca^{2+} binding to, and dissociation from, parvalbumin. Some other change takes place during stimulation which requires many minutes for reversal. The change has an energetic facet which contributes to labile heat production. From published values for the enthalpy change of Mg^{2+}–Ca^{2+} exchange on parvalbumin the magnitude of this 'unexplained' labile heat amounts to about 70% of total labile heat.

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