Differential effects of length on maximum force production and myofibrillar ATPase activity in rat skinned cardiac muscle

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1. The fall of maximum Ca^{2+}-activated force of cardiac myofibrils at short muscle lengths could be due to a reduction of cross-bridge cycling or to development of an opposing (restoring) force. To try to distinguish between these possibilities, we measured simultaneously myofibrillar force development and MgATPase activity (a measure of cross-bridge cycling) in rat skinned trabeculae at different muscle lengths. ATPase activity was measured photometrically from the utilization of NADH in a coupled enzyme assay. Muscle length was varied to give estimated 0·2 μm changes in sarcomere length (SL) over the range 1·4–2·4 μm.

2. Both Ca^{2+}-activated force development and ATPase activity were optimal at a muscle length (L_0) where the resting SL was 2·2 μm. At L_0 the maximum ATPase activity at 21 °C was $0{\cdot}56 \pm 0{\cdot}05$ mM s^{-1} (mean ± S.E.M., $n = 6$), which was equivalent to an ATP turnover per myosin S1 head of 3·3 s^{-1}.

3. The relationship between ATPase activity and SL was curved, with rather little change in ATPase activity over the SL range 2·0–2·4 μm, but significant falls at 1·8 μm and below. At 65 % of L_0 (corresponding to a mean active SL of approximately 1·4 μm), the ATPase activity was only 50 % of its value at 2·2 μm SL.

4. Force development decreased linearly as SL was reduced below 2·2 μm. Force fell by more than ATPase activity, particularly at SL 1·6 and 1·8 μm.

5. The fall of ATPase activity indicates that some of the decline of force production at short SL results from a fall in the net rate of cross-bridge cycling. This is probably the result of double overlap of thin filaments. However, the differential effect on force and ATPase reveals that, in the intermediate range of SL, decreased cross-bridge cycling can account for only part of the fall of force; the remainder is probably due to an increase in a restoring force, which may arise from deformation of the connective tissue in the muscle preparations used.

A decrease in the length of mammalian cardiac muscle results in a reduction in the force developed during the twitch. This length–tension relationship is a fundamental property of cardiac muscle and is the basis of the Frank–Starling mechanism, by which end-diastolic volume determines cardiac output. It has become increasingly clear that a major contribution to the cardiac length–tension relationship is due to length-dependent properties of the myofibrils within the myocardial cells (reviewed by Allen & Kentish, 1985). Experiments with skinned cardiac muscles (Hibberd & Jewell, 1982; Kentish, ter Keurs, Ricciardi, Bucx & Noble, 1986) have shown that myofibrillar force production is reduced at shorter sarcomere length (SL) and that two factors are responsible. The first is that the sensitivity of the myofibrils to $[Ca^{2+}]$ is reduced, probably due to a decrease in Ca^{2+} binding to troponin C in the myofibrils (Hoffman & Fuchs, 1987; Allen & Kentish, 1988). The second factor is that the maximum force-generating ability of the myofibrils is decreased at shorter SL. This effect is present at all Ca^{2+} concentrations, but it can only be clearly distinguished from the change in myofibrillar Ca^{2+} sensitivity if the myofibrils are fully activated with Ca^{2+}, so that the concomitant changes in Ca^{2+} sensitivity have no effect. In skinned cardiac trabeculae this fall of force is particularly marked. For example, a fall in SL over the physiological SL range of 2·3–1·6 μm in cardiac muscle (the 'ascending limb' of the length–tension relationship) decreased the maximum Ca^{2+}-activated force by about 50 %

(Kentish *et al.* 1986). The mechanism underlying this influence of SL on the maximum Ca^{2+}-activated force is unresolved and is the subject of the present study.

Two possible explanations for the fall of maximum force production at short SL were proposed by Gordon, Huxley & Julian (1966) to account for the ascending limb in tetanized frog skeletal fibres. They suggested myofilament interactions at short SL could cause (i) interference with force generation by the myosin cross-bridges, e.g. by double overlap of thin filaments, which may block the myosin-binding sites on actin and reduce the number of cycling cross-bridges, or (ii) production of a restoring force, e.g. by longitudinal compression of myofilaments, that could oppose shortening of the myofibrils. This restoring force would constitute an internal load in opposition to the force produced by the cross-bridges, thereby reducing the externally measured force. Both possibilities also apply to cardiac muscle, though in addition the substantial amounts of intra- and extracellular tissue elements in parallel with the sarcomeres in some cardiac muscle preparations, such as trabeculae, could constitute a non-myofibrillar source of restoring forces.

To distinguish between these two explanations for the fall of force at short SL in cardiac muscle – a decline in the number of cycling cross-bridges or the presence of a restoring force – we measured the actomyosin ATPase activity in cardiac muscles, as this reflects the overall rate of cross-bridge cycling. Our reasoning was that, if the fall of force is due to decreased cross-bridge cycling, the ATPase activity would fall in parallel with force as muscle length was reduced, whereas if the fall of force is due to an increased restoring force, cross-bridge cycling and thus ATPase activity should be unaffected by muscle length. In the present study, maximum Ca^{2+}-activated force production and ATPase activity were measured simultaneously in skinned cardiac trabeculae, with muscle length varied to change the SL over the physiological range in cardiac muscle.

Some of these results have been presented in preliminary form (Stienen & Kentish, 1992).

METHODS

Preparation of skinned trabeculae

Rats (~350 g) were killed by cervical dislocation. The hearts were excised and placed in oxygenated Tyrode solution with 1·8 mM Ca^{2+} at 4 °C. Langendorff perfusion was then started using Tyrode solution at 20 °C, in order to clear the vasculature of blood and so reduce the scattering of light by red cells during subsequent laser diffraction of the muscles. The hearts were opened up and 20 mM 2,3-butanedione monoxime was added to the Tyrode solution to inhibit cell contracture during dissection (Mulieri, Hasenfuss, Ittleman, Blanchard & Alpert, 1989). Suitable trabeculae (diameter ~150–250 μm) were cut out from the right ventricle and were skinned by 2–3 h immersion in relaxing solution (see below) containing 1 % Triton X-100, which effectively eliminates cell membranes and their constituent ATPases (Kurebayashi & Ogawa, 1991). One muscle was stored overnight in Triton at 4 °C; this produced no difference in the results obtained. The arms of aluminium T-clips were crimped firmly over the ends of each muscle and the muscle was transferred to the muscle bath.

Apparatus

The bath system consisted of a series of wells of volume 80 μl. The bath used for the ATPase assay had a volume of 30 μl and had quartz ends to allow the transmission of near-UV light for the measurement of NADH absorbance (see below). This bath was stirred by motor-driven vibration of a rubber diaphragm in its base. Water from a thermoregulator was circulated to hold the baths at constant temperature (21 °C). The muscle was transferred manually between baths. The T-clips on the muscle were attached to hooks on an isometric force transducer (AE801, SensoNor, Horten, Norway) and on a micromanipulator. Force and absorbance signals were filtered at 10 Hz and recorded on a chart recorder and, after A–D conversion, on a PC compatible computer. SL in the resting muscle was measured by laser diffraction (10 mW He–Ne laser, model 1125, Uniphase), with the position of the diffraction pattern measured by eye on a calibrated screen. The SL in the relaxed muscle was initially set to 2·2 μm, which was taken as the control SL in these experiments.

All solutions contained: 100 mM BES (*N*,*N*-bis[2-hydroxyethyl]-2-aminoethane sulphonic acid; pH 7·1 with KOH), 5 mM $MgATP^{2-}$, 1 mM free Mg^{2+}, 10 mM phosphoenolpyruvate, 0·9 mM NADH, 4 mg ml^{-1} pyruvate kinase, 0·24 mg ml^{-1} lactate dehydrogenase, and 0·2 mM diadenosine pentaphosphate (AP_5A). Potassium propionate was added to adjust the final ionic strength to 0·20 M. In most experiments 5 mM sodium azide was added to avoid interference by mitochondrial ATPase activity. Three types of solution were employed: relaxing solution with 20 mM EGTA (pCa = 9·0; pCa is $-\log_{10}[Ca^{2+}]$), pre-activating solution with 0·5 mM EGTA and 19·5 mM 1,6-diamino hexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (HDTA), and a maximal activating solution with 20 mM CaEGTA (pCa 4·3). Aliquots of these solutions (minus enzymes, NADH and AP_5A) were stored frozen. On the day of the experiment, the solutions were thawed, enzymes and other chemicals were added, and the solutions were kept on ice until required. Solutions were applied in the sequence: relaxing, pre-activating, activating, relaxing.

Enzymes were from Sigma and other chemicals from Sigma, Fluka and Boehringer.

Measurement of ATPase activity

The ATPase activity of the skinned cardiac muscles was measured by a coupled enzyme assay, described in detail previously (Stienen, Roosemalen, Wilson & Elzinga, 1990). In brief, the hydrolysis of ATP to ADP in the skinned muscle was coupled by two linked enzyme reactions to the oxidation of NADH to NAD^+: pyruvate kinase converted the ADP + phosphoenolpyruvate to ATP + pyruvate, then lactate dehydrogenase converted pyruvate + NADH + H^+ to lactate + NAD^+. The fall of NADH concentration was determined photometrically by recording the decrease in NADH absorbance of the solution. The reaction sequence is stoichiometric i.e. the hydrolysis of 1 mol of ATP results in the oxidation of 1 mol of NADH. Excitation light from a Hg arc lamp was directed horizontally through the solution below the muscle in the assay bath via the quartz bath ends. The emerging beam was split by a 50/50 beam splitter. One half of the beam passed to a photomultiplier via a 340 nm filter to measure NADH absorbance. The other half passed to a photodiode with a 400 nm

filter, which gave a reference signal independent of [NADH]. The NADH absorbance signal was divided by the reference signal, to correct for changes in excitation light intensity, and was fed into a logarithmic amplifier to give an output proportional to optical density.

The usual procedure for the assay is illustrated in Fig. 1. First, the rate of fall of NADH absorbance was measured with the muscle absent from the assay bath. The decrease in absorbance under these conditions was mostly due to photobleaching of NADH, with a minor contribution from contaminant ATPase activity in the enzymes used. During this period, the system was calibrated by injecting 0·1 μl of 10 mM ADP (i.e. 1 nmol) from a motor-driven Hamilton syringe; this caused a step decrease in absorbance (arrows). After a sufficient time (~1 min) for the baseline slope to be recorded, the muscle was moved into the bath. If the bath solution was the activating solution, as in Fig. 1, the muscle generated force and began to split ATP rapidly, as shown by the marked increase in slope of NADH absorbance. Stable levels of force and ATPase activity were attained in 5–10 s. Once force and ATPase rate (absorbance slope) had been steady for 30–40 s, the muscle was removed from the bath and placed in relaxing solution. Another 1 nmol of ADP was injected to check the reproducibility of the absorbance change. The rate of ATP hydrolysis due to the muscle was taken as the rate of absorbance change with the muscle present minus the mean of the rates before and after exposure of the muscle to the solution. This calculation was performed by computer, after fitting the absorbance with 1st order regression lines between selected points on the traces. To determine the resting ATPase activity in the muscle, the procedure of Fig. 1 was followed except that the assay bath contained relaxing solution rather than activating solution. To limit the bath accumulation of P_i to < 0·1 mM, the bath solution was replaced after every two or three assays.

Experimental protocol

The overall aim of the study was to measure the SL dependence of force production and ATPase activity. To this end, muscle length was varied in relaxing solution to give 0·2 μm alterations in the measured resting SL, and force and ATPase activity were then measured in the relaxed or activated muscle. (The SL in the *active* muscle at maximal [Ca^{2+}] could not be measured, owing to the laser diffraction pattern becoming too diffuse *viz.* Kentish *et al.* 1986.) To correct for a slight decline of force and ATPase activity with time during the experiment, force and ATPase activity at each 'test' resting SL were expressed relative to the mean values of force or ATPase activity in control contractures (SL 2·2 μm) that bracketed the test contracture. The muscle length required for each resting SL was noted using the micromanipulator vernier. If, on return to the control SL of 2·2 μm, the muscle length needed for this SL differed from the previous control value, the entire procedure (controls + test contracture) was repeated. Usually a change in muscle length for a given SL was needed only after the first contracture in an experiment. The reproducible muscle length for a resting SL of 2·2 μm is designated L_0. In most muscles the resting SL was

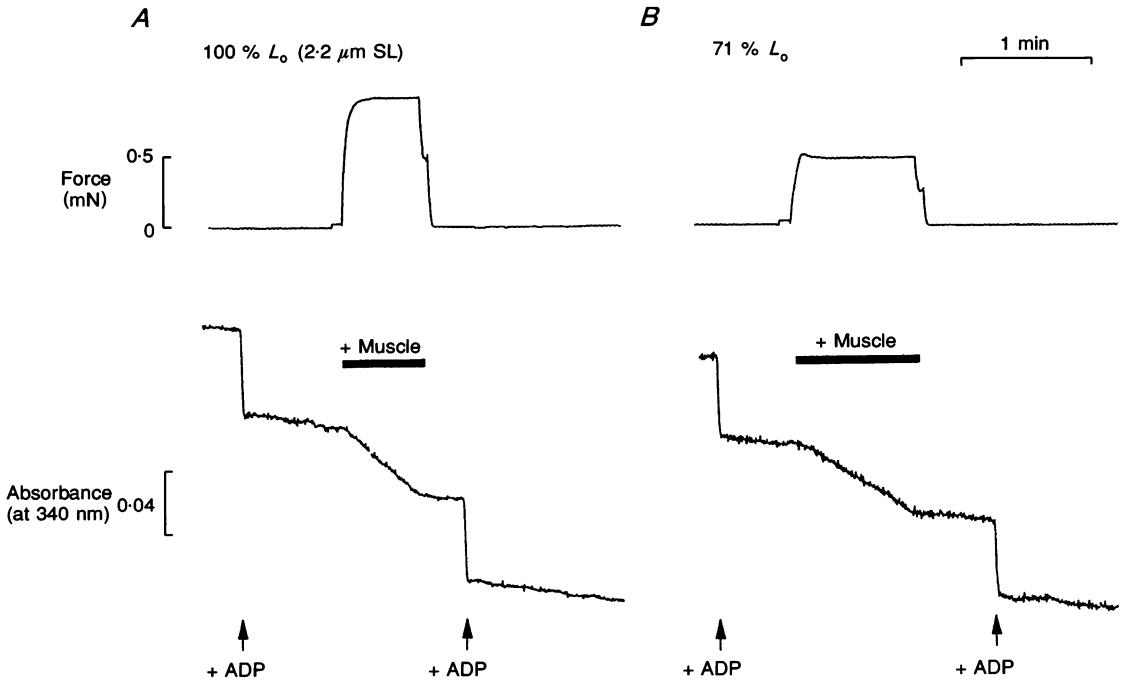

Figure 1. Chart records of force production (upper traces) and ATPase activity (lower traces) at two muscle lengths

L_0 is the muscle length for a resting SL of 2·2 μm. ATPase activity was measured from the decrease in light absorption as NADH was used up in the NADH–LDH coupled enzyme assay. The solution in the assay bath was activating solution (pCa 4·3). Bars indicate where the muscle was added to the assay bath after having been in pre-activating solution (pCa 9). At intervals 1 nmol of ADP was injected into the bath to calibrate the system (arrows).

found to be uniform (dispersion < 0·1 μm) as the laser beam was moved along the length of the muscle; if this was not so, the muscle was discarded. As the muscle was held isometric during contraction, the mean SL in the active muscle should be similar to the resting SL at the same muscle length (see Discussion). However, the resting SL could not be shortened below 1·8 μm, which was the SL in the muscle when slack. To try to produce 0·2 μm decrements of SL in the *active* muscle at SL < 1·8 μm, the following procedure was adopted. Readings of muscle length that gave changes in resting SL at 0·1 or 0·2 μm intervals in the range 2·2–1·8 μm were noted. The relationship between resting SL and muscle length was plotted and was found to be close to linear over this range (Fig. 2). This relationship was then used to estimate, by extrapolation, the lengths of the active muscle that would give SL values of 1·6 and 1·4 μm. Force and ATPase activity were then measured during Ca^{2+} activation at these muscle lengths.

At the end of the experiment, the length, width and depth of the muscle was measured with an eyepiece graticule in a dissecting microscope. The muscle was then allowed to dry in air at length L_0 and the weight of the muscle between the T-clips was determined with a Cahn electrobalance.

Electron microscopy was performed with some muscles. Trabeculae were first activated and then were shortened to give an 'assumed' SL of 1·6 or 1·4 μm. They were then transferred to rigor solution (activating solution minus MgATP) to keep the SL constant. After 10 min, rigor solution was replaced by 2·5% glutaraldehyde in buffer (0·1 M sodium cacodylate, pH 7·4), which was left for 2 h at room temperature. Once fixed, the muscles were disconnected from the apparatus, immersed in buffer and processed for electron microscopy.

Data values are given as means ± S.E.M. of n experiments. Statistical significance was taken as $P < 0·05$.

RESULTS

When measured at the control muscle length (L_0), at which the resting SL was 2·2 μm, the dimensions of the muscles used in the analysis were as follows (means ± S.E.M., $n = 6$): length, 2·19 ± 0·14 mm; depth, 177 ± 16 μm; width, 155 ± 7 μm. The force produced by these muscles in the first or second contracture of the experiment at full Ca^{2+} activation (pCa 4·3) was 58·3 ± 0·5 mN mm^{-2}; this substantial force production is similar to the value we usually find in skinned trabeculae that are homogeneous and visibly undamaged. The total ATPase activity in these contractures amounted to 27·1 ± 4·4 pmol s^{-1}. This value was obtained from five muscles measured at 21 °C and from one at 24·5 °C, corrected to 21 °C using a Q_{10} (temperature coefficient) of 4 (Burchfield & Rall, 1986). This ATPase activity corresponded to 0·56 ± 0·05 mM s^{-1} ($n = 6$) when divided by the calculated fibre volume, and to 3·53 ± 0·16 μmol s^{-1} (g dry wt)$^{-1}$ in four muscles in which the dry weight was determined (8·6 ± 2·0 μg).

Figure 3 shows how force and ATPase activity varied throughout the experiments. The resting ATPase activity (at pCa 9) was determined both at the start and the end of the experiment and did not change over this period. As Fig. 3 illustrates, if azide was present the resting ATPase activity was extremely low. At SL 2·2 μm the resting ATPase activity at the start of the experiment was 1·3 ± 0·8% ($n = 5$) of the total ATPase in the first Ca^{2+}-activated contracture; however, this rose to 8·7 ± 1·5% if azide was omitted ($n = 3$).

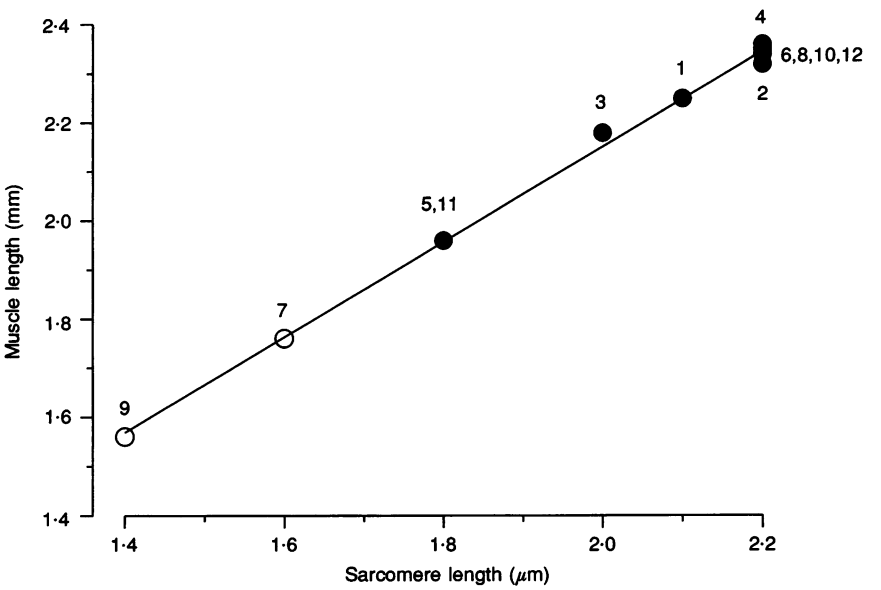

Figure 2. Relationship between muscle length and resting sarcomere length in a typical muscle
Sarcomere length was measured by laser diffraction (●) or estimated by extrapolation (○) to give the 'equivalent resting sarcomere length' in shortened muscles. Numbers indicate the sequence of observations. The line is the linear regression fit to the measured SL values.

In repeated control contractures (SL 2·2 μm, pCa 4·3), the force produced by the muscles declined a little throughout the experiment. This was particularly fast in the muscle shown in Fig. 3 (open symbols), since this muscle was studied at a higher temperature (24·5 °C) than the other muscles (21 °C), and the deterioration in skinned cardiac muscles has a high Q_{10} (~6; J.C. Kentish, unpublished observation). We found that the maximum ATPase activity (filled symbols) also declined during the experiment in parallel with force, suggesting that the decrease is a reflection of reduced cross-bridge activity. This may be due, for example, to wash-out of troponin C. The small degradation of contractile performance was corrected for by bracketing the 'test' contractions with the control contractions at SL 2·2 μm (see Methods), and should not affect the conclusions drawn in this study. The decline in maximal total ATPase activity was not associated with a noticeable change in the resting ATPase activity, so the myofibrils were well regulated by Ca^{2+} throughout the experiment.

In Fig. 3 there is some evidence that force and total ATPase activity were affected differentially as SL decreased. The influence of length on these parameters is shown in more detail in Fig. 4, which gives the mean results for the six muscles used. The abscissae are given as both muscle length and observed SL as we could not directly measure SL values below 1·8 μm (values we refer to below are extrapolated SL values). As SL was decreased, active force decreased in a near-linear fashion, and at SL 1·4 μm (muscle length = 65 % of L_0) was 42·0 ± 7·6 % ($n = 3$) of the control force at SL 2·2 μm. To check that the activating solution produced full Ca^{2+} activation of the myofibrils even at short SL, where it is known that myofibrillar Ca^{2+} sensitivity is reduced (Allen & Kentish, 1985), $CaCl_2$ was added to the activating solution in the bath. Force did not change, indicating that activation by Ca^{2+} was indeed maximal (results not shown).

The relationship for ATPase activity was clearly different from that for force: over the SL range 2·0–1·6 μm the total ATPase activity fell less than force as length decreased. At 1·8 and 1·6 μm the mean of the differences between percentage force and percentage total ATPase were 20·0 ± 2·5 % ($n = 6$) and 19·1 ± 2·8 % ($n = 5$), respectively; both differences were statistically significant (paired t test). However, at 1·4 μm relative force and ATPase activity were not significantly different. In most muscles the SL was not increased above 2·2 μm owing to the appearance of a high resting force, but in one muscle where this was possible the rise in SL to 2·4 μm produced no further increase in force or ATPase activity. These data indicate that, as SL was reduced over most of the physiological range of SL in cardiac muscle, force production generally fell more than total ATPase activity.

We also compared Ca^{2+}-activated force (Fig. 4) with the Ca^{2+}-activated ATPase activity, which was calculated as total ATPase activity minus resting ATPase activity. Resting ATPase activity was small (< 5 % of total) and was independent of SL over the range 1·8–2·2 μm (Fig. 4). We assumed that resting ATPase activity was constant over the entire SL range. As resting ATPase activity was so small,

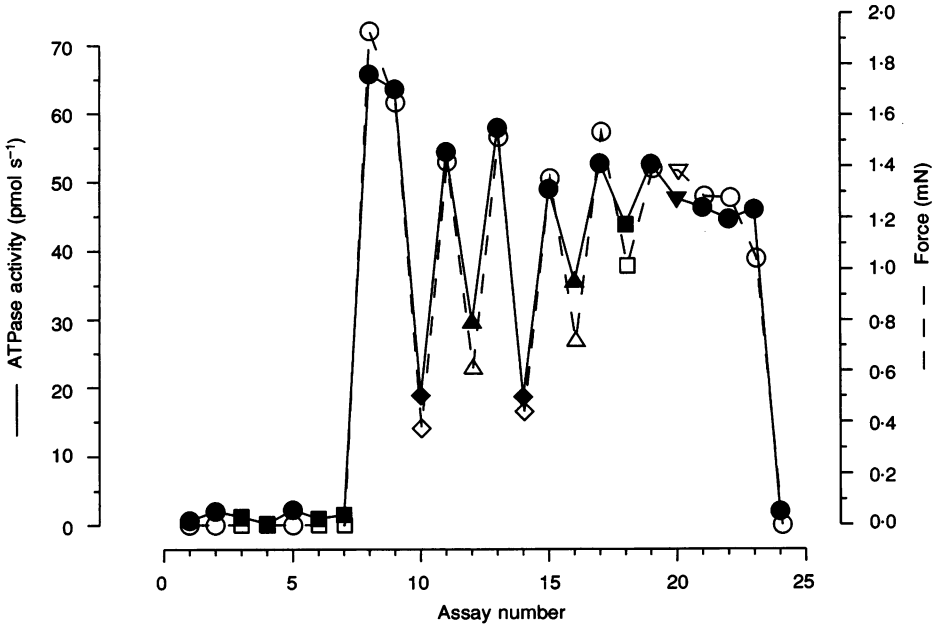

Figure 3. ATPase activity (filled symbols) and force production (open symbols) during an experiment

The muscle was bathed in relaxing solution at pCa 9 (assays 1–7 and 24) or in maximal Ca^{2+}-activating solution at pCa 4·3 (assays 8–23) at 24·5 °C. The resting SL values were 2·2 μm (○,●), 2·0 μm (▽,▼), 1·8 μm (□,■), 1·6 μm (△,▲; by extrapolation) and 1·4 μm (◇,◆; by extrapolation). Muscles were relaxed between each Ca^{2+}-activated contracture.

the calculated ATPase–SL relationship for Ca^{2+}-activated ATPase (not shown) was little different from that for total ATPase (Fig. 4). The maximum divergence was at the shortest length, where total and Ca^{2+}-activated ATPase activities were 51·2 ± 9·0 and 49·8 ± 9·4 % of their maximum values. As with total ATPase activity, there were significant differences between percentage force and percentage Ca^{2+}-activated ATPase activity at SL 1·8 μm (difference of 18·9 ± 2·8 %) and at SL 1·6 μm (17·1 ± 3·4 %). Thus the conclusion was the same as with the total ATPase data: muscle length had a differential action on force and ATPase activity.

A potential problem with the comparison of force and ATPase activity would arise if a significant fraction of the ATPase activity in the presence of Ca^{2+} was due not to the myofibrils but to the Ca^{2+}-activated ATPase activity of the sarcoplasmic reticulum (SR) Ca^{2+} pump. To estimate the extent of contaminating SR ATPase activity, in pilot experiments we used two inhibitors of the SR Ca^{2+} pump: 10 μM cyclopiazonic acid (Seidler, Jona, Vegh & Martonosi, 1989) dissolved in ethanol (final concentration 1 % v/v) or 0·12 mM quercetin (Kurebayashi & Ogawa, 1985) dissolved in polyethylene glycol (1 % v/v). After allowing for slight inhibition of ATPase activity due to the solvents themselves, the addition of cyclopiazonic acid decreased the Ca^{2+}-activated ATPase activity by 2·4 %, and quercetin decreased it by 6·9 % ($n = 2$). Thus the Ca^{2+}-activated SR ATPase contributed very little to the overall ATPase activity.

Ultrastructure of activated muscles

Sarcomere lengths below 1·8 μm could only be estimated during the experiment by extrapolation of the relationship between muscle length and SL (Fig. 2). To assess the ultrastructure in the more shortened muscles and to see if muscle length was a reasonable predictor of the active SL in these muscles, some muscles were fixed during rigor, after activation by Ca^{2+}, and were processed for electron microscopy. Force remained constant or increased by up to 20 % during the induction of rigor, and then did not alter appreciably during fixation. This suggests there was minimal sarcomere shortening during rigor or filament shrinkage during fixation (because the muscles became very stiff, even slight shrinkage would have produced a large force increment). Therefore no allowance was made for filament shrinkage during fixation. As a typical muscle illustrates (Fig. 5), there was good agreement between the predicted and actual SL in muscles at shorter SL. The SL was also found to be homogeneous throughout each

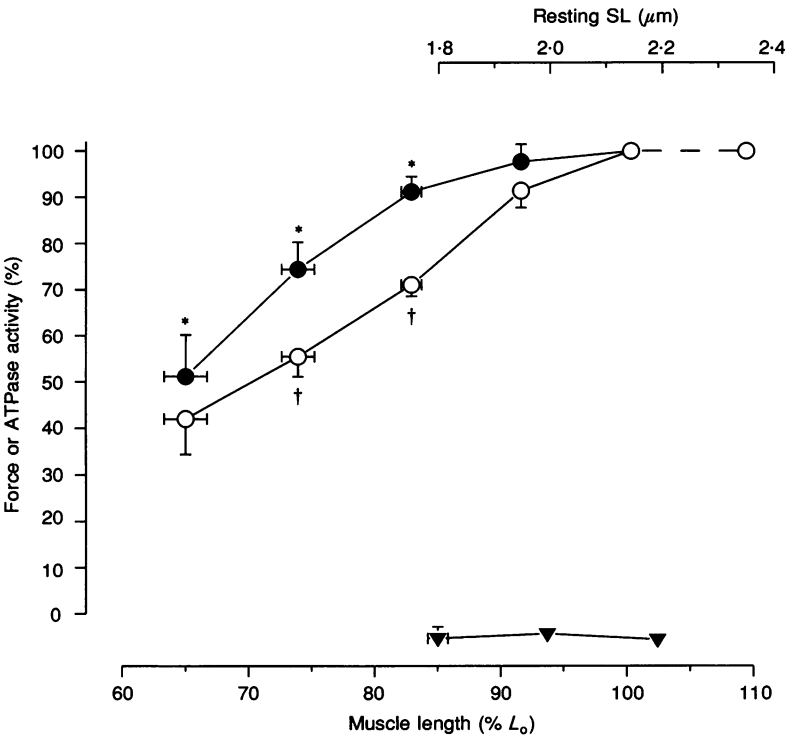

Figure 4. Length dependence of active force production at pCa 4·3 (○), total ATPase activity at pCa 4·3 (●) and resting ATPase activity at pCa 9 (▼)

Force and ATPase activities are given as a percentage of their maximum values at SL 2·2 μm. The abscissae shown are the measured SL in the relaxed muscle (top) and the muscle length needed to produce the 0·2 μm steps in SL (bottom, as a percentage of L_o, the muscle length at 2·2 μm SL). Means ± S.E.M. of 5 or 6 experiments, except at 65 % L_o ($n = 3$) and 109 % L_o ($n = 1$). Horizontal S.E.M. bars refer to the muscle length scale. *Relative ATPase: significantly different from 100 %; †relative force: significantly different from corresponding relative ATPase (both paired t test, $P < 0·05$).

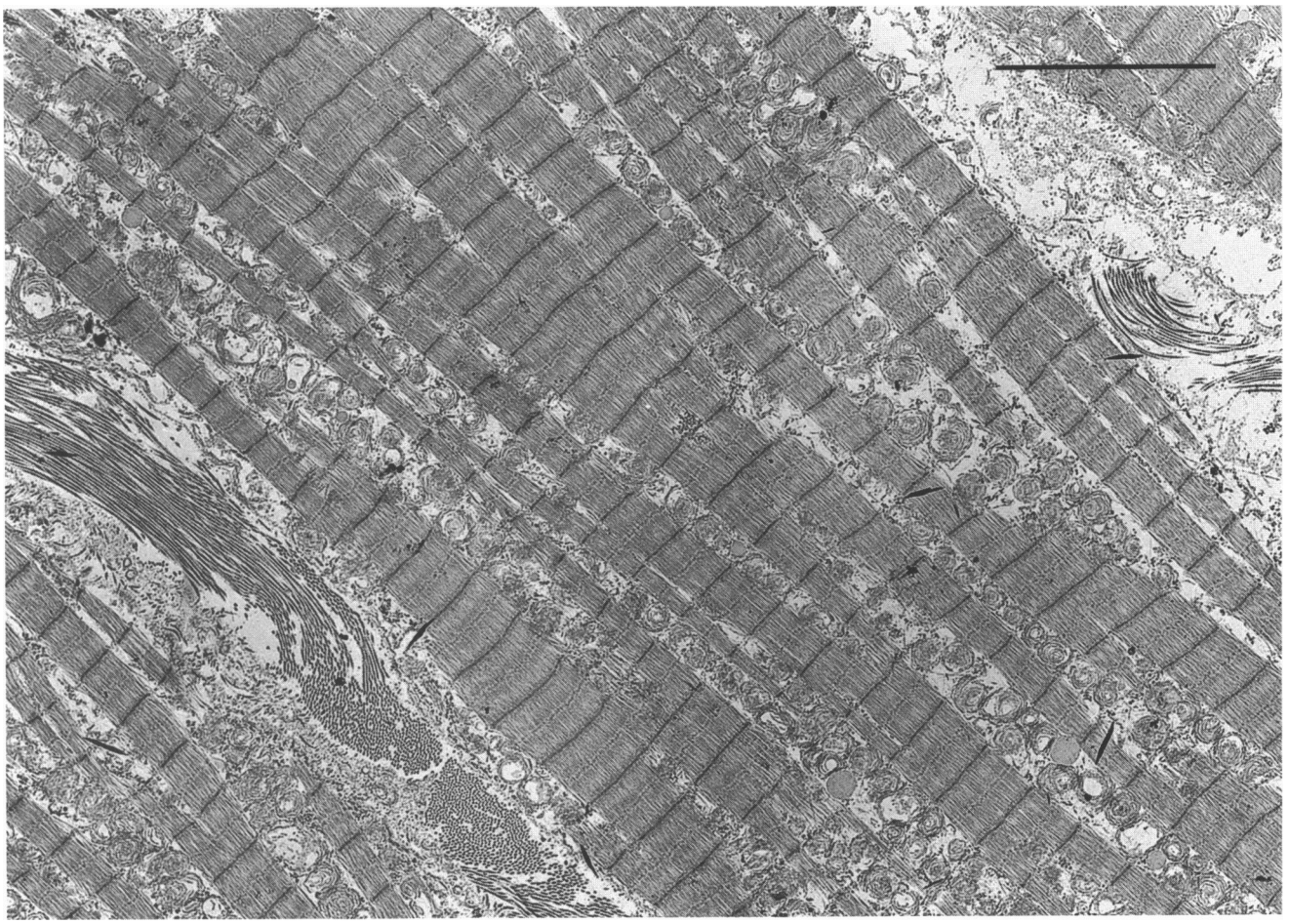

Figure 5. Electron micrograph of the core of a skinned trabecula
The muscle was fixed during activation at a muscle length that gave an extrapolated SL of 1·4 μm. Calibration bar, 5 μm. The mean SL measured from sarcomeres in this and other sections from the same muscle was 1·41 μm.

preparation, even though the muscle had been generating maximum force before being put into rigor. The micrograph also showed whorls of mitochondrial remnants in the core of the muscles. However, membrane was absent from the outer muscle layers (not shown). In addition, there was a substantial collagen matrix in these muscles.

DISCUSSION

Resting ATPase activity

With no azide in the solutions, the resting ATPase activity was 8·7 ± 1·5 % of the total ATPase activity at 2·2 μm SL. This was much less than found previously in skinned rat trabeculae (~45 % of total activity, Stienen & Elzinga, 1990; 30 % of total, J. C. Kentish & R. E. Godt, unpublished), probably because the prolonged skinning period of > 2 h increased the destruction of the cell membranes and their constituent ATPases. It is likely that mitochondrial remnants, visible in parts of the preparations (Fig. 5), accounted for this ATPase activity, since this activity was almost abolished by 5 mM azide (activity = 1·3 ± 0·8 % of total). The negligible resting activity in the presence of azide also shows that the actomyosin was well regulated by [Ca^{2+}], i.e. the addition of Ca^{2+} (pCa 4·3) induced a 50- to 100-fold increase in ATPase activity. This contrasts with results using suspensions of isolated cardiac myofibrils obtained by homogenization, which commonly show a Ca^{2+}-independent (basal) ATPase activity that is 10–20 % of the total activity (e.g. Solaro & Shiner, 1976; Kentish & Nayler, 1979). Our results suggest that this basal ATPase activity probably results from some damage to the myofibrils during homogenization.

Calcium-activated ATPase activity at SL 2·2 μm

Of the measured Ca^{2+}-activated ATPase activity (0·56 ± 0·05 mM ATP s^{-1} at 21 °C), more than 90 % was probably due to the activity of actomyosin ATPase because the contribution of SR Ca^{2+} pump to the ATPase activity was < 7 %, judged from the effects of SR inhibitors. Assuming that 95 % of the Ca^{2+}-activated ATPase activity was due to actomyosin and that the concentration of myosin S1 heads is 0·16 mM (Barsotti & Ferenczi, 1988), 0·56 mM s^{-1}

corresponds to a turnover of ATP per S1 head of 3·3 s^{-1}. This value is somewhat greater than in previous studies with other cardiac muscle preparations. For example, in pig skinned myocardium the turnover was 0·5 s^{-1} at 24 °C (Kuhn, Bletz & Rüegg, 1990) and in guinea-pig skinned trabeculae it was 0·4 s^{-1} at 12 °C (Barsotti & Ferenczi, 1988), which is equivalent to 1·4 s^{-1} at 21 °C (assuming a Q_{10} of 4; Burchfield & Rall, 1986). Our higher value for ATP turnover may be because we measured this at optimal SL and in a species (rat) of an age (1·5 months) where the myosin isozyme is almost exclusively the fast V_1 form (Horowits & Winegrad, 1987). It may be noted that the turnover rate of 3·3 s^{-1} approximates that for fast-twitch skeletal fibres from rat (3·8 s^{-1} at 22 °C; Stephenson, Stewart & Wilson, 1989), so it may be incorrect to consider that cardiac muscle is in every species a 'slow' type of muscle.

Length dependence of calcium-activated force and ATPase activity

ATPase activity is a useful measure of cross-bridge cycling rate in skinned muscle, although it does not distinguish changes due to alterations in the *number* of cycling cross-bridges from those due to alterations in the cycling *rate* of each cross-bridge. Potentially, the number of attached cross-bridges could be obtained from measurements of instantaneous stiffness, but in multicellular cardiac muscles the interpretation of stiffness measurements is complicated by a length dependence of passive forces, due to connective tissue, etc., at lengths above and below the slack length.

There have been previous studies on the length dependence of force and ATPase activity in skinned cardiac muscle (Kuhn *et al.* 1990) and skeletal muscle (Stephenson *et al.* 1989), using enzyme cascades involving NADH (discussed below). One disadvantage with both studies was that ATPase activity was measured in a short section of the muscle, the myosin content of which increased as the muscle was shortened. This would have tended to increase ATPase activity in that section. Mathematical corrections therefore had to be made to minimize this potential artifact. The present study avoids these uncertainties because ATPase activity was measured in the whole of the skinned muscle, so the mass of myosin studied was constant. Another advantage was that the bath system allowed continuous stirring, thus preventing artifactual changes in force due to unstirred layers (Kentish, 1991). However, one potential disadvantage of our procedure is that tissue within the T-clips could contribute to the measured ATPase activity but would not be subject to any length changes. For this reason, care was taken to select long trabeculae, to keep the tissue within the T-clips as small as possible (estimated to be < 20 % of total muscle volume), and to clamp the clips tightly so that this region was isolated from the bathing solutions.

In one muscle we tried to assess the contribution of the clamped ends to the ATPase activity by measuring the muscle's ATPase activity (at SL 2·2 μm), then taking out the central section of the muscle and remounting it with only a short segment visible. The visible length was reduced to 7·7 % of the original, while the ATPase fell to 11·9 %, suggesting that only about 4 % of the original ATPase activity was due to the clamped ends. In addition, some of the end section may have been subject to the length changes, so it would seem that the ATPase activity inside the clamped ends had a negligible effect on the results presented here.

In common with most other studies with skinned cardiac muscles, in this study it was not possible to measure the SL in the fully activated muscle because the laser diffraction pattern became too diffuse. The *mean* SL should be the same in the resting and activated muscle, provided that the sarcomeres extend the entire length of the muscle. Thus the resting SL could be taken as a measure of the mean SL in the activated muscle, at least for the SL range that could be measured in the resting muscle (1·8–2·4 μm); shorter SL values could not be measured directly, but the good correspondence in activated muscles between the 'extrapolated' SL (cf. Fig. 1) and the actual SL measured by electron microscopy (Fig. 5) suggests that we have a reasonable estimate of the mean SL in activated muscles. Mean SL may be the appropriate SL measurement for ATPase activity, but it is less clear whether the same is true for force. Developed force in sarcomeres in series may be determined by the strongest sarcomeres, which would shorten at the expense of the weaker sarcomeres (such as those in the damaged ends of the muscles). In this case the minimum SL would be the relevant independent variable. On the other hand, in preparations such as trabeculae radial as well as longitudinal variations of SL are possible, so it may be argued that mean SL, as recorded here, is the appropriate measure for both force and ATPase.

The problem that arises if ATPase activity is determined by the mean SL and force by the minimum SL is that, if the minimum SL was considerably less than the mean SL (i.e. there was substantial inhomogeneity of SL), then the force data should be shifted to shorter SL than for ATPase. This could account for the observed discrepancy between ATPase activity and force. However, we believe that the inhomogeneity of SL in the activated muscles was small, for several reasons: (i) sarcomere shortening may be large (> 10 %; see Allen & Kentish, 1985) in the centre of preparations that have compliant ends, e.g. if the muscles have been mounted using the valve at one end, but clamping the body of the muscle with T-clips, as used here, is likely to reduce end-compliance considerably; (ii) in the muscles that were fixed for electron microscopy during activation, there was rather little dispersion of SL (e.g. Fig. 5); (iii) the relationship between force and SL (Fig. 4), determined from our estimates of the mean SL in the activated muscle, was very similar to the relationship in rat skinned muscles measured under similar conditions but with SL control (Kentish *et al.* 1986). From these considerations we conclude that any non-homogeneity of SL would be too small to account for the discrepancy between the force–SL and ATPase–SL relationships shown in Fig. 4.

One major finding from the present study is that myofibrillar Ca^{2+}-activated ATPase activity decreased significantly as SL was reduced below 2·0 μm (Fig. 4). In

previous work using skinned ventricular muscle from pig, Kuhn *et al.* (1990) found that Ca^{2+}-activated ATPase activity and force were unaffected by an increase in resting SL from 2·1 to 2·4 μm, which is consistent with our results at this SL (Fig. 4), but Kuhn *et al.* did not study SL values below 2·1 μm. In skinned rat skeletal muscle, Stephenson *et al.* (1989) reported that, although force fell by about 80 % as SL was decreased from 2·7 to ~1·4 μm, there was no change in ATPase activity over the same SL range. Our ATPase results (Fig. 4) are clearly different from those of Stephenson *et al.* However, there may be true differences between muscle types in the effects of SL on ATPase activity, because Elzinga, Peckham & Woledge (1984) found in intact frog fibres that reducing SL below 2·2 μm caused the stable heat rate (due to actomyosin ATPase activity) to fall in parallel with force in extensor longus digiti, whereas it stayed constant (while force decreased) in sartorius.

The observed decrease in ATPase activity in cardiac muscle (Fig. 4) leads us to conclude that reduced cross-bridge cycling does contribute to the observed decrease in force at SL < 2·0 μm, but makes little or no contribution at longer SL values. This fall in ATPase activity is probably due to a fall in the number of attached cross-bridges. This could arise, not from changes in overlap of thick and thin filaments (as these are fully overlapped at SL < 2·2 μm), but from the double overlap of thin filaments, which would block some of the myosin-binding sites on actin. Calculations by Robinson & Winegrad (1979), based on the thin filament lengths in rat atria, indicated that cross-bridge number would begin to fall, as a result of double overlap, below 1·9 μm and would reach a 30 % decrease at 1·6 μm. This is similar to our finding of a 26 % reduction of ATPase activity at 1·6 μm (Fig. 4). This correspondence indicates that double overlap of thin filaments may completely account for the decline in the number of cycling cross-bridges, and hence in ATPase activity that we observed, over the physiological SL range (1·6–2·3 μm) in cardiac muscle.

A second finding from our work is that over much of the SL range studied, force decreased more than ATPase activity as SL was reduced (Fig. 4). We conclude that, over most of the SL range 2·2–1·6 μm, less than half of the decline of force may be attributed to a decrease in cross-bridge cycling (ATPase activity). What causes the remainder of the force decline is unclear. One possible mechanism is that at SL < 2·0 μm each cross-bridge produces less axial force per ATP molecule split, as a result of distortion of the myofilament lattice from its optimal configuration. Another possibility (for which there is some evidence; see below) is that there is a restoring force which opposes the shortening force produced by the cross-bridges and so decreases the measured force.

It is, however, necessary to consider two potential artifacts that could affect the force–SL and ATPase–SL relationships differentially; these arise from P_i accumulation inside skinned muscles during ATP hydrolysis.

(i) Since Pi reduces ATPase activity (Kawai, Güth, Winnikes, Haist & Rüegg, 1987), any changes in ATPase activity, and thus in P_i accumulation, will affect the ATPase activity recorded. For example, if the ATPase truly decreased at short SL, the reduced inhibition by the smaller internal [P_i] would blunt the fall of ATPase activity, i.e. the ATPase–SL relationship would be artifactually shallow. However, this effect would be even greater for force, which is inhibited more strongly than ATPase by P_i (Kawai *et al.* 1987), so that the force–SL relationship would be flatter than that for ATPase. This is opposite to what we observe and so cannot explain our results (indeed the difference between force and ATPase would have been underestimated if this effect is significant).

(ii) At shorter SL values, the muscle diameter may increase, which would raise the accumulated [P_i]. Since P_i inhibits force more than ATPase activity, this would depress force more than ATPase, as observed. Calculations using the diffusion equation (cf. Stienen *et al.* 1990) suggest that in a muscle of diameter 200 μm, splitting ATP at 0·56 mM s^{-1} at SL 2·2 μm (see Results), the mean [P_i] would be ~0·9 mM. If the muscle showed constant volume behaviour, the largest fall in SL (to 1·4 μm) would increase the diameter to 250 μm, and raise the mean [P_i] to 1·4 mM, which would decrease force by 8 % (from Kentish, 1991). This is the maximum change, and is less than one-sixth of the observed decrease in force. ATPase activity would fall by about one-half of this (Kawai *et al.* 1987). Almost certainly, however, this overestimates the effects on force and ATPase, as the muscle's diameter probably increases less than expected from constant volume behaviour. Thus the differential effect of length on force and ATPase activity is not likely to be due to an increase in muscle [P_i] at shorter SL values.

It seems likely that restoring forces will arise in trabeculae as a result of radial or axial deformation of elastic structures inside or outside the cells (see Allen & Kentish, 1985). Clearly intracellular structures do exert a passive restoring force, as skinned (and intact) cells relengthen after Ca^{2+} activation has been terminated (Fabiato & Fabiato, 1976) or ATP restored to cells in rigor (Niggli & Lederer, 1991). This intracellular restoring force is substantial at SL < 1·5 μm (Fabiato & Fabiato, 1976; Niggli & Lederer, 1991), and is probably due to compression of the thick filaments (length 1·55 μm). Such a restoring force may account for the uniformity of SL at SL ~1·4 μm (Fig. 5). At SL > 1·6 μm, however, the restoring force in skinned cells is too small (~4 % of maximum force; Fabiato & Fabiato, 1976) to account for much of the considerable force reduction that we saw in this SL range (up to ~20 % of maximum Ca^{2+}-activated force). With regard to the restoring force at SL > 1·6 μm, it is significant that the slope of the force–SL relationship is much steeper in multicellular preparations such as trabeculae (Hibberd & Jewell, 1982; Kentish *et al.* 1986; present results) than in skinned single cells (Fabiato & Fabiato, 1976). As restoring forces arising from intracellular structures should be similar in both types of muscle preparation, an additional restoring force in trabeculae probably arises from intercellular connections or from stiff extracellular connective tissue (shown in Fig. 5), which are absent from single cells. It is possible that deformation of extracellular elements may account, at least partly, for the differential effect of length on force production and ATPase activity.

One consequence of the discrepancy between force and ATPase activity is that muscle 'efficiency', in terms of external force produced per mole of ATP hydrolysed, decreases at intermediate SL. Relative to that at SL 2·2 μm, this efficiency falls to about 75 % at SL 1·6–1·8 μm. This would appear to be disadvantageous. However, if cross-bridge force is used to increase the potential energy stored in an elastic element responsible for a restoring force, then there is the possible benefit that the restoring force would produce faster elongation of the muscle cells when Ca^{2+} activation was ended, i.e. it would accelerate diastolic relengthening of the muscle (cf. also Robinson & Winegrad, 1979). Extrapolated to the whole heart, this could contribute to diastolic recoil, to aid ventricular filling by suction during diastole. Thus the apparent decrease in efficiency during contraction may be the price the myocardium pays for the advantage of diastolic recoil during relaxation.

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Acknowledgements

We should like to thank Dr Barbara J. Ward for performing the electron microscopy. Financial support by the British Heart Foundation and the Dutch Heart Foundation is gratefully acknowledged.

Received 12 October 1992; accepted 11 August 1993.