Active and passive forces of isolated myofibrils from cardiac and fast skeletal muscle of the frog

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1. Force measurements in isolated myofibrils (15 °C; sarcomere length, 2·10 μm) were used in this study to determine whether sarcomeric proteins are responsible for the large differences in the amounts of active and passive tension of cardiac *versus* skeletal muscle. Single myofibrils and bundles of two to four myofibrils were prepared from glycerinated tibialis anterior and sartorius muscles of the frog. Skinned frog atrial myocytes were used as a model for cardiac myofibrils.

2. Electron microscope analysis of the preparations showed that: (i) frog atrial myocytes contained a small and variable number of individual myofibrils (from 1 to 7); (ii) the mean cross-sectional area and mean number of myosin filaments of individual cardiac myofibrils did not differ significantly from those of single skeletal myofibrils; and (iii) the total myofibril cross-sectional area of atrial myocytes was on average comparable to that of bundles of two to four skeletal myofibrils.

3. In maximally activated skeletal preparations, values of active force ranged from 0·45 ± 0·03 μN for the single myofibrils (mean ± S.E.M.; $n = 16$) to 1·44 ± 0·24 μN for the bundles of two to four myofibrils ($n = 9$). Maximum active force values of forty-five cardiac myocytes averaged 1·47 ± 0·10 μN and exhibited a non-continuous distribution with peaks at intervals of about 0·5 μN. The results suggest that variation in active force among cardiac preparations mainly reflects variability in the number of myofibrils inside the myocytes and that individual cardiac myofibrils develop the same average amount of force as single skeletal myofibrils.

4. The mean sarcomere length–resting force relation of atrial myocytes could be superimposed on that of bundles of two to four skeletal myofibrils. This suggests that, for any given amount of strain, individual cardiac and skeletal sarcomeres bear essentially the same passive force.

5. The length–passive tension data of all preparations could be fitted by an exponential equation. Equation parameters obtained for both types of myofibrils were in reasonable agreement with those reported for larger preparations of frog skeletal muscle but were very different from those estimated for multicellular frog atrial preparations. It is concluded that myofibrils are the major determinant of resting tension in skeletal muscle; structures other than the myofibrils are responsible for the high passive stiffness of frog cardiac muscle.

Fast skeletal muscle develops much more force per cross-sectional area than cardiac muscle. Maximum isometric tension levels in vertebrate skeletal muscles typically range between 200 and 300 mN mm^{-2} (e.g. Bagshaw, 1993) while multicellular cardiac preparations can hardly develop 100 mN mm^{-2} (e.g. Brutsaert & Housmans, 1977; Kentish, ter Keurs, Ricciardi, Bucx & Noble, 1986). It is not clear whether the difference can be thoroughly accounted for by some combination of the larger myofibril density of fast skeletal fibres (Sommer & Johnson, 1979) and the damaged end compliance of papillary muscles and cardiac trabeculae that limits force generation (e.g. Krueger & Pollack, 1975).

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Alternatively, the difference in maximum tension levels between the two muscle types could be due to differences in the force-generating capacities of the contractile proteins. Data exist in the literature to suggest that myosin isoforms may generate different forces. Skeletal muscle fibres expressing different myosin isoforms can differ substantially in their maximum tension levels (e.g. Eddinger & Moss, 1987; Bottinelli, Schiaffino & Reggiani, 1991), although such differences have not been consistently reported (Greaser, Moss & Reiser, 1988). More recently, measurements of the average force developed by myosin molecules in an *in vitro* motility assay have shown that smooth muscle myosin generates much more force than skeletal muscle myosin while the comparison between cardiac and skeletal muscle myosins has given more complicated results (Harris, Work, Wright, Alpert & Warshaw, 1994; Van Buren, Harris, Alpert & Warshaw, 1995).

Techniques for the measurement of force from isolated single myofibrils are now available (Iwazumi, 1987; Okamura & Ishiwata, 1988; Bartoo, Popov, Fearn & Pollack, 1993; Colomo, Piroddi, Poggesi & Tesi, 1994*a*; Friedman & Goldman, 1996) and offer one direct means of comparing the strength of the cardiac and skeletal contractile apparatus without the need to sacrifice the integrity of the filament lattice.

Force measurements in single myofibril preparations can also be used to evaluate directly the contribution of myofibrillar structures to the large difference between the resting elasticity of cardiac and skeletal muscle tissues. It is, in fact, well established that the passive tension of a variety of preparations of cardiac muscle is higher than that reported for skeletal muscle at sarcomere lengths below 2·20 μm and increases much more markedly at longer sarcomere lengths (Brady, 1979). The source of the resting elasticity in striated muscle has been mainly attributed in the past to extracellular structures (Ramsey & Street, 1940; Gay & Johnson, 1967). This early view contrasts sharply with more recent demonstrations that the passive tension is borne largely by the myofibrils both in skeletal muscle (Magid & Law, 1985; Bartoo *et al.* 1993) and cardiac muscle (Linke, Popov & Pollack, 1994; Palmer, Brady & Roos, 1996), although in the latter the contribution of other cellular and extracellular structures may not be negligible (Granzier & Irving, 1995).

In the present paper the maximum active force and the sarcomere length–resting force relations of single myofibrils, or thin bundles of few myofibrils, isolated from frog cardiac and skeletal muscles have been compared. The results show that the two types of myofibrils do not differ significantly in their active force-generating abilities and passive force responses to sarcomere elongation. It is concluded that, at least in frogs, sarcomeric proteins are not responsible for the large active and passive tension differences between cardiac and skeletal muscle tissues.

Short preliminary reports of the results have already been published (Colomo *et al.* 1994*a*; Colomo, Piroddi, Poggesi & Tesi, 1995).

METHODS

The heart and individual hindlimb muscles (tibialis anterior and sartorius) were isolated from frogs (*Rana esculenta*), which had been decapitated, the brain destroyed, and spinally pithed.

Preparation and mounting of skeletal myofibrils

Single myofibrils and small bundles of skeletal myofibrils (Fig. 1) were prepared by homogenization of glycerinated muscles of the frog, following a procedure modified from Knight & Trinick (1985). All of the steps were carried out as near as possible to 0 °C and in the presence of protease inhibitors. Tibialis anterior and sartorius muscles were dissected in ice-cold Ringer-EGTA solution, tied to wooden sticks close to slack length, and then stored in the same solution at 0–4 °C for 12 h to ensure complete ATP depletion of the specimens. The muscles were then transferred into Rigor solution containing 50% glycerol. The preparations were left at 0–4 °C for 12 h to allow the glycerol to infiltrate the fibres; they were then stored at −20 °C for at least 24 h and used within 2 weeks. Myofibrils were prepared from the stored muscles on the day of the mechanical experiment. After removal of tendons and connective tissue, a glycerinated muscle was chopped into small pieces, placed in ice-cold Rigor solution and homogenized using a tissue tearer (model 985-370; Biospec Products Inc., Bartlesville, OK, USA) at medium speed for 15 s. A second homogenization for 15 s was performed after 1 min of rest. The myofibrils were recovered by low-speed centrifugation (2000 *g* for 10 min, 0–4 °C), washed twice in Rigor solution, resuspended in the same buffer and kept on ice.

A small volume of myofibril suspension was transferred to a temperature-controlled trough (15 °C) filled with relaxing solution (pCa 8). Selected single myofibrils or thin bundles of two to four myofibrils were mounted horizontally between two glass microtools (Fig. 1). As skeletal myofibrils strongly adhered to glass, firm attachment of the preparation to the microtools was made by careful micromanipulation to maximize the attachment area.

Preparation and mounting of cardiac myofibrils

Single myofibrils and small bundles of cardiac myofibrils were prepared by chemical skinning of individual atrial myocytes which had been enzymatically isolated from frog hearts. Frog atrial myocytes (Fig. 1) are the thinnest known single cell preparations of striated muscle; they sometimes contain only one myofibril and rarely contain more than four to five myofibrils (Hume & Giles, 1981; see also present study).

Intact frog atrial myocytes were isolated as described previously (Cecchi, Colomo, Poggesi & Tesi, 1992). A small volume of intact cell suspension was transferred to the experimental trough which was filled with relaxing solution. An intact, thin myocyte of regular shape was selected and mounted horizontally between two glass micropipettes that held the tapered ends of the preparation by suction (Fig. 1).

The myocyte was skinned by a brief and localized spray of skinning solution, delivered through a micropipette that was connected to a pressure reservoir via an electrovalve and positioned close to the myocyte, as shown in Fig. 2. The skinning solution (relaxing solution containing 0·05% (v/v) Triton X-100) was

sprayed on the myocyte for 5–10 s. Disruption of the membrane was often signalled by a sudden enhancement of the striation pattern and transient increase of the myocyte diameter. Too long an exposure or too high a concentration of Triton X-100 caused the myofibrils to deteriorate and slowly disperse.

Solutions

The solutions used to dissociate intact myocytes from frog atria have been described elsewhere (Cecchi *et al.* 1992). The Ringer-EGTA solution used in the early stages of the preparation of skeletal myofibrils had the following composition (mM): 1 EGTA, 100 NaCl, 2 KCl, 2 $MgCl_2$ and 50 Tris. The solution was adjusted to pH 7·0 by titrating with 1 M HCl. The Rigor solution used was identical to the above except that KCl was substituted for NaCl. A set of protease inhibitors (leupeptin, 10 μM; pepstatin A, 5 μM; phenylmethylsulphonyl fluoride, 0·2 mM), 0·5 mM NaN_3 and 0·5 mM dithiothreitol were added to both solutions just prior to use.

The standard relaxing and activating solutions used for mechanical experiments contained (all are Na^+ salts): 10 mM total EGTA; CaEGTA/EGTA ratio set to obtain pCa 8 and 4·75 in the relaxing and activating solutions, respectively; 3 mM MgATP; 3 mM free ATP; 10 mM Mops; propionate and sulphate to adjust the final solution to an ionic strength of 0·130 and a monovalent cation concentration of 90 mM. The concentrations of multivalent ionic species were calculated after solving the multiple equilibria of two metals (calcium and magnesium) and two ligands (EGTA and ATP), by using a computer program developed by P. W. Brandt (Columbia University, New York) and the following apparent association constants (log values at pH 7·00): CaEGTA, 6·3; MgEGTA, 1·6; CaATP, 3·7; MgATP, 4·1. Both solutions were adjusted to pH 7·0 with NaOH and the temperature was maintained at 15 °C.

The reagent grade chemicals used in this study were purchased from Sigma.

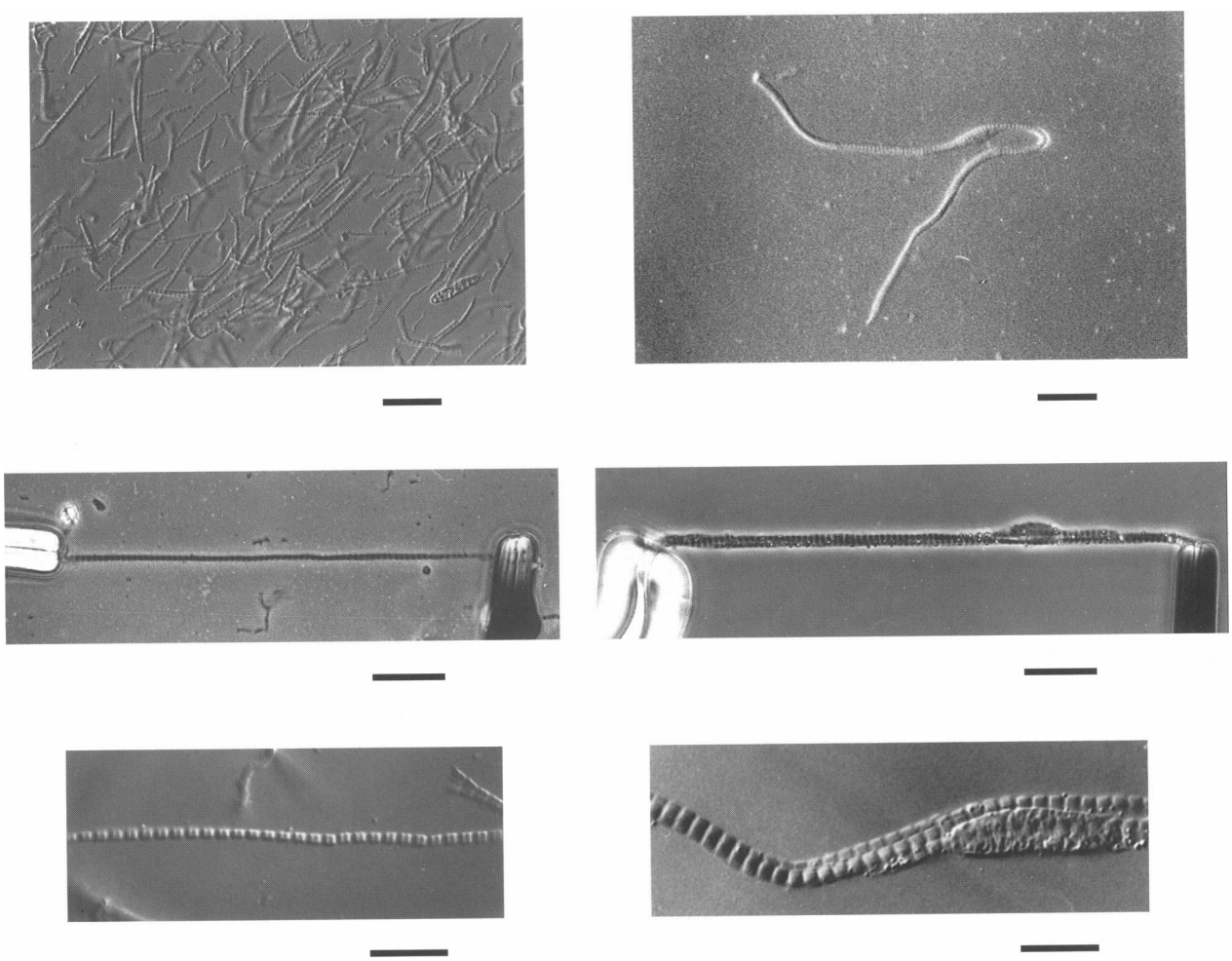

Figure 1. Photomicrographs of the skeletal and cardiac preparations used in this study

Top panels: suspension of isolated myofibrils obtained by homogenization of glycerinated frog tibialis anterior muscle (left) and a single intact atrial myocyte enzymatically isolated from the frog heart (right). Scale bars, 25 μm. Middle panels: phase-contrast images of a single skeletal myofibril (left) and a skinned atrial myocyte (right) mounted between the rigid motor lever arm and blackened cantilever force probe. In the case of the atrial myocyte, the suction micropipette connected to the motor had been bent to increase the efficacy of the attachment of the preparation. Scale bars, 25 μm. Bottom panels: high-magnification details, taken through Nomarski optics, of a single skeletal myofibril (left) and a skinned atrial myocyte (right). Scale bars, 10 μm.

Force transducer and experimental set-up

The experimental apparatus and force transducer used in the present experiments to measure and control force and the length of intact frog heart myocytes were essentially the same as those described previously (Colomo, Poggesi & Tesi, 1994*b*). Briefly (Fig. 2), one of the two glass microtools (suction pipettes in the case of cardiac myocytes) that held the myofibrils was relatively stiff and was mounted on the lever arm of a length-control motor. The other microtool doubled as a cantilever force probe of calibrated compliance. Force was measured by photoelectronically recording the elastic deflection of the tip of the probe. To increase the sensitivity of the photosensor, the tip of the force probe was blackened with micropigment ink. The force probes built for the present experiments had a compliance of 1–3 nm nN^{-1} and a frequency response of 2–5 kHz in the experimental solutions. In any given experiment, force probes were selected for compliance so that myofibril shortening during maximal force production was limited to less than 3% of the slack length (L_0).

The experimental apparatus was centred around an inverted microscope (Nikon Diaphot) placed on an air suspension table and equipped with conventional bright-field and phase-contrast optics. Nomarski differential interference contrast optics have been used in the most recent experiments. Images of the preparations at rest during ramp changes of resting length and during contraction were projected onto a high-resolution solid-state CCD camera (model KP-161E; Hitachi Denshi, Tokyo) and recorded using a conventional videotape recorder (model SVO-140PA; Sony, Japan).

Force and length signals, together with the switch signals to the electronic valve controlling the delivery of activating solution (see below), were continuously monitored throughout the experiment on a chart recorder (Rectigraph 8K; Nec San-ei, Tokyo). The same signals were also recorded, during relevant experimental events, on a digital oscilloscope (Pro20; Nicolet, Madison, WI, USA) and stored on floppy disks for subsequent analysis.

Measurement of preparation dimensions and sarcomere length

Overall length and width of both types of preparations and sarcomere length were measured by employing video images or photomicrographs taken through phase-contrast or Nomarski optics. The total magnification was usually ×4000 for measurements of myofibril width and sarcomere length; lower-power magnification was used to determine the overall length of the preparations mounted between the glass microtools.

The width of both types of preparations was measured in relaxing solution at slack sarcomere length. In the case of the atrial myocytes, width was measured in the intact preparation prior to skinning. As atrial myocytes were composed of a bulging central region that contained the nucleus and two spindle-shaped side regions (Fig. 1), widths varied significantly along the preparation. The mean width of the side regions of the myocyte was used to calculate the cross-sectional area of the cell assuming it to be a right cylinder. The cross-sectional area of skeletal myofibrils was calculated by assuming a circular shape and using as diameter the mean of the largest and the smallest width values along the preparation. As a control for the validity of the optical measurement of preparation width, calibrated diffraction gratings (1–2·5 μm spacings) were used. No systematic error was found in the measurement method, although precision was limited by the optical resolution of the microscope. More precise estimates of the typical size of the cross-sectional area of both types of preparations was obtained using electron microscopy (see below).

The striation spacing was usually determined for both types of preparations by averaging the measurements of sequences of ten sarcomeres displayed in the central region of the video images. In the case of short skeletal myofibrils displaying less than twenty sarcomeres, the overall length of the preparation was measured and divided by the total number of sarcomeres in the myofibril.

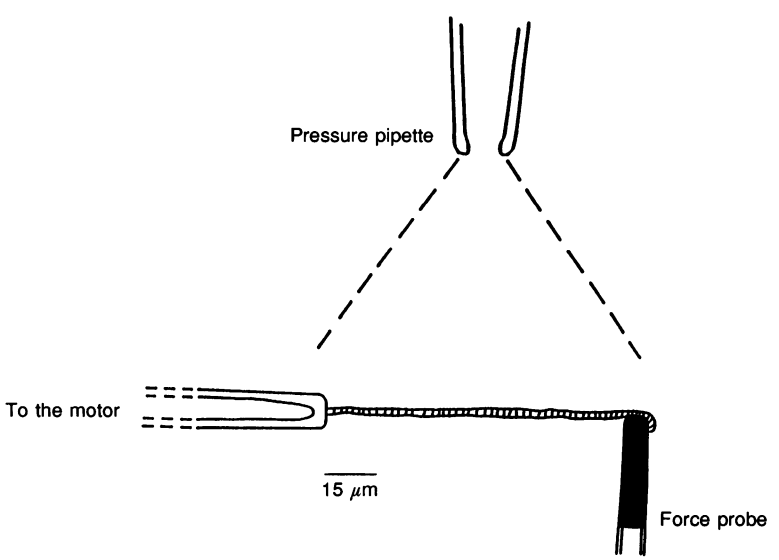

Figure 2. Method for effecting fast solution changes on mounted myofibril preparations

A stream of solution was delivered from a pressure pipette either to skin intact frog myocytes or to activate skeletal and cardiac myofibrils mounted between a cantilever force probe and stiff motor lever arm. The effectiveness of the solution change was tested: (i) by observing the profile of the stream after adding a dye to the injected solution, and (ii) by measuring the conductance between two microelectrodes placed in the same position as the preparation ends when a high-resistivity solution was injected into the experimental trough filled with a low-resistivity solution.

Table 1. Electron microscope measurements on cross-sections of individual myofibrils from skeletal and cardiac muscle

Preparation	Cross-sectional area (μm^2)	Thick filament spacing (nm)	Thick filament density (μm^{-2})
Skeletal myofibrils	1·22 ± 0·10 (23)	38·6 ± 1·0 (30)	844 ± 18 (30)
Cardiac myofibrils	1·29 ± 0·11 (12)	39·9 ± 0·9 (30)	805 ± 20 (30)

Values are means ± S.E.M.; values of n are given in parentheses.

Myofibril activation and experimental protocol

All experiments were carried out at 15 °C. The mean resting sarcomere length of the mounted cardiac and skeletal myofibrils was set at about 2·10 μm; for both types of preparations this corresponded to a length at which myofibrils were not visibly slack and did not generate any significant resting force. Activation of the myofibril was induced by perfusing the preparation transversely with a localized stream of pCa 4·75 solution; this activation method produced a [Ca^{2+}] well above the minimum required to maximally activate frog cardiac and skeletal myofibrils (see also Results). The activating solution was delivered through a pressure pipette with a tip bore of 10–20 μm, which was positioned at a distance of about 200 μm from the central region of the preparation, perpendicular to its longitudinal axis (see Fig. 2). The pipette was connected to a pressure reservoir via an electrovalve. The stream of activating solution progressively widened with the distance from the pipette. Pressure was regulated in order to obtain a stream width much larger than the preparation length at a distance of 200 μm from the pipette. For test purposes, the stream of solution could be made more visible by running a solution containing a dye into a trough filled with solution that did not contain any dye. The responses of the myofibrils to several subsequent activations were usually recorded. In a few control experiments, force was measured from skeletal myofibrils before and after exposure to a brief jet of the solution used to skin the atrial myocytes. This brief Triton X-100 treatment did not affect the force developed by maximally activated skeletal myofibrils ($P = 0·95$, Student's paired t test, $n = 5$).

In some experiments, the passive force responses of the relaxed myofibrils to ramp lengthenings were recorded after the viability of the preparation had been tested with a single and brief jet of activating solution. The relation between sarcomere length and passive force of cardiac and skeletal myofibrils was determined by imposing length ramps of different speeds (0·01–10 L_0 s^{-1}) and amplitudes (10–75% L_0) on the relaxed preparations and measuring the quasi-steady passive force levels attained 20 s after the end of the elongation when most of the stress relaxation was over. The mean sarcomere length before and after each length change was determined as described previously. In the range of sarcomere lengths tested (from slack length to 3·60 μm), the relation between overall length of the preparation and mean sarcomere length at rest was highly linear implying the absence of regions along the preparations with significantly different resting compliance.

Electron microscopy

Samples of skeletal myofibril preparations in glycerol or newly prepared cardiac myocytes in relaxing solution were centrifuged in the presence of 1% bovine serum albumin for 5 min at 13000 g. The pellets were fixed in a mixture of 1% OsO_4, 2% glutaraldehyde, 1% $K_2Cr_2O_7$ in 0·1 M cacodylate buffer at pH 7·2 for 1 h at 0 °C. Subsequently, pellets were washed in double-distilled water, dehydrated in alcohol and propylene oxide, and embedded in Epon 812. Ultra-thin sections were cut on an ultramicrotome (Ultracut 5; Reichert, Vienna), routinely stained with uranyl acetate and lead citrate and examined in a Philips 208 electron microscope. Micrographs of single skeletal myofibrils and cardiac myocytes at ×19500 were redrawn and the cross-sectional area of individual myofibrils and cardiac myocyte total area was measured with a planimetric method. The density of thick filaments was measured in six cardiac and skeletal myofibrils in five regions of 0·05 μm^2 at ×101000. The spacing between thick filaments was estimated at the same magnification by measuring the distance between five to ten aligned myosin filaments 5 times.

Results are given as means ± S.E.M.

RESULTS

Specimen structure and size

It was important in the present study to compare the size and structural features of the two types of myofibril preparations used for the experiments especially because of their different nature: isolated skeletal myofibrils *versus* skinned cardiac myocytes. When viewed by phase-contrast or differential interference contrast microscopy (e.g. Fig. 1), the skeletal muscle preparations appeared as single myofibrils 40–150 μm long, 1–1·5 μm wide, or thin bundles of two to four myofibrils. The larger fibre fragments present in the suspensions were not used for mechanical experiments. Skinned atrial myocytes were 100–300 μm long and appeared to be made of very few individual myofibrils, which did not differ in width from the single skeletal myofibrils. Sarcomere slack lengths were essentially the same in both types of myofibrils, averaging 2·07 ± 0·05 μm in the cardiac myofibrils ($n = 50$) and 2·04 ± 0·04 μm in the skeletal myofibrils ($n = 31$).

To better judge differences and similarities between the two types of preparations, pellets of skeletal myofibrils and atrial cells were examined by electron microscopy (Fig. 3). Analysis of the skeletal muscle pellets confirmed that the preparation mostly consisted of single myofibrils and thin bundles of two to four myofibrils. Although far from being perfectly cylindrical, individual skeletal myofibrils appeared in cross-section as discrete organelles which well retained their *in situ* structure and shape. Mean data from structural measurements on cross-sections of individual skeletal myofibrils are given in Table 1. Direct measurements of

myosin filament density (Table 1) gave essentially the same number of filaments per unit cross-sectional area as that calculated from the average lateral spacing between thick filaments (831 μm^{-2}). This indicates that the filament lattice in the electron micrographs was quite regular. Comparison of the thick filament spacing found in single skeletal myofibrils with the 1,0 lattice spacing typically reported in X-ray diffraction studies on frog skeletal muscle (42 nm; e.g. Bagshaw, 1993) suggests that the amount of shrinkage resulting from the fixation conditions was relatively small (less than 10%). It is worth noting that the mean cross-sectional area given in Table 1 for single skeletal myofibrils,

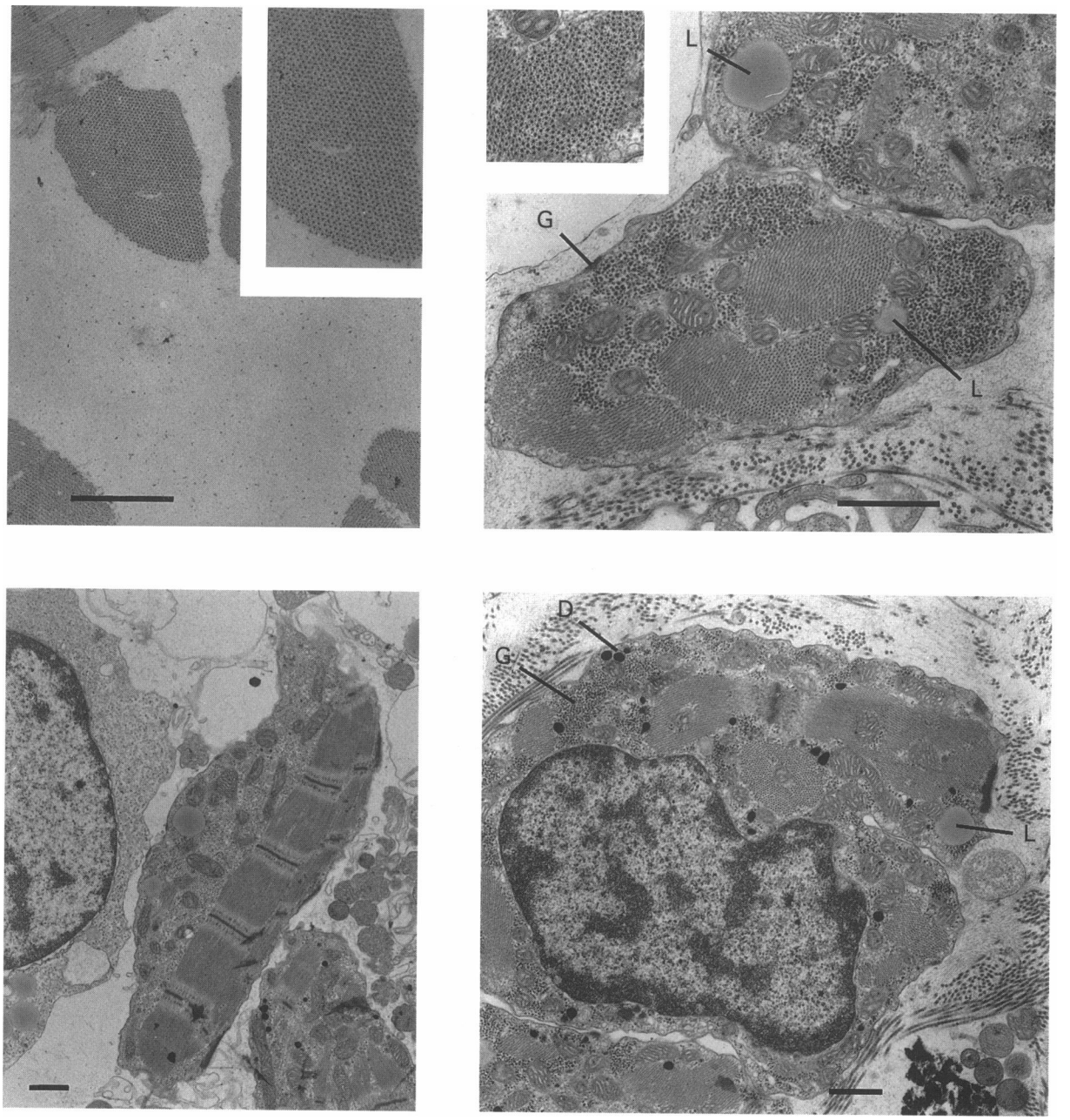

Figure 3. Electron micrographs of frog skeletal myofibrils and atrial myocytes

Top panels show a cross-section of a single skeletal myofibril isolated from tibialis anterior muscle (left) and a cross-section of the end region of a single atrial myocyte (right). The myocyte contained three individual myofibrils. Insets show higher magnification details of the skeletal myofibril and of one of the cardiac myofibrils, respectively. Scale bars, 1 μm (0·62 μm for the insets). Bottom panels show a longitudinal section of the end region of an atrial myocyte (left), which may contain a single myofibril, and a cross-section of the nuclear region of another myocyte containing several myofibrils (right). Scale bars, 1 μm. L, lipid droplets; G, glycogen particles; D, dense bodies.

even if corrected for shrinkage, is not significantly different from that estimated from video images of the single myofibrils used for mechanical experiments ($1{\cdot}29 \pm 0{\cdot}11\ \mu m^2$; $n = 16$). Serial electron micrographs of eighteen cross-sectioned atrial cells revealed that the number of individual myofibrils in a myocyte varied from one to seven among different cells. Only two of the cells examined contained a single myofibril while most of them contained two to four myofibrils. In some cells a larger number of myofibrils were found in the nuclear region compared with the end regions of the myocyte indicating that not all myofibrils spanned the whole length of the cells. The mean

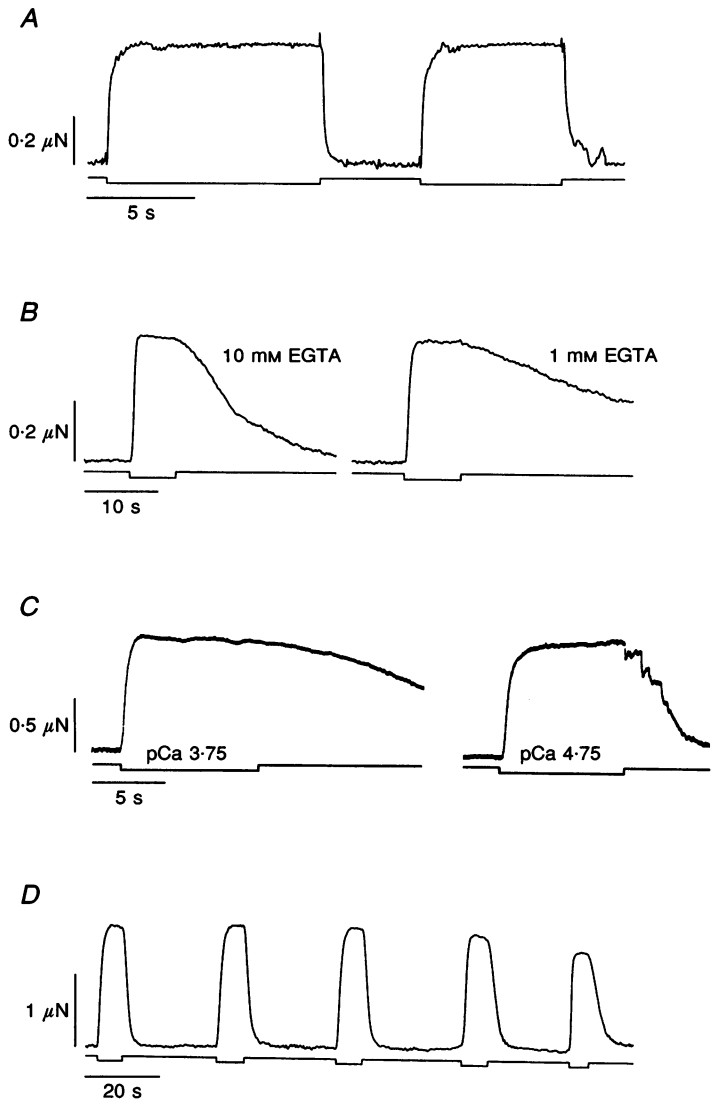

Figure 4. Force records in contractions of frog skeletal and cardiac myofibrils

In each panel, top traces are force signals and bottom traces are the switch signals to the electronic valve controlling the delivery of activating solution. *A*, single skeletal myofibril; mean diameter, 1·24 μm. Standard relaxing solution in the trough (pCa 8); standard activating solution in the pressure pipette (pCa 4·75). In this particular experiment, the standard relaxing solution was sprayed on the myofibril through a second pressure pipette after the delivery of activating solution had been stopped. This resulted in a fast relaxation. *B*, single skeletal myofibril; mean diameter, 1·10 μm. Standard relaxing and activating solutions were used in the contraction shown on the left (10 mM EGTA); relaxing and activating solutions with a lower total EGTA concentration (1 mM) were used in the contraction shown on the right. *C*, skinned atrial myocyte; mean cell diameter, 3·39 μm. Standard activating solution was used in the contraction shown on the right; pCa 3·75 activating solution was used in the contraction shown on the left; the trough was filled with standard relaxing solution in both contractions. Traces in this panel were taken from the chart recorder. *D*, skinned atrial myocyte; mean cell diameter, 3·72 μm; contractions from 5 sequential activations. Standard relaxing solution in the trough; standard activating solution in the pressure pipette.

value of the whole cross-sectional area of the myocytes, measured in the end regions of ten cells, was $9{\cdot}83 \pm 1{\cdot}20\ \mu m^2$, a size very close to that roughly estimated from video images ($10{\cdot}18 \pm 0{\cdot}74\ \mu m^2$; $n = 45$). On average, in the non-nuclear regions the total myofibril area of the cardiac cell cross-section was $3{\cdot}68 \pm 0{\cdot}61\ \mu m^2$ and accounted for only $40{\cdot}8 \pm 3{\cdot}3$ % of the whole cross-sectional area of the myocyte. Individual myofibrils observed in cross-sections often revealed a less regular shape than single skeletal myofibrils. In addition, the border between myofibrils could not be well distinguished in all sections and outlines of myofibrils were often fuzzy. As shown from the data in Table 1, however, individual cardiac myofibrils were perfectly equivalent in the mean number of myosin filaments to single skeletal myofibrils. In fact, the small difference in cross-sectional area between the two types of myofibrils is well matched by the small difference in myosin filament density. Data shown in Table 1 seem consistent with the occurrence of a smaller amount of shrinkage of atrial myocytes during fixation compared with skeletal myofibrils.

In conclusion, these results indicate that: (i) frog atrial cells are comparable in mean number and size of myofibrils to the thin bundles of two to four skeletal myofibrils used for mechanical experiments (only a few cells are equivalent to single skeletal myofibrils); and (ii) measurements of the whole cross-sectional area of the myocytes led to a 2·5-fold overestimation of the effective cardiac myofibril area.

Maximum active force of skeletal and cardiac myofibrils

As shown in Fig. 4, when frog atrial myocytes and skeletal myofibrils were activated by a localized stream of pCa 4·75 solution, force rose rather quickly to a maximum that was maintained as long as the activating solution was flowing. In both types of preparations relaxation started upon cessation of the jet of activating solution but was rather slow. It could be greatly accelerated if, at the end of the activating solution jet, the preparation was perfused by a stream of relaxing solution delivered by a second pressure pipette (Fig. 4*A*). The contractions elicited with the pressure-pipette method represented the responses of maximally activated preparations. In fact, neither a decrease in the Ca^{2+}-buffering capacity of the relaxing solution in the experimental trough nor a decrease in the pCa of the activating solution in the pressure pipette could increase the force developed by the myofibrils (Fig. 4*B* and *C*). The force developed by both types of myofibrils was already close to maximum when the pCa of the activating solution that filled the pressure pipette was 5·50. Contractions were usually well reproduced over a few activation cycles before there was a significant decline in active force (Fig. 4*D*). Myofibrils undergoing marked run-down of active force exhibited some deterioration with evident decrease of sarcomere length homogeneity. Due to this behaviour, only the force values measured in the earliest contractions were used to estimate the maximum active force of each preparation.

The absolute values of the maximum force developed by skeletal myofibrils depended upon the number of myofibrils in the preparation. The traces in Fig. 5*A* show examples of contractions recorded from a single myofibril and a thin bundle of two to three myofibrils. On average, the values of the maximum force developed by the skeletal preparations were $0{\cdot}45 \pm 0{\cdot}03\ \mu N$ ($n = 16$) for single myofibrils and $1{\cdot}44 \pm 0{\cdot}24\ \mu N$ ($n = 9$) for small myofibril bundles. Normaliz-

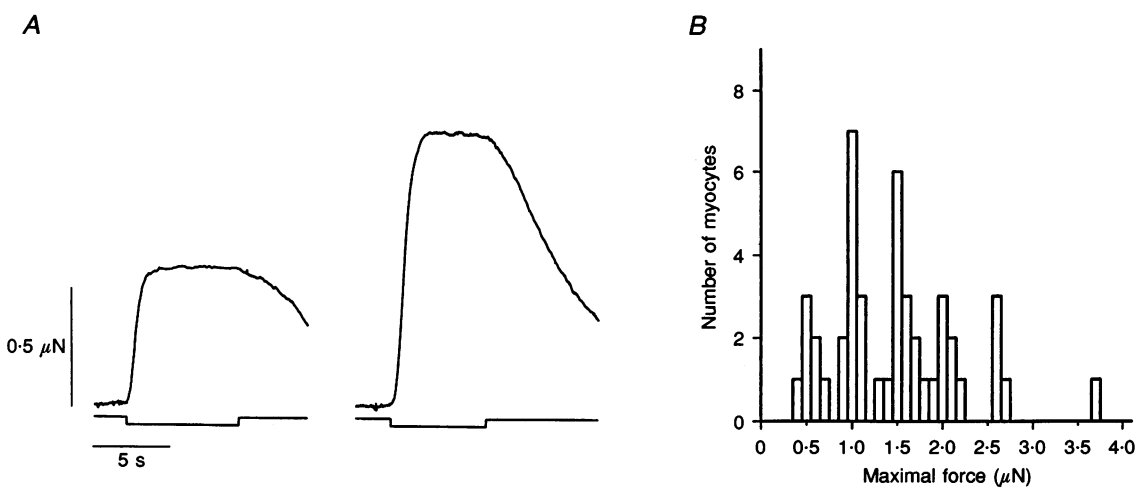

Figure 5. Maximum active force of isolated myofibrils from frog skeletal and cardiac muscle

A, force responses (top traces) to maximal activation of a single skeletal myofibril (left) and a bundle of 2–3 skeletal myofibrils (right). Bottom traces, delivery signal of the standard pCa 4·75 activating solution. Single myofibril mean diameter, 1·39 μm; myofibril bundle mean diameter, 1·91 μm. *B*, distribution of maximum active forces of 45 skinned frog atrial myocytes. The histogram shows the number of preparations per class (class division, 100 nN).

ation of maximum force values for the cross-sectional area of the preparations yielded a mean tension of 376 ± 21 mN mm^{-2} ($n = 25$); this is higher than the tetanic tension usually measured in intact frog skeletal fibres at 0–5 °C (about 250 mN mm^{-2}; e.g. Edman, 1979).

The results obtained from forty-five maximally activated frog atrial myocytes are summarized in Fig. 5*B*. Developed force averaged 1·47 ± 0·10 μN, essentially the same mean value as that obtained for bundles of two to four skeletal myofibrils. Absolute force values for individual myocytes were scattered widely, ranging from less than 0·5 μN to more than 3·5 μN. Since frog atrial myocytes contain a variable number of myofibrils, it is reasonable to assume that the maximum force that can be generated by an individual frog cardiac myofibril is close to the lowest levels of the force values found, i.e. 0·4–0·5 μN. Moreover, as shown in Fig. 5*B*, the distribution of the maximum force values measured in different myocytes revealed a non-continuous trend with peaks at intervals of about 0·5 μN. This supports the suggestion that the average maximum force developed by individual cardiac myofibrils is around 0·5 μN and that frog cardiac and skeletal myofibrils develop the same amount of active force.

The absolute force values found in frog atrial cells were significantly related to the overall cross-sectional area of the myocytes and normalization yielded a mean maximum tension value of 148·9 ± 5·6 mN mm^{-2} ($n = 45$). Although this is one of the highest values reported for cardiac muscle, it is nevertheless about 2·5 times smaller than the mean active tension found for frog skeletal myofibrils. The difference, however, can be completely accounted for by the low density of myofibrils per unit cross-sectional area of frog atrial myocytes (see above).

Passive force of skeletal and cardiac myofibrils

The force responses of relaxed skeletal and cardiac myofibrils to ramp elongations of various extents and velocities were investigated. In both types of preparations the velocity of lengthening did affect the force during elongation but did not significantly affect the steady force level reached after most of the stress relaxation was over. Static resting force, therefore, depended only on the extent of elongation.

In skeletal myofibril preparations, the absolute values of static force measured at various degrees of sarcomere elongation strongly depended on the number of myofibrils in the preparation. Figure 6*A* shows typical examples of the sarcomere length–resting force relations obtained from a single skeletal myofibril and from a thin bundle of skeletal myofibrils that, as inferred from its size, was probably made of two to three myofibrils. In the skinned frog atrial myocytes, the absolute values of resting force measured at any given sarcomere length exhibited a rather large scatter among different preparations but were in the same range of values as those of the skeletal preparations. In fact, the average relation between sarcomere length and resting force of the atrial myocytes lies very close to the relation obtained from the thin bundle of skeletal myofibrils (Fig. 6*A*). The results suggest that individual myofibrils from cardiac and skeletal frog muscle bear essentially the same amount of resting force at any given sarcomere length.

As in the case of active tension, however, normalization of the measured resting force values for the estimated cross-

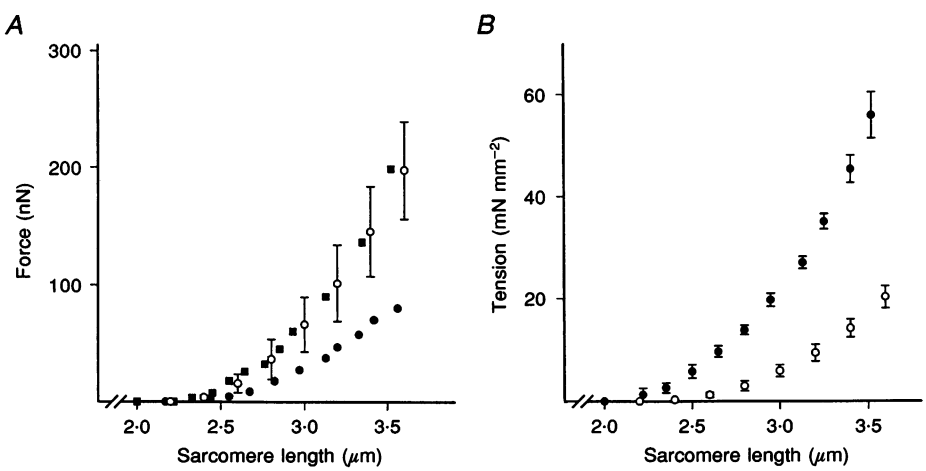

Figure 6. Relations between sarcomere length and passive force or tension in isolated myofibrils from frog skeletal and cardiac muscle

Force and tension levels were measured 20 s after elongation to the indicated sarcomere length. *A*, sarcomere length–passive force relations for a single skeletal myofibril (●) and a thin bundle of 2–4 skeletal myofibrils (■) are compared with the mean length–force relation obtained from 6 skinned atrial myocytes (○). Data points represent individual measurements for the skeletal myofibril preparations and means ± S.E.M. for the cardiac preparations. *B*, sarcomere length–passive tension relations for 6 skeletal myofibril preparations (●) and 6 skinned atrial myocytes (○). Data points are given as means ± S.E.M.

Table 2. Curvature index (α) and initial elastic modulus (E_0) values for the length–resting tension curve of isolated myofibrils and larger preparations of frog skeletal and cardiac muscle

Preparation	α	E_0 (mN mm^{-2})	n	Reference
Skinned atrial myocyte	4·14 ± 0·47	3·62 ± 0·65	6	Present study
Skeletal muscle myofibril	4·37 ± 0·28	9·16 ± 1·38	6	Present study
Single skeletal muscle fibre (skinned)	4·13 ± 0·10	5·40 ± 0·75	12	Magid & Law (1985)
Single skeletal muscle fibre (intact)	4·04	5·01 *	—†	Rapoport (1973)
Whole skeletal muscle	4·28 ± 0·19	2·60 ± 0·25	11	Magid & Law (1985)
Atrial trabecula	8·70	27·19	—‡	Winegrad (1974)

Values are means ± S.E.M. of the values of α and E_0 that best fitted experimental data to eqn (1). In eqn (1), σ is Lagrangian stress (force per cross-sectional area estimated at the slack sarcomere length, S_0) and ϵ is sarcomere strain ($S/S_0 - 1$). All E_0 values, except that of the atrial trabecula, have been calculated at a sarcomere length of 2·15 μm. The E_0 value for the atrial trabeculae refers to a sarcomere length of 2·00 μm. * The value of 9·08 for E_0, originally reported by Rapoport (1973), was related to an initial sarcomere length of 2·33 μm; the value in the table has been recalculated for a sarcomere length of 2·15 μm to better compare with the present values and those reported by Magid & Law (1985). † Pooled data; ‡ typical experiment.

sectional area of each preparation resulted in rather large differences between the tensions calculated for the two types of preparations. In fact, as shown in Fig. 6*B*, at any given sarcomere length the mean resting tensions of the skeletal myofibrils were 2–3 times higher than those of the skinned cardiac cells. As before, the tension difference in this case can also be accounted for by the low effective myofibril area per unit cross-sectional area of frog atrial myocytes.

A more quantitative comparison between the resting elasticity of the two myofibril types was obtained by fitting the sarcomere length–resting tension data of each preparation to the exponential equation (Rapoport, 1973; Magid & Law, 1985):

$$\sigma = E_0/\alpha(e^{\alpha\epsilon} - 1), \quad (1)$$

where σ is Lagrangian stress (force per cross-sectional area estimated at the slack sarcomere length S_0), ϵ is sarcomere strain ($S/S_0 - 1$), and E_0 and α are constants representing the initial elastic modulus and curvature index, respectively. Mean values of α and E_0 estimated for single skeletal myofibrils and skinned atrial myocytes are shown in Table 2 together with the same parameters found for larger preparations of frog skeletal and cardiac muscle. Values of α are practically the same for frog cardiac and skeletal myofibrils indicating that the curvature of the length–tension relation is the same for both types of myofibrils. The mean value of the initial elastic modulus, E_0, for the cardiac myocytes is about 2·5 times smaller than that found for the skeletal myofibrils, exactly as expected from the difference in myofibril density per unit cross-sectional area between the two types of preparations.

DISCUSSION

Skeletal myofibrils isolated from rabbit psoas muscle can be easily prepared with excellent mechanical results (Okamura & Ishiwata, 1988; Bartoo *et al.* 1993; Friedman & Goldman, 1996). Here we report a method that provides good quality isolated frog skeletal myofibrils for mechanical studies. Frog skeletal myofibrils, prepared as described in Methods, display normal structure and retain all the mechanisms of contraction regulation as can also be shown by rapid-flow quench measurements of ATPase activity (C. Tesi, N. Piroddi, R. Stehle, C. Lionne, C. Poggesi, F. Travers & T. Barman, unpublished results).

Although Linke *et al.* (1994) recently reported a successful mechanical study on myofibrils isolated from rabbit heart, in our and other laboratories (S. Ishiwata, personal communication), attempts to isolate cardiac myofibrils of suitable length for mechanical experiments often lead to unsatisfactory results. Skinned frog atrial myocytes, as used in the present study, are a suitable alternative for the investigation of the mechanical properties of cardiac muscle down to the level of individual myofibrils.

The results of this study suggest that frog cardiac sarcomeres generate the same amount of force in response to both maximal activation and passive elongation as that of fast skeletal muscle. This conclusion may be questioned because: (i) the cardiac and skeletal preparations used for the experiments were obtained by different methods, (ii) the size and nature of the two types of preparations were different (single cells *versus* isolated myofibrils), and (iii) there were limits in the precision of the measurement of the cross-sectional area of these thin preparations. Results from control mechanical experiments, however, provided some

evidence against the different preparation procedures having a significant effect on the measured forces. Moreover, the electron microscope results demonstrated a substantial equivalence in myofibril number and size of the cardiac and skeletal preparations used for the mechanical experiments. Measurements of absolute force can therefore be used to compare the strength of cardiac and skeletal contractile systems without concern about the precision of the measurements of the cross-sectional area of the preparation.

Active force measurements

It is also difficult to compare present results in myofibrils with those obtained in intact muscle preparations because the solutions used in this study may not be the most appropriate substitute for the myoplasm (e.g. Na^+ was the major cation, $[Mg^{2+}]$ was much lower than that in the myoplasm). Nonetheless, the agreement between the active tension levels of frog skeletal myofibrils and the tetanic tension values of intact frog muscle fibres (e.g. Edman, 1979) is surprisingly good. In fact, the rather large difference between 376 mN mm^{-2} (mean active tension of myofibrils at 15 °C) and 250 mN mm^{-2} (typical tetanic tension of intact fibres at 0–5 °C) can almost be entirely accounted for by considering: (i) the effect of temperature on force development in frog skeletal muscle, and (ii) the size of the effective myofibrillar space in the whole cross-sectional area of muscle fibres. According to the Q_{10} value reported by Edman (1979), tetanic tensions of intact frog fibres at 0–5 °C should be scaled up by at least 25% to compare them with present results at 15 °C. Furthermore, using the estimate obtained by Mobley & Eisenberg (1975) of the fractional fibre volume that consists of myofibrils in frog skeletal muscle (83%), tensions reported for intact fibres should be scaled up by another 20% to compare them with tensions measured in isolated myofibrils.

The absolute values of active force found here for skinned frog atrial myocytes are consistent with those reported for bullfrog atrial myofibrils (Iwazumi, 1987) and for maximally activated skinned *Xenopus* atrial cells (Hofmann & Moss, 1992). The active tension values estimated for frog atrial myocytes in this study (149 mN mm^{-2}) is substantially higher than the highest values (90–100 mN mm^{-2}) reported for intact and skinned multicellular preparations of cardiac muscle (Brutsaert & Housmans, 1977; Kentish *et al.* 1986), even when these latter values are scaled up by 10–15% to account for extracellular space. According to Fabiato (1981), submaximal activation may be responsible for low force generation in the case of intact preparations, because full saturation of the contractile apparatus with Ca^{2+} cannot even be achieved. On the other hand, diffusion limitations may compromise the maximum force-generating ability of relatively large skinned cardiac specimens.

The finding that the active tension developed by individual myofibrils of both cardiac and skeletal muscles of the frog is essentially the same suggests that the maximal force-generating capacities of cardiac and skeletal contractile proteins do not differ. This conclusion is in contrast with

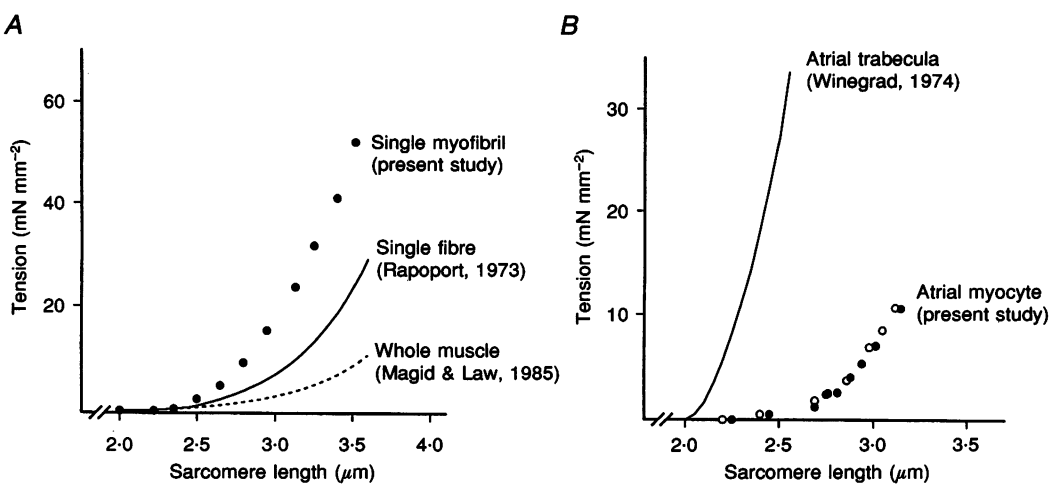

Figure 7. Sarcomere length–passive tension relations for isolated myofibrils and larger preparations of frog skeletal and cardiac muscle

A, the mean tension values measured in 6 skeletal myofibril preparations after 20 s equilibration at each length (●) are compared with data reported by Magid & Law (1985) for whole frog semitendinosus muscles and by Rapoport (1973) for single intact fibres of frog semitendinosus muscle. The dashed line was drawn using eqn (1) and the mean parameter values obtained by Magid & Law from 11 muscles. The continuous line is Rapoport's best fit of pooled data obtained from 10 fibres to the same equation. *B*, the typical sarcomere length–resting tension relation reported by Winegrad (1979) for a frog atrial trabecula (continuous line) is compared with data obtained from an intact frog atrial myocyte (○) and from the same myocyte after skinning (●).

that of a recent study by Linke *et al.* (1994) employing isolated myofibrils from mammalian striated muscles, although the differences in results between this study and that of Linke *et al.* (1994) may be due in part to differences in the methods used for the preparation of the myofibrils and composition of the activating solutions. Linke *et al.* (1994) reported that in myofibrils from rabbit psoas muscle active force was up to 4 times higher than that of myofibrils from the heart of the same species and concluded that a difference in the force-generating abilities of the myosin isoforms expressed in the two types of muscles could account for their findings. Such a difference, however, has not been confirmed by direct comparison of the mean force per cross-bridge measured in cardiac and skeletal myosin molecules in an *in vitro* assay (Van Buren *et al.* 1995). Although fast cardiac myosin isoforms seemed to be somewhat weaker than those of the fast skeletal muscle, the slow cardiac myosin, V_3, which is the predominant isoform expressed in the adult rabbit heart (e.g. Pagani & Julian, 1984), was found to generate the same amount of force as the fast skeletal myosin. To our knowledge, it has not yet been firmly established which kind of myosin is expressed in frog atrial myocytes. However, immunohistochemical staining and immunoblotting analysis with a set of different anti-myosin heavy chain (anti-MHC) monoclonal antibodies indicate that the MHC isoforms expressed in the frog atrial myocardium share most epitopes with the mammalian β/slow MHC isoform, while they differ substantially from the isoforms expressed in frog fast skeletal muscle (D. Mornet, personal communication). These findings suggest that frog atrial myocytes express a V_3 type of myosin and, therefore, that the results of the present study are consistent with the *in vitro* force measurements of Van Buren *et al.* (1995). The equivalence in force of myofibrils of patently different isozyme composition does not imply that the molecular mechanics of different myosins are the same. According to current knowledge of cross-bridge mechanisms, in fact, both cross-bridge unitary force and fraction of cycle time during which force is generated (duty cycle) contribute to the determination of the mean force per cross-bridge. Therefore, combinations of changes of these two factors can account for the present results.

Passive force measurements

The resting elasticity of individual skeletal myofibrils is similar to that described for larger preparations of both frog tibialis (Bagni, Cecchi, Colomo & Tesi, 1988; Claflin, Morgan & Julian, 1989) and semitendinosus (Rapoport, 1973; Magid & Law, 1985) muscles. In Fig. 7*A*, the mean sarcomere length–passive tension relation found for skeletal myofibrils is compared with typical relations reported for larger preparations of frog semitendinosus muscles. The exponential shape of the curve for isolated myofibrils is similar to that measured for intact fibres and whole muscles. As shown in Table 2, the curvature index, α, is practically the same for all the skeletal muscle preparations, suggesting that the source of passive tension is the same from the whole muscle to the single myofibril. The values of the initial elastic modulus, E_0, progressively increase from the whole muscle to the single myofibril as expected from the upwards shift of the length–tension relations compared in Fig. 7*A*. These changes can be accounted for in part by the variations in the myofibril content per unit cross-sectional area of the different preparations. Moreover, the higher resting tension found in the present study can also be the consequence of incomplete stress relaxation. Resting tension was measured 20 s after the end of elongation when the fast components of stress relaxation had disappeared; however, according to Magid & Law (1985), slow components of the relaxation phenomenon may last for several minutes. It is worth noting that the static passive tension data shown in Fig. 7*A* were collected 5–10 min after elongation of intact fibres and at even later times (30 min) for whole muscles. In spite of the above uncertainties, the present results do indicate that whole muscles and whole fibres are not any stiffer than the isolated myofibrils. This is consistent with the idea that in frog skeletal muscles resting tension is almost entirely generated in the elastic structures in the myofibrils, at least in the tested range of sarcomere lengths.

The values of passive force and tension found at different sarcomere lengths in frog atrial myofibrils are very similar to those reported in a pioneering study on intact frog atrial cells by Tarr, Trank, Leiffer & Shepherd (1979). Such values are much lower than those usually reported for larger preparations of mammalian (e.g. Krueger & Pollack, 1975; Kentish *et al.* 1986) and amphibian (Winegrad, 1974; Matsubara & Murayama, 1977) hearts. In Fig. 7*B*, the sarcomere length–passive tension relation of a skinned frog atrial myocyte is compared with that obtained in the same cell before skinning and with the relation reported for intact frog atrial trabecula by Winegrad (1974). The data shown in Fig. 7*B* together with the values of α and E_0 estimated for multicellular and myofibril preparations from the frog heart (Table 2) support the conclusion that, in the frog myocardium, at variance with skeletal muscle, myofibrils are responsible for very little of the passive tension of the whole tissue. Passive stiffness in frog cardiac muscle seems to reside primarily in structures external to the individual myocytes. On the other hand, cardiac myofibrils, like the skeletal myofibrils, seem to be the major determinant of the resting elasticity of individual myocytes since the elastic properties of intact and skinned cells do not differ significantly (Fig. 7*B*).

The present study indicates that individual myofibrils from frog cardiac muscle exhibit the same elastic resistance to passive elongation as single myofibrils from skeletal muscle. Since the most likely candidate for the source of resting tension in single myofibrils is the I-band portion of the titin filaments (e.g. Trinick, 1991; Wang, McCarter, Wright, Beverly & Ramirez-Mitchell, 1991), the findings suggest that the elastic properties of titin molecules from frog cardiac and skeletal sarcomeres do not differ.

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