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Force generation and shift of mass between myosin and actin in skinned striated muscle fibres at low calcium concentrations

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Abstract Skinned muscle fibres from the gracilis muscle of the rabbit were used to record small angle X-ray diffraction spectra under various contractile conditions. The intracellular calcium concentration, expressed as pCa, was varied between 8.0 and 5.74. Equatorial diffraction spectra were fitted by a function consisting of five Gaussian curves and a hyperbola to separate the (1.0), (1.1), (2.0), (2.1) and Z-line diffraction peaks. The hyperbola was used to correct for residual scattering in the preparation. The ratio between the intensities of the (1.1) and (1.0) peaks was defined as the relative transfer of mass between myosin and actin, due to crossbridge formation after activation by calcium. The relation between the ratio and the relative force of the fibre (normalized to the force at pCa 5.74 and sarcomere length 2.0 μm) was linear. At high pCa (from pCa 6.34 to 8.0) no active force was observed, while the ratio still decreased. Sarcomere length was recorded by laser diffraction. The laser diffraction patterns did not show changes in sarcomere length due to activation in the high pCa range (between 8.0 and 6.34). From these results the conclusion is drawn that crossbridge movement occurs even at subthreshold calcium concentrations in the cell, when no active force is exerted. Since no force is generated this movement may be related to crossbridges in the weakly bound state.

Key words Actin · Myosin · Muscle fibre · X-ray diffraction

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Introduction

Since the introduction of the crossbridge model for muscle contraction efforts have been made to link the existence of crossbridges to the level of active force generation. Active force in this context is taken to mean force generated after activation by calcium from different sources inside and outside the muscle cell. Different direct and indirect techniques have been developed to visualze the crossbridges. Geeves (1991) reviewed the different states occurring during crossbridge cycling.

Insight into the transitions between the different states of the crossbridge can be obtained by measuring ATP-ase activity during crossbridge cycling. Kuhn et al. (1985) showed the effects of attachment and detachment of crossbridges in relation to the rate of MgATP hydrolysis. Kraft et al. (1992) used MgATP[γS] to control crossbridge turnover between different states. They combined the technique with small angle X-ray diffraction and showed that at low calcium (pCa = 8.0) crossbridges in the presence of this nucleotide analogue have properties of the weakly bound state.

Small angle X-ray diffraction is used to study the regular crystalline arrangement of the actin-myosin filaments. The position of the diffraction peaks provides information about the lattice spacing between the thick and thin filaments. The intensities of the equatorial diffraction peaks change during activation due to a stronger sampling of the S1 heads around the myosin filaments by the transformed (1.1) reflection and a weaker sampling by the (1.0) reflection. This is interpreted as a shift of mass towards the vicinity of the actin filament. The shifts have been studied using radiation, either at low intensities, originating from a rotating anode (Brenner and Yu 1985; Schoenberg 1980; Yu and Brenner 1989) or at high intensities from a synchrotron source (Brenner and Yu 1993; Huxley et al. 1982; Jung et al. 1989; Konishi et al. 1991; Schiereck et al. 1992). Changes in active force should correspond to the shift of mass between the actin and myosin layers. To relate the process of activation to the different steps in the cross-

bridge cycle and the generated force, time resolved X-ray diffraction patterns have been recorded (Huxley et al. 1982, Konishi et al. 1991). In these studies the spectra are averaged over tens to hundreds of repeated contractions. The ratio between the intensities of the (1.1) and (1.0) equatorial reflections express the relative change in mass between the actin and myosin layers. Yu et al. (1979) and Brenner and Yu (1985) showed that a linear or slightly upwards curved relation was recorded between this ratio and the active force as a consequence of the change in the number of strongly bound crossbridges. In a study on chemically skinned papillary muscle Matsubara et al. (1989) showed that at maximal activation (pCa 4.4) about 80% of the myosin heads were transferred to the thin filament. At high pCa they observed a relatively larger percentage (about 40%) of myosin heads transferred to the thin filaments than at low pCa. Brenner and Yu (1993) showed that changes in the intensities of the (1.0) and (1.1) reflections brought about by activation did not follow the reciprocal concept. They suggest that the structure of weakly bound crossbridges is different from that of force generating crossbridges or those in rigor.

In this study we report experiments carried out on rabbit gracilis freeze dried skinned muscle fibres at room temperature (20 °C). Only low calcium activation is used, as higher activation levels result in fibres pulling themselves apart. The displacement of the myosin heads towards actin and the generation of force at low intracellular calcium concentration appear to occur independently of each other.

Methods

Muscle preparation

Gracilis muscle were obtained from rabbits. The rabbit was anaesthesized with sodium-pentobarbital (30 mg/kg). Gracilis muscles were removed and cut into long small strips (2 cm long, 4 mm width). The animal was euthanized after removing the heart, for other experimental purposes. Preparations were immediately immersed in liquid nitrogen for about 15 minutes. They were freeze dried in closed containers on dry silicagel at –25 °C, for at least 2–3 weeks (deBeer et al. 1988). After freeze drying the membranes of the sarcoplasmic reticulum and the mitochondria were disrupted. Single fibres could easily be dissected from the freeze dried preparations (diam. 50–100 μm, length >5 mm). Fibres were rehydrated before use. Their structure and physiological performance were found to be compatable to those of permeabilized muscle fibres prepared by other methods (Jung et al. 1988).

Bathing solutions

Several bathing solutions were used to control and maintain different mechanical activations such as relaxation and activation at different levels. The composition of the various solutions is calculated according to the method described by Fabiato and Fabiato (1979). We used a pKa of 7.5 for imidazole and a $\log(K_{1,\mathrm{CaEGTA}})$ of 11.045 in order to account for the temperature and ionic strength at which the experiments were performed (Moisescu and Thieleczek 1979). Different calcium concentrations were obtained by mixing the relaxation and the activation solutions. To eliminate the effect of Ca^{2+} uptake and release by the membrane fragments of the SR and mitochondria we used a strongly buffered Ca^{2+}-EGTA solution.

The relaxation solution contained a high concentration of EGTA (20 mM) and did not contain free Ca^{2+}. Its pCa is calculated to be higher than 8.0. All solutions contain 60 mM imidazole (pH = 6.8, set with KOH), 10 mM PCr and 50 U/ml CPK. The free Mg^{2+} concentration was 1 mM, the MgATP concentration was 5 mM and the ionic strength was 160 mM (adjusted with KCl). The compositions are adopted from Jung et al. (1989). The experiments were carried out at a temperature of 20 °C.

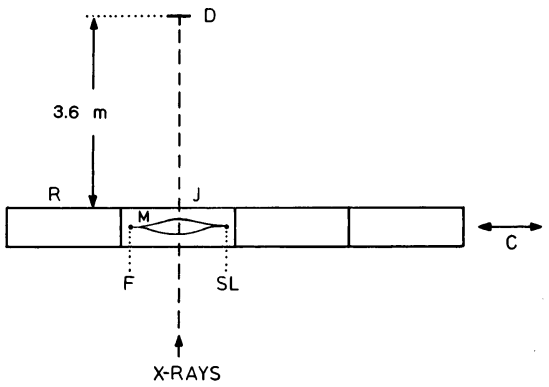

Fig. 1 Experimental setup. A schematic drawing of the position of the muscle fibre (*M*) in a particular superfusion solution in one of the bins of the cuvette (*C*): relaxation (*R*), and activation with different [Ca] in the other bins. X-rays are diffracted by the fibre and recorded 3.6 m behind the preparation by a photon detector (*D*)

X-ray diffraction measuring apparatus

A schematic drawing of the experimental setup is shown in Fig. 1. The skinned muscle fibre was mounted between two pairs of tweezers which served as clamps. One pair of tweezers was connected to a force transducer (KG3, Scientific Instruments, Heidelberg; specifications: resol. 0.1 mg at 10 Hz; res.freq. 700 Hz), the other to a moveable plateau. The length of the fibre and thus the length of the sarcomeres was set by moving the tweezers relative to each other. The mounting device, with the fibre attached, was connected to a table bearing a holder with 4 cuvettes mounted in line. The holder could be moved so that the muscle fibre was immersed in one of the cuvettes.

The apparatus was placed in the beamline of a small angle x-ray diffraction station (#8.2) of the Synchrotron Radiation Laboratory at Daresbury (UK). The wavelength of the incident beam was 0.15 nm. Photon flux at the sam-

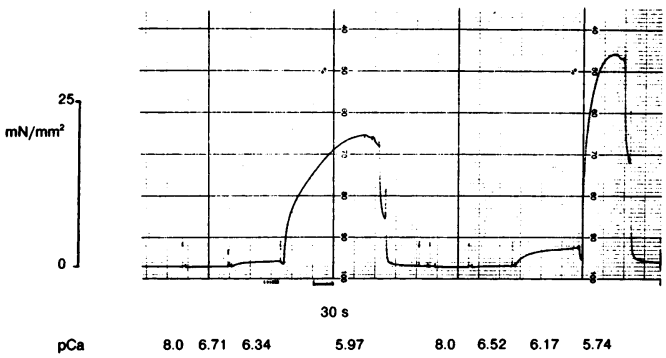

Fig. 2 Force development at different values of pCa during a protocol. A typical paper recording of force development of a muscle fibre when bathing solutions are changed. The time bar represents 30 s. The diffraction spectrum is recorded during 10 s at steady state tension. The value of the particular pCa's are given for each level of force development. The force transients between each pCa value are due to the penetration of the fibre through the fluid surface of the cuvette

ple was 8.10^{10} photons/s · mm^2 at 2.0 GeV and 200 mA (Daresbury Annual Report, 1992). The two-dimensional photon detector was mounted 3.6 m behind the muscle fibre. The equatorial diffraction patterns were obtained by integrating the 2D spectrum symmetrically around the midchannel of the detector. The force signal and beam intensity signal were sampled during the radiation period and the data were stored on disk. Sarcomere length was measured by laser diffraction and the uniformity of the preparation along the axis of the fibre was checked. The first order diffraction pattern was monitored on a screen placed 500 mm behind the muscle preparation. Only spatially uniform (within 0.05 μm) preparations with symmetric first orders were used (deBeer et al. 1988). Slack length of the preparation after rehydration was between 1.8 and 1.9 μm. The sarcomere length was set at 2.0 μm.

Experimental protocol

After the fibre was mounted, it was rehydrated in relaxation solution for at least 15 minutes. Then the fibre was activated and relaxed twice before it was used and exposed to the protocol below.

A protocol consisted of relaxation (pCa = 8.0), 3 activation levels: pCa = 6.71, 6.34, and 5.97 respectively, relaxation (pCa = 8.0), 3 activation levels: pCa = 6.52, 6.17, and 5.74 respectively, relaxation (pCa = 8.0). We did not use the maximal activation level (pCa = 4.4) in order to protect the preparation against too high internal stress and to prevent slip of the attached ends in the tweezers. The duration of the radiation to obtain one diffraction spectrum was restricted to 10 s to minimize the radiation damage during the series of protocols. Data sampling occurred at steady state tension. A typical chart recording of the force during such a protocol is shown in Fig. 2.

During x-ray diffraction pattern recording it was impossible to record simultaneously the sarcomere length by laser diffraction. To verify the changes in sarcomere length during a protocol of different activation interventions, the laser diffraction pattern was continuously recorded from a different fibre of the same preparation in the same apparatus without X-ray diffraction. The length of the sarcomere during the activation protocol differs maximally by about 0.1 μm at the higher activation level, but returns to the starting value within 0.05 μm at pCa = 8.0.

Data analysis

The diffraction pattern was corrected for differences in beam intensity due to the continuous loss of energy in the storage ring. Also, the contribution of the background due to scattering of bathing solutions, the mica windows of the cuvette, and the sensitivity differences in the detector have been taken into account. For that purpose background scattering spectra and detector response spectra were recorded before each new preparation was mounted. To account for the decrease in x-ray intensity during each recording, the signal from an ionisation chamber was sampled. Rat tail collagen fibres (wet) were used for calibration of the interfilament distance. The peak intensities were determined by fitting the experimental diffraction patterns in which the positions of the (1.0), (1.1), (2.0) and (2.1) reflections are arranged together based upon the hexagonal arrangement of actin and myosin filaments and in which the reflection of the Z-line was accounted for. The diffraction peaks were fitted by a combined multi-Gaussian and a hyperbolic function using the Marquardt-Levenberg steepest gradient method (Schiereck et al. 1992, Brenner and Yu 1985; Jung et al. 1989; Yu and Brenner 1979). The positions of the (1.0)- and (1.1)-peaks and the ratio of the areas of the peaks were calculated by integration of the areas of the Gaussian fits of the peaks. Conclusions were drawn on the differences in data obtained for the different pCa values by means of the sign-ranking test or Student's t-test ($P<0.05$).

The fitted peaks should be located symmetrically around the zero order peak, which represents the not-diffracted beam (stopped by the beamstop to protect the x-ray camera). Spectra were discarded when a 4-channel difference (full scale of the detector = 512 channels) or when the intensity difference between the left and right part of the corrected spectrum was more than 10%.

Results

Representative recordings of equatorial diffraction spectra are shown in Fig. 3. In the case of two pCa values, 8.0 and 5.74, the output of the detector after 10 s is plotted.

The typical drop of the signal in the middle of the spectra is caused by the lead beamstop that protects the detector against direct irradiation from the X-ray beam. A typical result of the fitting procedure on the diffraction pattern is shown in Fig. 4. Corrected equatorial diffraction patterns at three different values of pCa (8.0, 6.34 and 5.74)

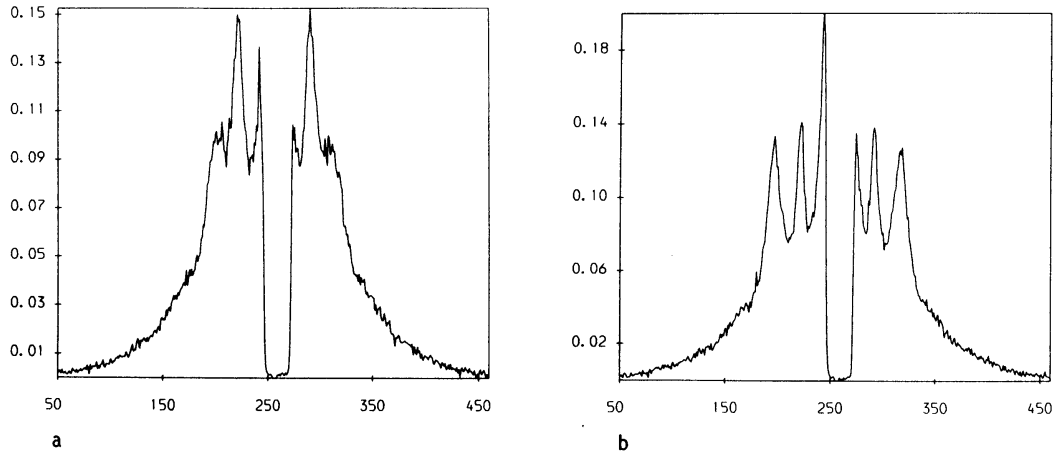

Fig. 3a, b Equatorial diffraction spectra. Equatorial diffraction spectra at two pCa values: **a** at pCa=8.0, **b** at pCa=5.74. The spectra are corrected for the differences in sensitivity in the channels of the detector. The not-diffracted beam is stopped by a lead beamstop to prevent detector damage, as shown in the middle part of the spectra

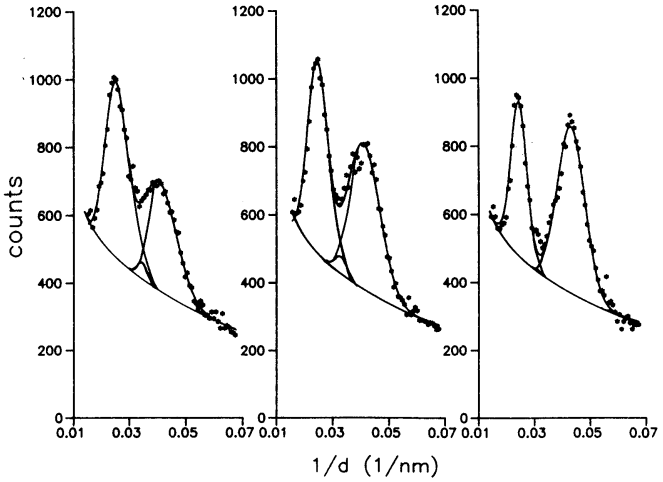

Fig. 4 Equatorial diffraction spectra. Equatorial diffraction spectra at three different values of pCa (8.0, 6.34 and 5.74) are shown. (The force recordings are shown in Fig. 2). The *dots* represent the (already corrected; see Methods) counts in the corresponding detector channels. The *curves* show the fitted relations through the datapoints shown

are plotted (dots). The solid lines represent the fitted curves. We used the same algorithm as Jung et al. (1989). The individual Gaussian curves and the hyperbola are also plotted in the case of pCa=5.74. At pCa=6.34 only a few percent of the force at pCa=5.74 is generated as can be judged from Fig. 2. The increase of the intensity of the (1.1) peak is much more obvious than the decrease of the (1.0) peak. This is clearly shown in the inset of Fig. 5, where I(1.0) and I(1.1), normalised at pCa=6.52, are plotted versus the relative developed tension. The ratio between (1.1) and (1.0) intensity (left and right spectrum combined) is plotted versus the relative active force (relative to force at pCa=5.74) in Fig. 5. Data from 15 fibres with sarcomere length=2.0 μm are combined in this figure. The three data points at the ratio axis are the values of the ratios for pCa; 8.0, 6.71, and 6.52 respectively. At these activation levels, when no active force is observed, information will be lost if the mean value of these data points is presented. These three data points indicate that shift of mass already occurs, suggesting a clear difference in activation level. When skinned trabeculae of the right ventricle of the rat are used, the same result is obtained (data not shown).

If the relative force is plotted against pCa the lower part of the well known S-shaped curve shown in Fig. 6a is found. In Fig. 6b the ratio of the equatorial intensities is plotted as a function of pCa. It is clearly seen that at high pCa the shift of mass already occurs while the force is still zero (i.e. about 20% of the shift of mass at pCa 5.74). These two curves generally reach their saturation level at pCa=4.4. The final increase in developed tension from pCa=5.74 to 4.4 is about 20 to 30% (Schiereck et al. 1993). The laser diffraction pattern recordings did not show changes in sarcomere length during activation at high pCa values. At lower pCa values, when substantial tension was also generated, laser diffraction patterns faded and the position of the first order diffraction peak changed, indicating a small internal shortening of the sarcomeres (about 0.1 μ) and inhomogeneities in the middle of the preparation. From these recordings we conclude that it is unlikely that sarcomere length is changed at high pCa when no force is generated. From these results we conclude that weakly bound crossbridges in skeletal muscle, responsible for shift of mass between actin and myosin, are already present at high pCa although the sarcomere exerted negligible active force.

Discussion

In this study we relate the ratio of the intensities of the equatorial (1.1) and (1.0) x-ray diffraction peaks to the shift of mass between myosin and actin filaments as a consequence of activation by calcium ions. The values of the intensities are calculated after fitting the diffraction pattern by a number of Gaussian curves and a hyperbola. The ac-

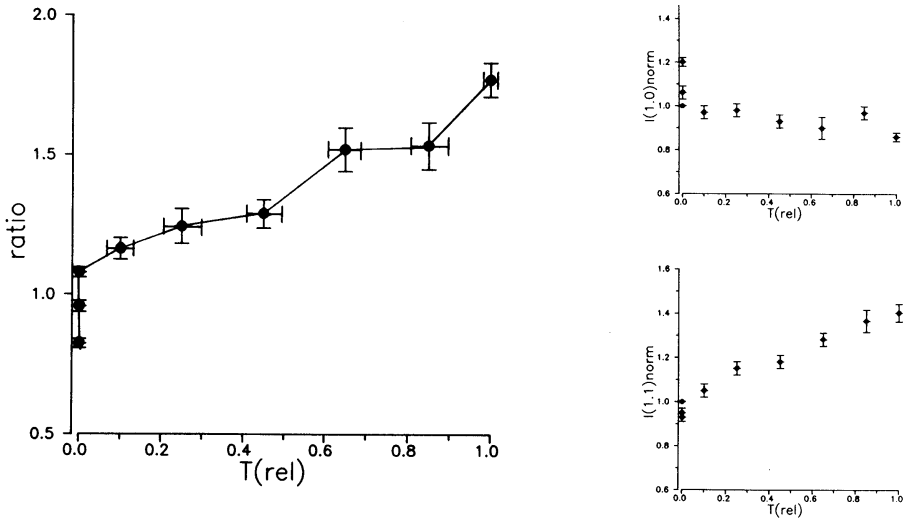

Fig. 5 Ratio between I (1.1) and I (1.0), versus relative active force in skeletal muscle. The ratio between (1.1) and (1.0) diffraction peak intensities, representing shift of mass between myosin and actin is uniquely related to active force, only when active force is not zero. At zero force a shift of mass at subthreshold calcium concentrations is observed. Sarcomere length was 2.0 μm. The *bars* represent the SEM; total number of fibres included was 15. *Inset:* I(1.1) and I(1.0) (normalised at the values at pCa = 6.52) are plotted versus relative tension

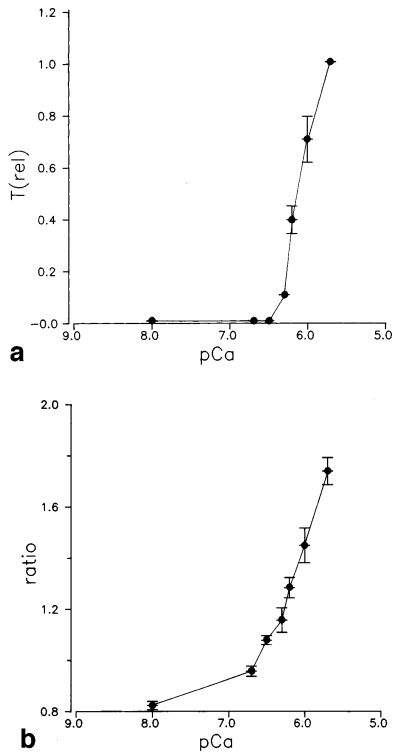

Fig. 6 Relative tension and ratio related to pCa. **a** shows the T (rel) – pCa relationship, **b** shows the ratio I(1.1)/I(1.0) – pCa relationship. A clear increase in shift of mass occurs while force is still zero at high pCa. Sarcomere length was 2.0 μm. The *bars* represent the SEM; total number of fibres included was 15

curacy of the fitting procedure depends on the quality of the experimental data. A longer period of irradiation of the sample increases the signal to noise ratio of the spectra, but also increases the radiation damage of the preparation. For that reason we designed a protocol that minimized the radiation such that radiation damage of the preparation was as low as possible while the signal to noise ratio of the spectra is acceptable. Our protocol lasts for about 9 times 10 s radiation per fibre.

The accuracy of the fitting procedure also depends slightly upon the position of the subsequent peaks. Especially the position of the (1.1) peak depends upon actual geometric parameters that will change by a change in sarcomere length and force generation (Griffiths et al. 1993; Jung et al. 1989; Matsubara et al. 1989; Schiereck et al. 1992; Schoenberg 1980; Yu et al. 1979). In this study we set sarcomere length to a fixed value (2.0 μm). However, after force generation internal redistribution can occur owing to small differences in local stress strain relations. A change in the (1.1) peak position is to be expected after force generation occurs. In Fig. 4 it is seen that at pCa 5.74, indeed, the position of both peaks are shifted towards smaller internal distances. In the case of subthreshold calcium, when no force is generated, the positions of the peaks remain unchanged.

A change in sarcomere length is also responsible for a change in sensitivity of the actomyosin complex to calcium. A rightward shift (to lower sensitivity) is seen at decreasing sarcomere length. If sarcomere length in our preparation is decreased by internal shortening due to extension of the clamped endings of the muscle fibre after activation, the values of force and ratio as shown in the present study are underestimated. The corrected values should be more pronounced than shown here, although in the case of 0.05 μm shortening I(1.0) decreased only by a few percent (Schiereck et al. 1992).

Because no internal force is generated, and no external force is applied it is assumed that no change in sarcomere length occurred. We therefore conclude that the change in intensities of the two diffraction peaks is due to a change in mass distribution between actin and myosin.

The level of calcium activation can be set and controlled throughout the contraction when skinned muscle fibres are used. The concentration of EGTA is chosen such that at very low calcium concentrations a possible contribution of calcium release from fragments of the sarcoplasmic reticulum or mitochondria to the final calcium concentration is unlikely. The levels used in this study (pCa between 5.74 and 8.0) are those generally occurring in twitch contrac-

tions in muscle cells. In these twitches the [Ca^{2+}] at the level of myofilaments undergo a cycling process between the extreme levels as seen in the force-calcium relation. The finding that at 'subthreshold' calcium level, when no active force is generated, a large portion of attached crossbridges is already present, leads to the conclusion that during a normal contraction cycle, crossbridges are cycling between weakly and strongly bound states.

In skinned muscle cells, the intracellular skeleton will be affected. This is the reason that at higher tension damage of the myofilament matrix can occur. Therefore, in the present study pCa was restricted to minimally 5.74. This concentration is about 80% of that at which maximal tension is developed (deBeer et al. 1989). Without recording maximal tension it is impossible to quantify the tension – pCa relationship, although the finding that at high pCa (> 6.52) tension is zero will not be affected by different levels of maximal tension.

At rest, under normal physiological conditions Granzier and Wang (1993) showed that a substantial proportion of crossbridges are coupled. If an intact muscle fibre is stimulated after a long period of rest, under normal metabolic conditions, a depressed force generation is shown. At increasing stimulation interval, the total amount of weakly bound crossbridges is also decreased as a consequence of further decrease of intracellular calcium. This conclusion supports the hypothesis of crossbridge induced crossbridge coupling as proposed by Gordon et al. (1988).

The change in ratio between (1.1) and (1.0) reflection is attributed to the change in crossbridge mass distribution, resulting in an almost linear relationship between this ratio and developed tension (Brenner and Yu 1985, 1989). When tension is generated, the presence of the parallel connected tension generating crossbridges can be proved by stiffness measurements, showing a linear relationship between stiffness and active tension (Blangé et al. 1972; Jung et al. 1988). In the case of weakly bound crossbridges only, at almost zero tension, their presence is not simply proved by stiffness measurements because of the presence of passive elastic material (intracellular skeleton) and the fact that weakly bound crossbridges can detach and reattach at a very high rate (Jung et al. 1989). The results of the present study support the findings in the study of Brenner and Yu (1985), although at low calcium concentrations our results appear to be in contradiction with that study. The differences between methods and preparations used may account for the differences. Brenner and Yu (1985) used fibres from psoas muscles, while our fibres originated from gracilis muscles. Also differences in temperature (6–9 °C and 20 °C), differences in skinning (chemically and freeze drying) and differences in bathing procedure before starting a protocol (relaxing versus rehydrating and several preconditioning contractions) may cause the differences in active tension development at higher calcium concentrations.

The sarcomere length in the present study was kept at 2.0 μm while in the study of Brenner and Yu (1985) the length was kept at 2.3–2.4 μm. The advantage of a sarcomere length at optimal overlap especially accounts for the fact that internal shortening has less influence on the tension generation although changes in calcium sensitivity still occur. In our preparation the sensitivity of troponin-C to calcium is less than at longer (2.4 μm) length. That means that at very low calcium concentrations in our preparation still no active tension is developed while at longer length due to increased sensitivity, active tension will already be developed (deBeer et al. 1988). We restricted our study to 2.0 μm only.

The main difference is that at very low calcium concentrations, in our study no active force is generated, in contrast to the study of Brenner and Yu (1985). Like Brenner and Yu (1985) we find a linear relationship between the ratio I(1.1)/I(1.0) when tension has been developed. In the present study this line is shifted parallel upwards and intercepts the ratio axis at 1.05 instead of 0.8 in the Brenner and Yu study. If no tension is developed at higher pCa we observed a further decrease in ratio towards 0.8. The main methodological difference between the present study and the Brenner and Yu study is that we used a high intensity synchrotron radiation source. The time necessary to obtain enough statistics in our experiment was restricted to 10 s. Therefore, we did not use the method of periodical isotonic shortening at almost zero load and restretch of the preparation for stabilization of the sarcomere pattern as done in the Brenner and Yu study. Such a periodical change in length also distorts the crossbridge distribution in the different states. At high pCa, when no developed force is recorded, this sarcomere length intervention can only distort the weakly bound state. As a consequence, there will be no mass transfer towards the thin filament. In the present study the weakly bound state is not disturbed. This is reflected in the observation that at high pCa the main change is found in the I(1.0), indicating the departure of the myosin heads from the relaxed lattice as shown in the inset of Fig. 5.

We conclude that our results are additional to the results of Brenner and Yu (1985), adding the presence of weakly attached connections to the crossbridge cycle.

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