EQUATORIAL X-RAY DIFFRACTION FROM SINGLE SKINNED RABBIT PSOAS FIBERS AT VARIOUS DEGREES OF ACTIVATION

Changes in Intensities and Lattice Spacing

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ABSTRACT Equatorial x-ray diffraction patterns were obtained from single skinned rabbit psoas fibers during various degrees of activation under isometric conditions at ionic strength 170 mM and 6–9°C. By direct calcium activation, contraction was homogeneous throughout the preparation, and by using a cycling technique (Brenner, 1983) integrity of the fiber was maintained even during prolonged steady activation. The intensity ratio of the two innermost reflections I_{11}/I_{10}, and the normalized intensities I^*_{10} and I^*_{11} varied linearly with increasing force. Thus the result agreed qualitatively with an earlier finding, obtained from the whole sartorius muscle, that intensity changes in *10* and *11* are directly correlated with isometric force level (Yu et al., 1979).

Spacing of the myofilament lattice (d_{10}) was found to decrease with increasing isometric tension. With the filaments in full overlap, maximum shrinkage was 14%. The lattice spacing started to level off when the degree of calcium activation was ≥50%, approaching a limit approximately at 380–360 Å. This decrease of the lattice spacing indicates that there is a radial force produced by force generating cross-bridges, but the net radial force appears to become insignificant as lattice spacing approaches 380–360 Å.

INTRODUCTION

Equatorial x-ray diffraction from skeletal muscle has been shown to vary in a characteristic way depending on the physiological state. For an intact frog sartorius muscle, the intensity ratio I_{11}/I_{10} of the two inner-most reflections *10* and *11* changed from ~0.5 to 2.5 (Haselgrove and Huxley, 1973; Podolsky et al., 1976) in transition from the relaxed state to full activation. The ratio increased further in the rigor state (Huxley, 1968). Two-dimensional Fourier synthesis (Haselgrove and Huxley, 1973) based on these two reflections showed that an increase in the intensity ratio could be explained by mass movement away from the thick filament region to the thin filament region. This mass shift has been generally interpreted as formation of cross-bridges, namely attachment of the myosin heads (mainly myosin subfragment-1) to the thin filaments. Since then, changes in intensity ratio I_{11}/I_{10} were frequently used as indications of changes in cross-bridge attachment. An implicit assumption in this interpretation was that changes in I_{11}/I_{10} were correlated with the number of attached cross-bridges. Later, it was shown that indeed the intensity ratio increased with isometric force in direct proportion in an intact frog sartorius muscle (Yu et al., 1979). The intensity of the reflection *10* was found to decrease and intensity *11* to increase in a graded way throughout the range of force studied, which suggested that the observed changes in I_{10} and I_{11} were directly correlated with the number of cross-bridges formed in the isometric state.

The interpretation, however, was complicated by the fact that the results were obtained from whole sartorius muscles (~3 cm long and 0.6–0.8 mm thick), and inhomogeneous activation among the fibers could not be ruled out completely. Furthermore, it is conceivable that threshold for calcium activation among individual fibers varies widely, such that the graded force levels might be a result of changes in number of fully activated fibers rather than changes in the number of cross-bridges formed within the sarcomere.

To resolve this uncertainty, skinned single rabbit psoas fibers where the sarcolemma was made permeable to large particles were used in the present study. The advantage of this preparation is that it is activated directly in solutions with various Ca concentrations controlled by an EGTA buffer system, thus ensuring a homogeneous degree of activation. Single fibers were used since in this case the

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problem of limited diffusion of ATP is minimized. Furthermore, by applying a novel cycling technique (Brenner, 1983) to a single fiber during contraction, uniformity of the striation pattern was maintained throughout several hours of various degrees of activation.

Another important piece of structural information that is not readily explored by studying intact muscle is the changes in the lattice spacing during contraction. For some configurations of the attached cross-bridges, such as those proposed by H. E. Huxley (1969), one might expect a radial force component directly correlated with axial force (Huxley, 1969; Schoenberg, 1980), which could alter the interfilament spacing. In skinned fiber preparations, decrease in lattice spacing was observed in transition from relaxed state to activation (Shapiro et al., 1979; Matsubara et al., 1980; Matsubara et al., 1984*a*, 1985), or to rigor state (Maughan and Godt, 1981; Matsubara et al., 1984*b*). However, the relationship between active axial force and lattice spacing has not been reported in detail other than at the two end points, i.e., the relaxed state and the fully activated state. Consequently, it is yet to be determined if radial force is correlated with the active axial force.

Preliminary accounts of this work have been reported briefly (Brenner and Yu, 1983).

METHODS

Fiber Preparation

Single skinned rabbit psoas fibers were prepared according to Brenner (1983). Experiments were performed at ionic strength μ = 170 mM at 6–9°C. Relaxing solution contained in millimoles per liter: 1 ATP, 3$MgCl_2$, 1 EGTA, 1 DTT, 10 imidazole, 150 KCl, pH 7.0. Activating solution contained in millimoles per liter: 1 ATP, 3$MgCl_2$, 1 CaEGTA, 10 CrP, 250–300 u CPK (Sigma Chemical Co., St. Louis, MO), 10 caffeine, 1 DTT, 10 imidazole, 120 KCl; pH 7.0. Pre-activating solution had the same composition as the activating solution except that no calcium was included. Solutions for partial activation were mixtures of the activating solution and preactivating solution to vary the free calcium concentration.

Sarcomere length was between 2.3 and 2.4 mm and was monitored by laser diffraction during relaxation and activation. In addition, homogeneity of the striation pattern was monitored by an inverted microscope throughout the experiment (Brenner et al., 1984).

Activation

Skinned fiber segments were mounted between a force transducer and the lever tip of a modified moving coil galvanometer by using cyanoacrylate glues (Brenner, 1980). Before activating solution was introduced, the fiber was first incubated in the pre-activating solution for ~15 min. Force levels, recorded on a storage oscilloscope (model 5103N; Tektronix, Inc., Beaverton, OR), ranged between 1 and 1.2 kg/cm^2 at 5°C and 1.8–2.2 kg/cm^2 at 15°C.

To keep the striation pattern well ordered and stable during calcium activation, the fibers were cycled between isometric contraction and lightly loaded isotonic shortening. After every 5–20 s of isometric contraction, fibers were allowed to shorten under light (3–5%) relative loads. After a shortening period of 250 to 500 ms, fibers were restretched directly out of isotonic shortening and resumed isometric contraction at their original lengths (Brenner, 1983). Each fiber was activated to give three to four force levels, which included at least one maximal level P_o at pCa 4.5. When several maximal force levels were produced during one experiment, maximal levels did not vary >10%. Otherwise, the fiber was discarded. The order of various degrees of activation was random.

X-Ray Diffraction

The fine focus x-ray camera set up and data reduction procedures were identical to those described previously (Brenner et al., 1984), except that only cursor stripping was used in obtaining the peak intensity. Exposure time generally was 500 s except in cases where fibers broke before reaching the pre-set time.

To compare individual reflections, *10* and *11*, obtained from different fibers, a normalization procedure was adopted, such that data fluctuations due to fiber diameter, beam intensity, etc. were minimized. Immediately before and after each activation, patterns of the relaxed state were obtained. I_{10} and I_{11} of those two patterns were first normalized with respect to the intensity of the transmitted beam I_c. The average of the intensities I_{11}/I_c and I_{10}/I_c from the two patterns were then used as normalization for the active state. That is

$$I^*_{10} = (I_{10}/I_c)_{\text{active}}/(I_{10}/I_c)_{\text{relaxed}},$$

with I_{11}* similarly defined.

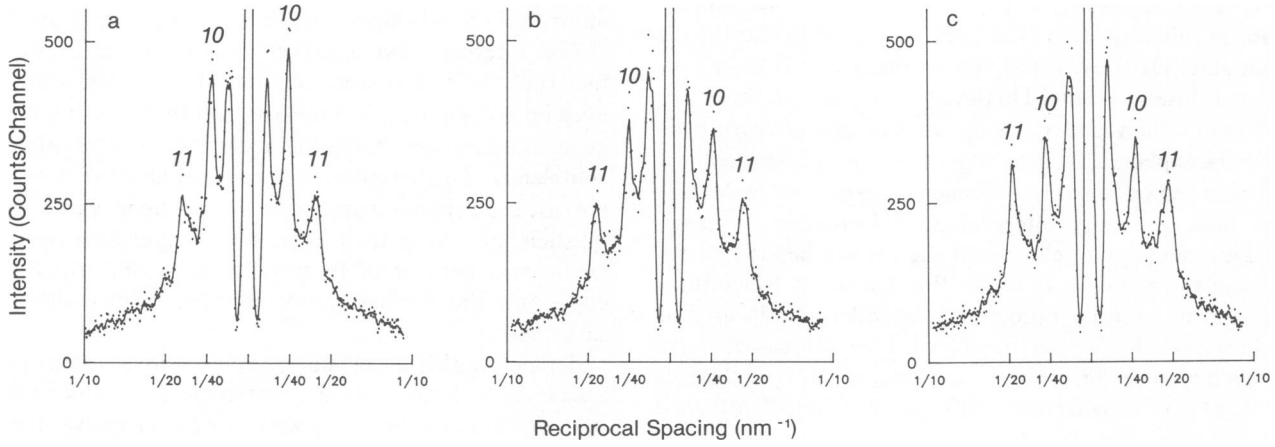

FIGURE 1 Equatorial x-ray diffraction patterns from a single rabbit psoas fiber during various degrees of activation at μ = 170 mM and 7°C: (*a*) relaxed in preactivating solution; (*b*) force = 48% of maximal level; (*c*) force at maximal level. Dots correspond to original data; lines correspond to data smoothed by a three-point weighted average. Sarcomere length = 2.3 μm. Exposure time = 500 s. Specimen-to-detector distance = 33 cm.

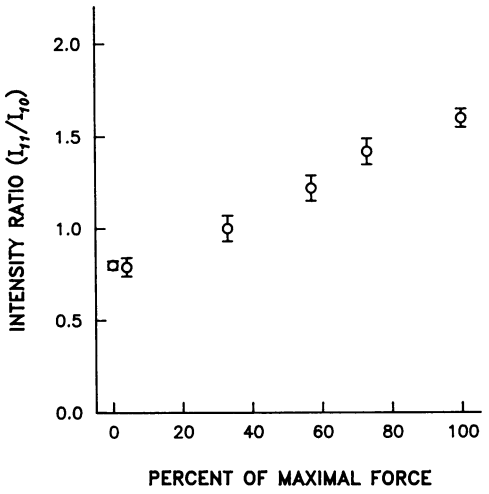

FIGURE 2 Summary of intensity ratio of I_{11}/I_{10} obtained from nine fibers. Error bars are standard errors of the mean (SEM) in the intensity ratio, while SEM of force levels are smaller than the radius of the circles. The ratio increased from 0.80 for the relaxed state to 1.60 at maximal level.

RESULTS

Typical equatorial patterns from a single fiber during various levels of isometric contraction are shown in Fig. 1. Generally, force levels were maintained within a few percent during exposure time. There was no discernable deterioration of patterns after several cycles of activation, since the widths of the reflections remained unchanged. That the integrity of the fibers was well maintained throughout the experimental procedure was also evidenced by the reversibility of the diffraction patterns: the relaxed patterns before and after activation cycles gave similar intensity ratios with variations within experimental errors. Twelve fibers were used. The data from one fiber, which showed sarcomere dispersion during activation, were discarded.

Intensity Ratio I_{11}/I_{10}

Relationship between the intensity ratio I_{11}/I_{10} and force level is summarized in Fig. 2. The ratio increased from 0.80 for the relaxed state to 1.60 at the maximal force level in a linear way. Of course, if intensity ratio is defined as I_{10}/I_{11} instead of I_{11}/I_{10}, the variation of this ratio with respect to force level is hyperbolic. Thus an hyperbolic behavior of I_{10}/I_{11} (Tanaka et al., 1983) is not in conflict with our present or the previous data (Yu et al., 1979).

Based on the intensity ratio, the amount of mass transfer from the thick filament region to the thin filament region during maximum activation was calculated by two-dimensional Fourier synthesis (Haselgrove and Huxley, 1973). The transfer is 50% of that occurring when a relaxed fiber at μ = 170 mM goes into rigor (Brenner et al., 1984).

Intensity Changes in I^*_{10} and I^*_{11}

Variations in the individual reflections were examined in detail. Fig. 3 *a* and *b* showed that the normalized I^*_{10} decreased from 1 (normalization value) to 0.75 as force was raised to maximal level, while I^*_{11} increased from 1 to 1.49. Both I^*_{10} and I^*_{11} vs. isometric force are described adequately as linear functions of isometric force level.

Changes in Lattice Spacing d_{10}

Changes in the lattice spacing between the *10* planes, d_{10}, were a highly nonlinear function of force level (Fig. 4). In the relaxed state at μ = 170 mM, d_{10} was 441 Å, (± 2 Å SEM with n = 30), same as that reported in an earlier work (Brenner et al., 1984). d_{10} monotonically decreased with increasing force. The decrease appeared to level off when isometric force levels were >50% of the maximal force. At 100% activation, d_{10} was 380 Å. The 14% shrinkage in lattice spacing accompanying full activation was in agreement with results reported by Shapiro et al. (1979), but larger than the 8% observed by Matsubara et al. (1984, 1985).

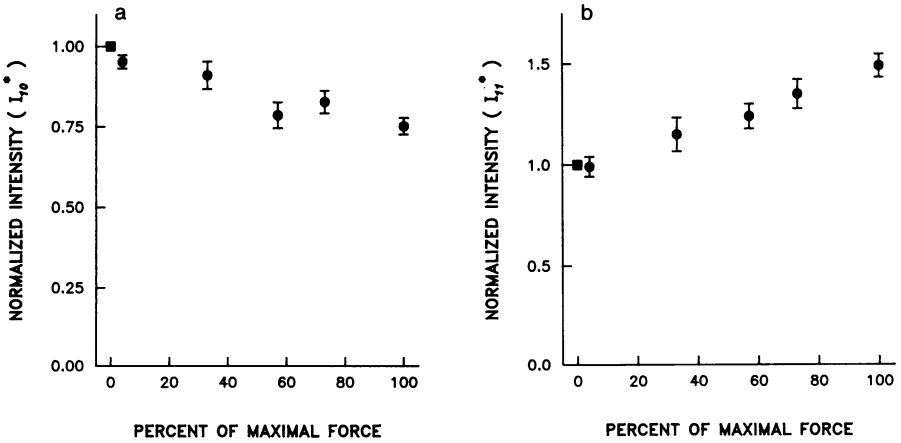

FIGURE 3 Intensities of individual reflections *10* and *11* normalized with respect to the transmitted beam. (■) from relaxed state, which is defined to be 1; (●) from activated state with force ranged between 4 and 100%. Error bars are standard errors of the mean in intensities.

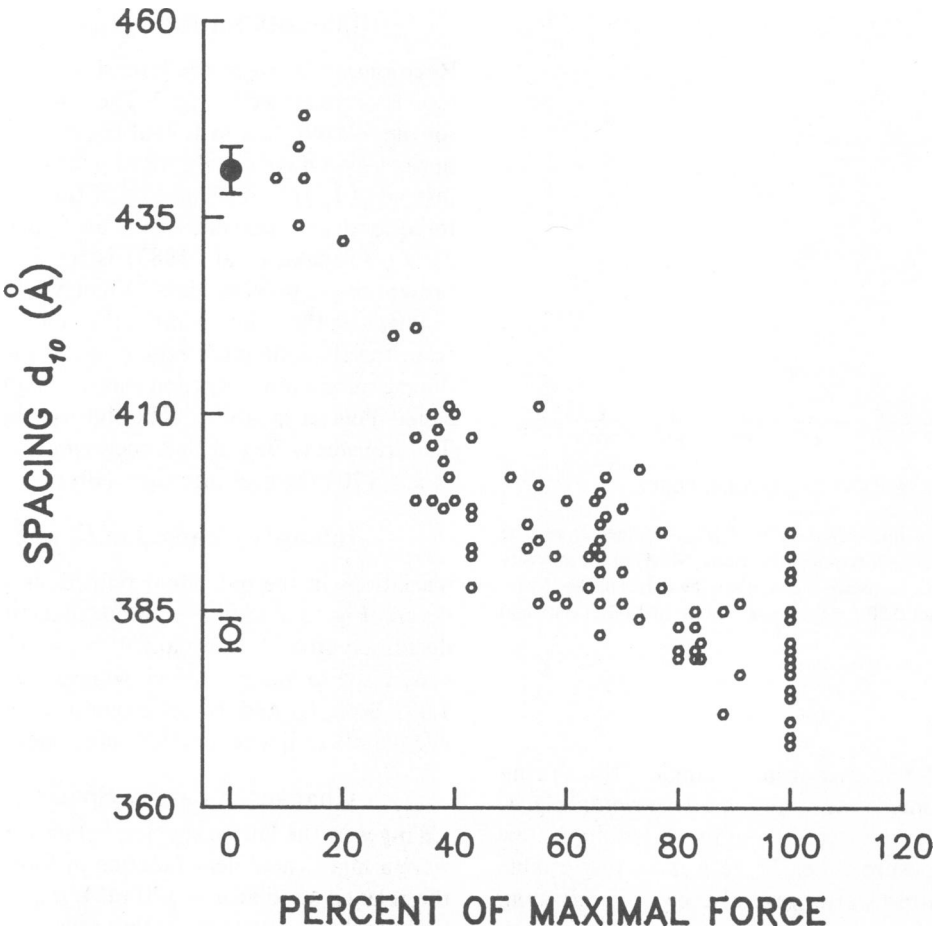

FIGURE 4 Lattice spacing d_{10} as a function of force levels. Large filled circle (●): average value of d_{10} of the relaxed state at μ = 170 mM; open large circle (○): average value of d_{10} of the relaxed state at μ = 20 mM; small open circles (○): d_{10} of each individual experiment. Note that d_{10} starts to level off when force is >50% of maximal level.

DISCUSSION

The changes in intensities as a function of force level observed in the present study are in qualitative agreement with the study on whole intact sartorius muscle (Yu et al., 1979). Similar to the present result, the intensity ratio I_{11}/I_{10} was found to be almost linear with respect to force level and I_{10} decreased and I_{11} increased throughout the range of force studied. The advantage of the present study is that by using a single skinned fiber preparation, activation is more likely to be homogeneous throughout the specimen, and the degree of activation is controlled directly by known free calcium concentration in the bathing solution. With the cycling technique, the integrity of the fiber is maintained during sustained contractions. The possibility that the graded change in intensities was due to inhomogeneity in activation is thus unlikely. The qualitative agreement between results from the fibers and the whole muscle indicates that the correlation between intensity ratio and force level observed in whole intact muscle is also directly attributable to the number of cross-bridges in the isometric force generating state at the sarcomere level, rather than changes in the ratio of activated vs. nonactivated fibers as suggested by Tanaka et al. (1983).

Upon activation, the lattice spacing of skinned fiber decreases by 14% whereas in intact muscle the spacing is unchanged. One might expect that the behavior of intensity ratio vs. force level to be qualitatively different upon activation in the two preparations, since intensity ratio might depend on lattice spacing. However, in a relaxed fiber at μ = 170 mM where few cross-bridges are attached, it was shown that intensities were little affected by lattice spacing between 440 and 380 Å (Brenner et al., 1984). In a rigor fiber where all cross-bridges are attached, the intensity ratio remained unchanged with d_{10} between 380 and 370 Å (Brenner and Yu, unpublished results). Based on these two results it is rather unlikely that the intensity ratio could be affected by lattice spacing in a force generating fiber, although one cannot completely rule out such a possibility.

It was pointed out by Lymn (1978) that changes in configurations of cross-bridges could cause substantial changes in intensities. However, by studying muscle fibers activated isometrically at various degrees, ambiguities in

interpreting the intensity data are avoided. There is strong evidence that the function of calcium activation is to recruit an increasing number of force-generating cross-bridges (Podolsky and Teichholz, 1970; Goldman and Simmons, 1984; Brenner, in preparation). Although the force-generating cross-bridges may assume a distribution of attachment configurations, this distribution most likely remains unchanged if calcium only affects the number. Therefore, changes in intensities observed in the present experiments are directly related to the number of cross-bridges formed in the main force-generating state.

In spite of the substanital similarity in the general characteristics of intensity changes obtained from the whole sartorius and from single psoas fibers there are quantitative differences in the two results. The magnitude of change in I_{11}/I_{10}, I_{10}*, and I_{11}* was greater in the case of frog sartorius. The differences may be explained by the lower force levels generated by the psoas at ~7°C. At 2–4°C, the frog sartorius generates at 2–3kg/cm^2 forces, while the psoas at 7–9°C produces 0.8–1.2 kg/cm^2. Preliminary results obtained from psoas fibers during full activation at higher temperatures (10 and 15°C) where force levels were higher showed further changes in intensities ($I_{11}/I_{10} \sim 2$). This suggests that the low intensity ratio could reflect a lower number of cross-bridges formed in psoas, rather than any basic differences in structure.

Another difference is found in the decrease in I_{10} at low force levels. It was noted in the previous study (Yu et al., 1979) that there appeared to be a somewhat steeper drop in I_{10}^* when tension was below 50%. It was suggested that there might be disordering involving the entire thick filament or just the myosin heads, as calcium was introduced. Another suggestion was that there might be an activation step before cross-bridge attachment (Squire, 1979). However, variation in I_{10}^* observed in the present study showed little steep drop at low force level, with the overall relation being only slightly nonlinear. The activation process appears to be adequately explained by an increasing number of cross-bridges formed. The discrepancy could be due to more uniform activation in the single fibers, but it remains puzzling why I_{11}^* is not affected similarly. Therefore, the reason for the discrepancy is not clear.

The intensity ratio of 1.60 at full activation at 7°C for the psoas is about one-half that of a toe muscle of a mouse ($I_{11}/I_{10} = 3.1$) activated fully at 19–21°C for 5–10 min (Matsubara et al., 1984*b*, 1985). Difference in temperature could be a contributing factor, although our preliminary results at 15°C indicated that intensity ratio was ~2. More significantly, perhaps, are the different experimental conditions. In the reported experiments, a saponin-skinned whole muscle was activated without a backup system. It is not clear whether under those conditions there was sufficient ATP present throughout the muscle during the time of exposure. In fact, the high intensity ratio of 3.1 is close to their reported rigor value (3.4). The mechanical properties and light microscopic appearance of a single psoas fiber during cycling, when maximally activated in the absence of a backup system, showed that only a layer of ~10–20 μm is sufficiently saturated at 1 mM ATP and 5°C (Brenner, unpublished observation).

The shrinking of lattice spacing associated with rising force provides evidence that the active force-generating cross-bridges exert a radial force. However, this radial force is not proportional to the axial force as indicated by the nonlinear behavior of d_{10}, which starts to level off when force is >50% of the maximal force. The present study does not distinguish whether the nonlinear behavior is caused by a passive lattice component resisting compression below ~380 Å or by structural hinderance caused by the attached cross-bridges, or by decreasing radial force per cross-bridge. Our recent preliminary result suggests that the nonlinear behavior is not caused by passive lattice component. Rather, the radial elasticity of the active cross-bridges is approximately linear with equilibrium position at 360 Å.

In summary, changes in intensities of *10* and *11* reflections at various degrees of Ca^{++} activation at 6–9°C are directly correlated with changes in the number of cross-bridges in the main force generating state. In addition, the changes in lattice spacing suggest that a net radial force is produced by the force generating cross-bridges. This radial component is not a constant fraction of the axial force.

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REFERENCES

Brenner, B. 1980. Effect of free sarcoplasmic Ca^{++} concentration on maximum unloaded shortening velocity: measurements on single glycerinated rabbit psoas muscle fibers. *J. Muscle Res. Cell Motil.* 1:409–428.

Brenner, B., M. Schoenberg, J. M. Chalovich, L. E. Greene, and E. Eisenberg. 1982. Evidence for cross-bridge attachment in relaxed muscle at low ionic strength. *Proc. Natl. Acad. Sci. USA.* 79:7288–7291.

Brenner, B. 1983. Technique for stabilizing the striation pattern in maximally calcium-activated skinned psoas fibers. *Biophys. J.* 41:99–102.

Brenner, B., and L. C. Yu. 1983. Equatorial x-ray diffraction from single rabbit psoas fibers during various degrees of activation. *Biophys. J.* 41(2, Pt. 2):257*a*. (Abstr.)

Brenner, B., L. C. Yu, and R. J. Podolsky. 1984. X-ray diffraction evidence for cross-bridge formation in relaxed muscle fibers at various ionic strenghts. *Biophys. J.* 46:299–306.

Goldman, Y. E., and R. M. Simmons. 1984. Control of sarcomere length in skinned muscle fibers of *rana temporaria* during mechanical transients. *J. Physiol. (Lond.).* 350:497–518.

Haselgrove, J. C., and H. E. Huxley. 1973. X-ray evidence for radial cross-bridge movement and for the sliding filament model in actively contracting skeletal muscle. *J. Mol. Biol.* 77:549–568.

Huxley, H. E. 1968. Structural difference between resting and rigor muscle. Evidence from intensity changes in the low-angle equatorial x-ray diagram. *J. Mol. Biol.* 37:507–520.

Huxley, H. E. 1969. The mechanism of muscular contraction. *Science* (*Wash. DC*). 164:1356–1366.

Lymn, R. W. 1978. Myosin subfragment-1 attachment to actin. *Biophys. J.* 21:93–98.

Matsubara, S., L. C. Yu, and R. J. Podolsky. 1980. X-ray diffraction from activated skinned muscle fibers. *Fed. Proc.* 39:1729. (Abstr.)

Matsubara, I., Y. Umazume, and N. Yagi. 1984*a*. Lateral shrinkage of the myofilament lattice in chemically skinned muscles during contraction. *In* Contractile Mechanisms in Muscle. G. Pollack and H. Sugi, editors. Plenum Publishing Corp., New York. 711–720.

Matsubara, I., Y. E. Goldman, and R. M. Simmons. 1984*b*. Changes in the lateral filament spacing of skinned muscle fibers when cross-bridges attach. *J. Mol. Biol.* 173:15–33.

Matsubara, I., Y. Umazume, and N. Yagi. 1985. Lateral filamentary spacing in chemically skinned murine muscles during contraction. *J. Physiol. (Lond.)*. 360:135–148.

Maughan, D. W., and R. E. Godt. 1981. Radial forces within muscle fibers in rigor. *J. Gen. Physiol.* 77:49–64.

Podolsky, R. J., and L. E. Teichholz. 1970. The relation between calcium and contraction kinetics in skinned muscle fibers. *J. Physiol.* (*Lond.*). 211:19–35.

Podolsky, R. J., R. St. Onge, L. C. Yu, and R. W. Lymn. 1976. X-ray diffraction of actively shortening muscle. *Proc. Natl. Acad. Sci. USA.* 73:813–817.

Schoenberg, M. 1980. Geometrical factors influencing muscle force development II. Radial forces. *Biophys. J.* 30:69–77.

Shapiro, P. J., K. Tawada, and R. J. Podolsky. 1979. X-ray diffraction of skinned muscle fibers. *Biophys. J.* 25(2, Pt. 2):18*a*. (Abstr.)

Squire, J. M. 1979. Structure and force generation in muscle. *Nature* (*Lond.*). 281:99–100.

Tanaka, H., T. Tameyasu, and H. Sugi. 1983. Relation between equatorial x-ray intensities and isometric twitch forces in frog skeletal muscle. *Proc. Jpn. Acad. B. Phys. Biol. Sci.* 59:13–16.

Yu, L. C., J. E. Hartt, and R. J. Podolsky. 1979. Equatorial x-ray intensities and isometric force levels in frog satorius muscle. *J. Mol. Biol.* 132:53–67.