X-Ray Evidence that in Contracting Live Frog Muscles there Exist Two Distinct Populations of Myosin Heads

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ABSTRACT Using synchrotron radiation and whole muscles, 2 ms time-resolved x-ray diffraction patterns were recorded at 8°C. The results show that in both isotonic and isometric contractions, as well as in length changes imposed at maximum tension [P_o], the meridional third myosin layer line consists of two distinct reflections with different intensities and spacings that measure ~14.623 and 14.412 nm at P_o. Although the intensity behavior of the two reflections is strikingly different during quick releases, it is very similar during stretches. Study of the time courses indicates that myosin heads diffracting at P_o with the ~14.623 nm periodicity are actively involved in tension production. Those diffracting at P_o with the periodicity of ~14.412 nm appear not be associated with tension production during isometric contraction and releases, but the results suggest that they are recruited during stretches and here contribute to tension production. Our most important conclusion is that under all conditions of contraction we have investigated there exist two populations of myosin heads, each with a well defined axial disposition and configuration.

INTRODUCTION

It is generally agreed that the meridional third myosin layer line (3M) is mostly due to the axial repeat of the myosin heads along the filament which, on average, measures ~14.340 nm in resting frog muscles. During the past 20 years, many attempts have been made to obtain structural information from studies of this reflection using whole muscles and low-angle x-ray diffraction techniques. It was found that in the transition from rest to maximum isometric tension [P_o] the intensity of the 3M first decreases and then increases (Huxley et al., 1982), whereas its spacing increases by about 1% (Huxley and Brown, 1967). On the basis of further results (Bordas et al., 1993; Martin-Fernandez et al., 1994), it was found that the intensity changes could be attributed to an order/disorder transition in the muscle structure occurring after activation and to the subsequent process of tension generation, respectively. Additional data came from experiments that showed that in response to a quick stretch or release imposed from P_o the intensity of the 3M can decrease by as much as 80% (Huxley et al. 1983), and that although after a quick release its spacing also decreases by ~0.6%, it did not appear to change in a quick stretch (Huxley et al., 1989).

We have repeated and extended many of the experiments with whole muscles described by Huxley et al. (1982, 1983, 1989). Our results, obtained with long cameras (~7.5 m), reveal that in both isotonic and isometric contractions, as well as during length changes imposed from P_o, the 3M actually consists of two distinct reflections with different intensities and spacings. The individual time courses of the intensity and spacing changes could be determined from the data using peak stripping procedures.

METHODS

The methods used for handling and mounting the muscles, and the techniques for data collection and reduction, were essentially identical to those published previously (Martin-Fernandez et al., 1994). The release and stretch protocols are described in the legends of Figs. 2 and 3. We followed the peak stripping procedures used the Levenberg-Marquardt method (Press et al., 1987) in which linear backgrounds and Gaussian functions were used to fit the data.

RESULTS

Records of the 3M at P_o

Our records show that in contracting muscles the spacing of the 3M increases and its half-width becomes about 1.6 times wider relative to the value in the rest pattern. Using image plate detectors, we also find that the 3M clearly appears as a split peak at P_o (Fig. 1 *a*). This demonstrates that at P_o there are two reflections that—from here onwards and unless a more accurate value of the spacing is required—we will call the 14.4 and 14.6 nm reflections. Using proportional gas chambers (which have less resolution than the image plate system but enable collection of time-resolved diffraction data), we produced records in which the two overlapping peaks are still sufficiently resolved to strip out the periodicities and relative contributions to the total intensity of the two overlapping peaks. This was done by fitting Gaussians with a half-width equal to that of the reflection at rest. At P_o the periodicities of the two reflections measure ~14.416 and 14.623 nm (i.e., increases of ~0.500 and 1.975%), and their intensities come to ~0.38 and 0.62, respectively. The intensities were normalized to a value of 1 for the total intensity recorded at P_o in the diffraction band, which includes the two reflections.

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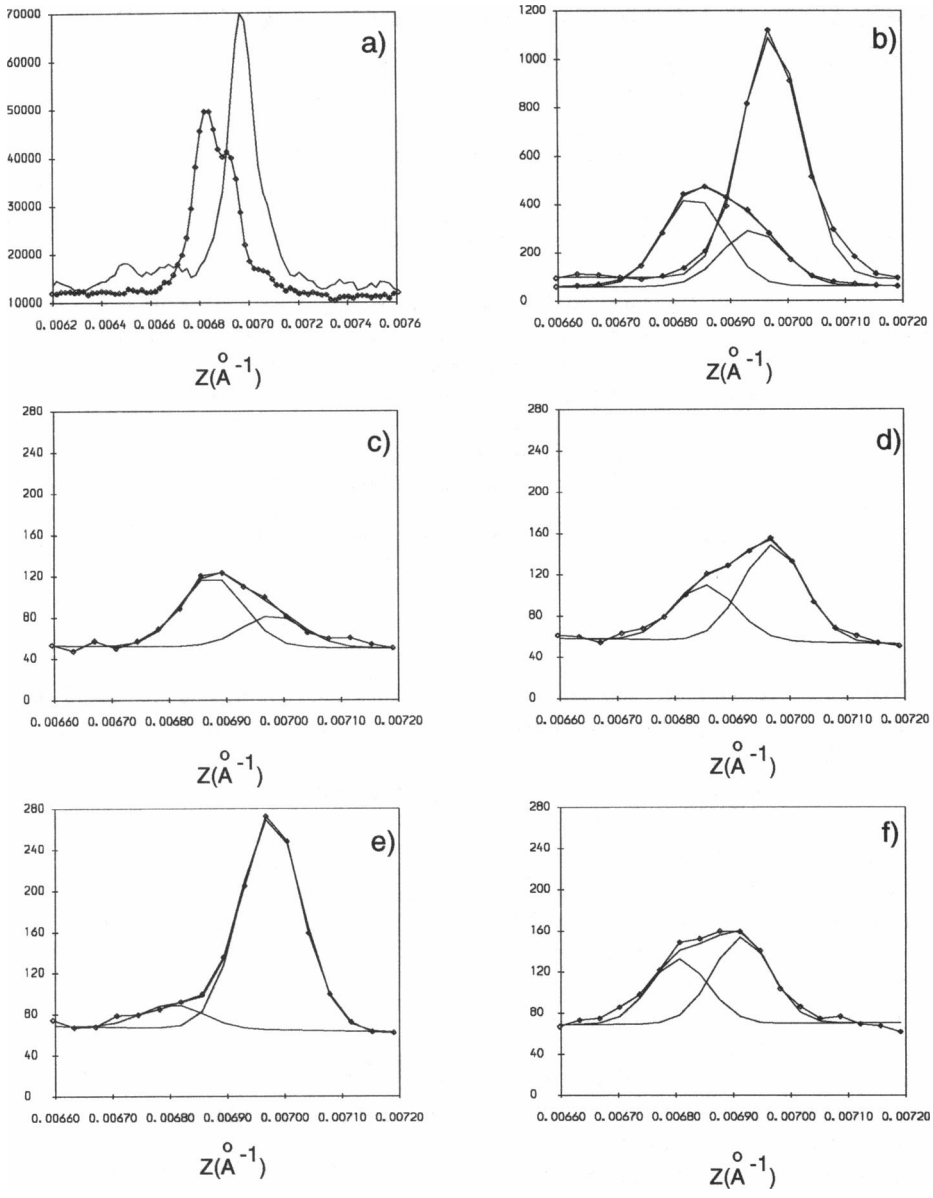

FIGURE 1 Meridional diffraction diagrams from muscles at rest and in various states of contraction recorded in the region of the third myosin layer line. All diagrams have been constructed by radial integration of muscle patterns in the region comprising the meridional peak on the third myosin layer line at rest. A spacing of 14.34 nm for the main diffraction maximum at rest has been used for the calibration of the axial reciprocal space coordinate Z. The traces in *a* were collected with an image plate detector using two different muscles; therefore, the relative intensities of the trace at rest (——) and at P_o (—◆—) are not strictly comparable. The data in all other traces were collected with the proportional gas chamber (—◆—) on the same muscles and under identical conditions. Therefore, the relative intensities of all of these data can be compared (note different *y* axis values in *c–f* compared with those in *b*). The two peaks underneath the traces from contracting muscle in *b–f* represent the relative contribution to the total intensity obtained by fitting two Gaussians, each with a half-width equal to that of the reflection at rest. The unlabeled line going through the experimental data is the total fit corresponding to the sum of the two Gaussians and a linear background. (*a*) In addition to the prominent 14.34 nm meridional peak, the trace from resting muscles shows a number of other reflections at spacings of ~15.38, 14.99, 13.87, and 13.54 nm and a shoulder at a spacing of ~14.14 nm. The trace at P_o shows that the center of gravity of the reflection moves toward longer spacings and that it contains a maximum and a shoulder at spacings of ~14.6 and 14.4 nm, respectively. (*b*) Similar traces collected with the multiwire proportional chamber. The two overlapping peaks at P_o confer an asymmetric shape to the diffraction band. The spacing of the two fitted maxima at P_o are ~14.412 and 14.623 nm, respectively. (*c*) Trace obtained 3 ms after the onset of a quick release from P_o, which produced a ~1% change in the muscle length. In this situation, the two reflections have periodicities of ~14.34 nm (i.e., the value at rest) and 14.591 nm, respectively. (*d*) Trace obtained during the 8 ms subsequent to the collection of the data shown in *c* while the muscle is undergoing unloaded shortening according to the protocol described in Fig. 2. The spacings of the reflections are ~14.34 and 14.591 nm, respectively. Note the intensity increase undergone by the 14.34 nm reflection relative to that in *c*. (*e*) Trace collected at ~30 ms after an initial quick release during a period of unloaded shortening according to the protocol described in Fig. 2. The intensity of the 14.4 nm reflection has increased substantially, and its spacing has settled at a value of ~14.328 nm, which is shorter than the rest spacing by ~0.08%. Note that a weak reflection is detectable at a spacing of ~14.684 nm that corresponds to the position expected for the third order of the 44.0 nm C-protein repeat. (*f*) Trace collected at ~3 ms after the onset of a fast stretch from P_o that produced a ~1% change in the muscle length. In this situation, the spacing of the reflections has increased to ~14.46 and 14.678 nm, respectively. Note that the intensity of these reflections is subtantially lower than that at P_o and is comparable with that seen in *d*.

Changes in the 3M during releases

The changes in the total integrated intensity (Fig. 2 *a*) follow the time course described previously by Martin Fernandez et al. (1994). It is known that the initial rapid intensity decrease associated with a fast release lags slightly behind that of the tension drop (Huxley et al., 1983; Irving et al., 1992). Even with a time resolution of 2 ms, this delay can be discerned also in time expanded plots obtained from our data (traces not shown here).

Fig. 2 *b* shows how the complex time course seen in Fig. 2 *a* arises from the different responses of the 14.4 and 14.6 nm reflections. Furthermore, the tracings in Fig. 2 *c* demonstrate that the initial delay in the total intensity decrease

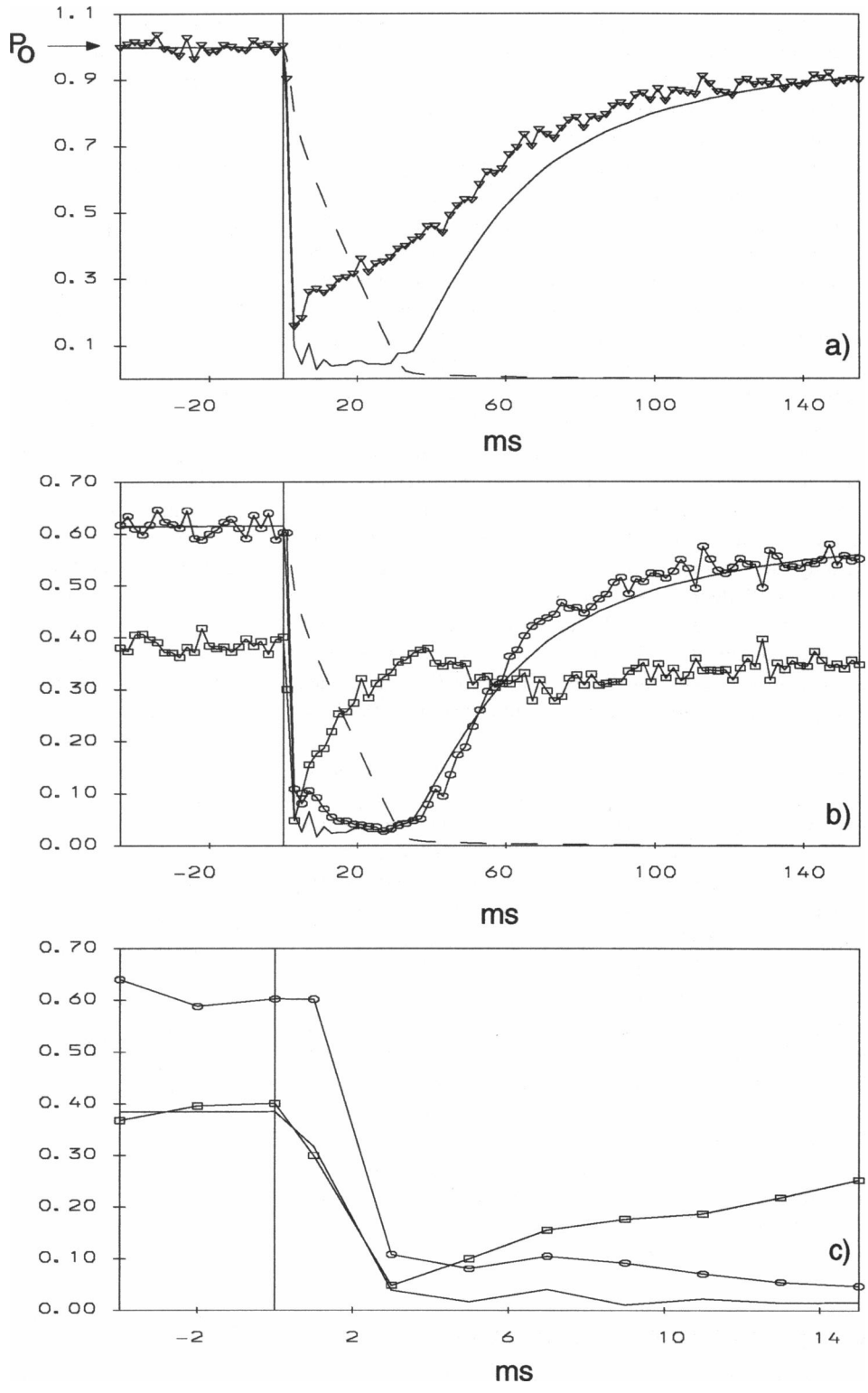

FIGURE 2 Muscles were stimulated isometrically from rest by applying a 600-ms-long train of pulses; they were released 400 ms after the start of stimulation when tension had reached P_o. The mechanical maneuvers involved an initial quick release amounting to a ~1% change in the muscle length, followed by a ramp release whose speed was adjusted to keep tension at an almost negligible level. The total release amounted to ~6.5% in the muscle length. Tension and length curves are shown by the continuous and dashed lines, respectively. The vertical line in all figures indicates the onset of the release. (*a*) Time course of the total integrated intensity in the 3M (—△—) compared with tension and length. The intensity and tension have been normalized to unity at P_o. (*b*) Intensity changes of the contributions from the 14.4 and 14.6 nm reflections to the total time course (□ and ○, respectively) compared with the time courses of tension and length, which have been scaled down for purposes of comparison. (*c*) Expanded view of *b* showing the response to the quick release of the intensity in the 14.4 and 14.6 nm reflections (□ and ○, respectively).Tension has been scaled to the magnitude of the intensity change in the 14.4 nm reflection during the quick release. Note that although 1 ms after the release (first point on the right of the vertical line) the intensity of the 14.4 nm reflection has decreased to ~80% of its P_o value, the intensity of the 14.6 nm reflection has not changed at all.

after a quick release is mostly due to the delay in the response time of the 14.6 nm reflection. Thus, within our time resolution, although the tension drop and the intensity decrease in the 14.4 nm reflection start almost simultaneously, the intensity in the 14.6 nm reflection does not begin to change until ~1 ms later. However, these initial intensity changes in the 14.4 and 14.6 nm reflections are completed simultaneously within 3 ms of the onset of the release, when their intensity has decreased to ~0.05 and 0.1, respectively, of the total intensity at P_0. Thereafter, the differences in the time courses of the intensity recovery in the two reflections are very substantial (Fig. 2 *b*).

During the unloaded shortening phase, the intensity of the 14.4 nm reflection increases rapidly (half-time 8–10 ms) and has practically recovered its P_0 value at the stage when the muscle's length is clamped. As tension redevelops, the intensity of the 14.4 nm reflection decreases within 30–35 ms to ~0.3 of the total intensity at P_0, and then increases again very slowly. In contrast, the intensity of the 14.6 nm reflection remains low, and it reaches a minimum value of ~0.035 of the total intensity at P_0. When tension redevelops at the end of shortening, the intensity of the 14.6 nm reflection increases with a half-time very similar to that of tension rise.

We also studied the time courses of the spacing changes in the 14.4 and 14.6 nm reflections, and those measured from the center of gravity of the composite diffraction band (traces not shown here). The changes in the latter have been described previously by Martin Fernandez et al. (1994). Regarding the spacings of the 14.4 and 14.6 nm reflections, we find that both decrease without any detectable delay relative to the rapid tension drop. The 14.4 nm reflection returns to its rest spacing of 14.34 nm, whereas the one at 14.6 nm decreases to 14.543 nm. Relative to their P_0 values, these changes correspond to spacing decreases of ~0.50 and 0.55%, respectively.

During the unloaded shortening phase, the 14.4 nm reflection remains at 14.34 nm, or perhaps at a slightly lower spacing. As tension redevelops, this spacing returns to its P_0 value with a time course similar to that of tension rise.

Eight milliseconds after the initial quick release, during which the spacing of the 14.6 nm reflection decreases abruptly by ~0.55%, its value stabilizes at ~14.591 nm, i.e., a spacing shorter by ~0.22% than that at P_0. Thereafter, the intensity of this reflection becomes so low that it is difficult to follow its behavior. Fig. 1 *e* shows that at the end of unloaded shortening a reflection is present at a spacing of ~14.684 nm, which is longer by ~0.42% than that at P_0. This reflection probably does not correspond to a new position of the 14.6 nm reflection. It seems more likely that as the latter disappears, an underlying reflection can be detected. In fact, a periodicity of 14.684 nm is very close to that expected from the third order of the 44.0 nm C-protein repeat (Squire, 1981).

Changes in the 3M during stretches

Fig. 3 *a* shows that during the initial quick stretch tension increases to ~1.7 P_0, whereas the total integrated intensity in the 3M decreases to ~40% of its P_0 value. Within our time resolution, the onset of this intensity drop is simultaneous with the rise of tension, and the completion of both changes also occurs simultaneously within 3 ms of the start of the quick stretch. During the subsequent slower ramp stretch, when tension increases almost linearly with time to ~2 P_0, the intensity continues to decrease in a similar manner, eventually reaching ~0.2 of its P_0 value.

The time course of the relative intensity contributions of the 14.4 and 14.6 nm reflections is illustrated in Fig. 3 *b*. The initial change in the 14.6 nm reflection takes the form of a sudden intensity drop to 0.2 of the total P_0 value. Within our time resolution, this event is simultaneous with the rapid rise of tension. Thereafter, for the duration of the ramp stretch, the intensity of the 14.6 nm reflection diminishes very slowly with a time course similar to that of the tension rise, eventually reaching ~0.1 of the total intensity at P_0. Compared with the initial time course of the intensity drop in the 14.6 nm reflection, that of the 14.4 nm reflection is somewhat slower: it takes ~8 ms to decrease to ~0.2 of the total intensity value at P_0. Subsequently, as in the case of the intensity of the 14.6 nm reflection recorded during the ramp stretch, that of the 14.4 nm reflection also decays very slowly. At the end of the ramp-lengthening phase, the intensities of both reflections are practically identical, having reached ~0.1 of the total intensity at P_0.

We have also studied the spacing changes of the two reflections. The tracings (not shown here) reveal that during the quick stretch the spacing of the 14.4 nm reflection increases relative to its P_0 value by 0.33–14.46 nm. The corresponding figures for the 14.6 nm reflection are 0.38 and 14.678 nm. In both cases, the spacing increase is simultaneous with the rapid rise of tension. Thereafter, throughout the duration of the slower ramp stretch, the spacing of the 14.6 nm reflection remains unchanged, whereas that of the 14.4 nm reflection appears to increase very slowly toward a value of 14.427 nm.

DISCUSSION

The results presented here reveal that in both isotonic and isometric contractions, as well as in length changes imposed at P_0, the 3M consists of two distinct reflections with different intensities and spacings. Furthermore, we find that although the intensity behavior of the two reflections is strikingly different during releases, it is very similar during stretches. The fact that the time course of the intensity changes in the 14.6 nm reflection is similar to that of the tension changes suggests that myosin heads diffracting with this periodicity are actively involved in tension production. This idea receives further support from our observation that after stimulation from rest the intensity of this reflection also increases in parallel with tension rise (data not shown here). On the other hand, the heads diffracting with the 14.4 nm periodicity appear not be associated with tension production during isometric contraction and releases. However, the intensity of the 14.4 nm reflection follows a similar time course to that of tension during the stretches, which suggests that the

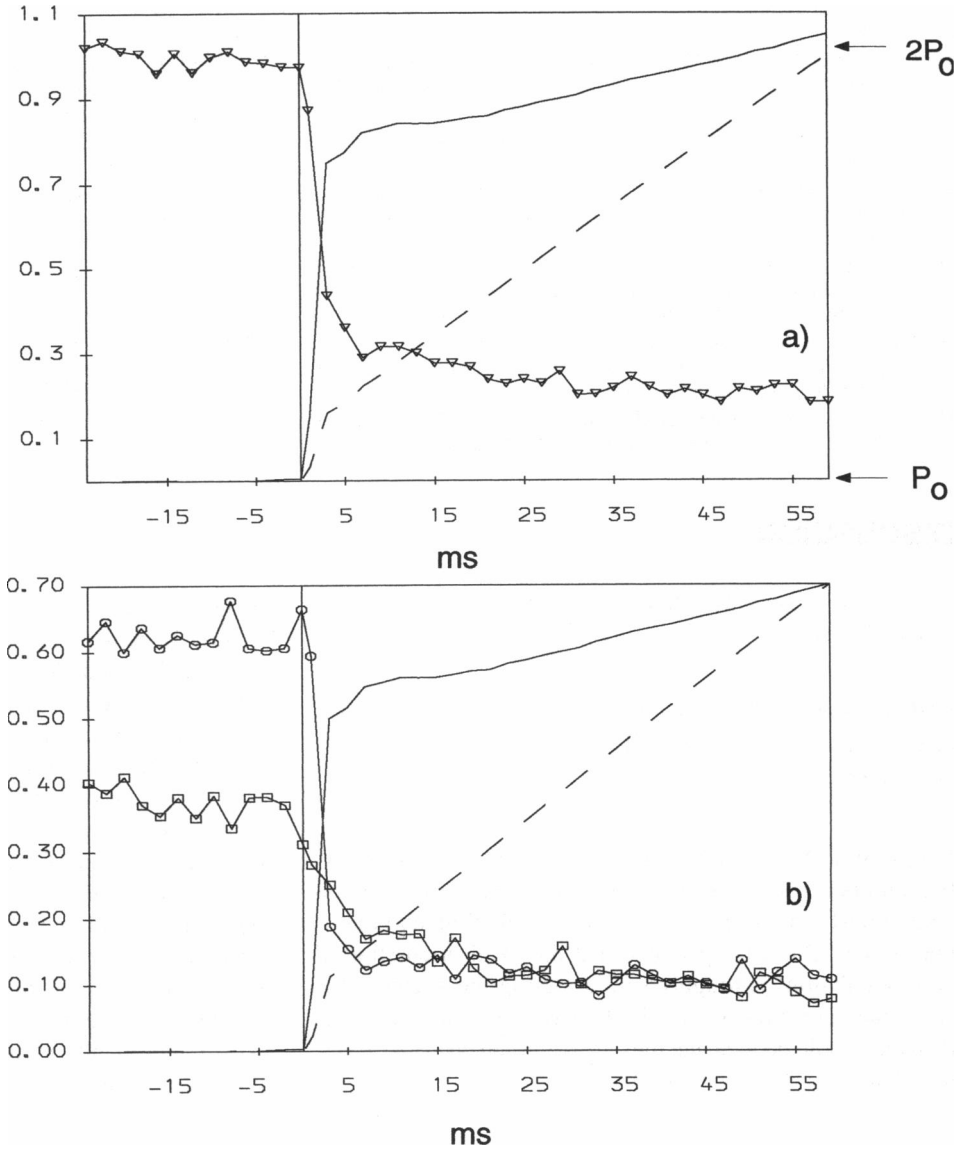

FIGURE 3 Muscles were stimulated isometrically from rest by applying a 600-ms-long train of pulses; they were stretched 400 ms after the start of stimulation when tension had reached P_o. The mechanical maneuvers involved an initial quick stretch that produced a ~1% change in the muscle length, followed by a ramp stretch adjusted so that tension gradually increased from ~$1.8P_o$ to ~$2.05P_o$. The total stretch amounted to ~6.5% change in the muscle length. Tension and length curves are shown by the continuous and dashed lines, respectively. The vertical line in all figures indicates the onset of the stretch. (*a*) Time course of the total integrated intensity in the 3M (—▽—) compared with tension and length. The intensity, tension, and length have been normalized to unity at P_o. The tension and length are displayed relative to a *y* axis running from a value of 1 to 2.1. Note that the time course of the intensity changes is similar to that of tension. (*b*) Contributions to the total intensity changes arising from those in the 14.4 and 14.6 nm reflections (□ and ○, respectively) compared with the time course of tension (——) and length (- - - - -). The tension and length changes have been scaled down for the purpose of comparison. The intensities of both reflections correspond to their relative contribution to the total integrated intensity normalized to unity at P_o.

heads diffracting with this periodicity are recruited to produce tension during stretches.

Our most important conclusion is that under all conditions of contraction we have investigated there exist two populations of myosin heads, each with a well defined axial disposition and configuration. From this it follows that it may be necessary to reassess the work of Huxley et al. (1983), Bordas et al. (1993), and Martin-Fernandez et al. (1994) with whole muscles, and of Irving at al. (1992) and Piazzesi et al. (this meeting) with single fibers, because all of the conclusions put forward by these investigators regarding the 3M were based on an interpretation of its behavior in terms of the activity of one myosin head population diffracting with one periodicity.

Traditionally, the interpretation of results from quick release and stretch experiments has been based on the assumption that all elasticity resides on the myosin heads (Huxley and Simmons, 1971). The amount of release per half-sarcomere required to discharge the elastic element in the heads has been estimated to be to ~4.0 nm (Ford et al., 1977). Our unpublished data show that in full muscle overlap ~0.3% extensibility occurs on the thin filament as monitored by the position of the 2.73 nm actin meridional reflection. This, together with the spacing changes of the 3M reflections reported here for releases and stretches, which probably reflect the combined extensibility of the thick and thin filaments, suggests that filament compliance accounts for a major part of the elasticity. If that were so, it would mean that although the heads might rotate in response to stretches and releases, they are significantly less elastic than has been believed hitherto. Clearly, this will have to be taken into account in any future attempts to reinterpret the significance of mechanical data that involve sudden length changes.

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DISCUSSION

Session Chairperson: Ivan Rayment
Scribe: David Lawson

RHEA LEVINE: Can you tell me if you have any indication of what percentage of the heads might be in each of the two populations?

JOAN BORDAS: Well, that's the problem. In order to get that number from the diffraction data, one has to know unambiguously the form factor of each population, and that is not known. One can speculate that the axial orientation of both populations is roughly similar during the stretches because they are subjected to similar strain. If that is the case, then one would deduce that they are occupied on a 50% basis. But that is the best one can do.

HUGH HUXLEY: The separation of the centers of the two halves of the A-band is just less than 0.9 μm, and the separation between the two peaks you've seen is a little over 1 μm. Those two are rather close together, and because of the shape of the peak and the way you sample it, I think you could get errors in that direction in the measured separation depending on how you measure the peak position. So I was wondering whether at least part of what you're seeing mightn't be interference between the two halves of the A-band. In that case, one would get extremely complicated effects when you took the tension off and the two halves came slightly closer together and at the same time some heads left actin and started diffracting at the shorter spacing.

BORDAS: There are a number of reasons why we do not believe this is the case. First of all, the distance between the two interfering units would have to be of the order of 10,000 Å to explain the presence of this splitting.

HUXLEY: 1 μm.

BORDAS: 1 μm, right. Now for this to happen, it would obviously be dependent on the sarcomere length, which we haven't observed. We also have noticed that other reflections which arise from these kind of interference effects vanish during contraction. This is because there is a certain amount of axial translation between filaments that makes them vanish. That argues against it also. Why would these particular reflections be preserved? The third reason why we have not considered an interference effect as an explanation is why would these reflections have such different time courses? And why would one of the time courses follow tension so accurately while the other one doesn't? In addition, we see similar effects in the high order reflections. For instance, the 72 shows the same effects. The one important difference, which is probably interesting, is that its total intensity does not change, but the two contributions behave the same way. Also, other higher order reflections appear to do the same thing. Putting all these things together, it would be very difficult to explain the observations on the basis of interference effects. One would expect, at least, to have different effects on different reflections.

HUXLEY: One of those I don't think would give any difficulty—the effective change of sarcomere length—because that won't effect the interference between the 2 halves of the given A-band.

BORDAS: If the interference is across the M-line, that's true. But isn't that a little bit too short?

HUXLEY: Well, that's the one that's 0.9 μm.

BORDAS: What we have measured is about 1000 Å longer than that. So it doesn't fit in.

HUXLEY: If you think of shape of the sampling as the curve you're sampling, you could get a small error in that direction.