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CROSS-BRIDGE MOVEMENT IN RAT CARDIAC MUSCLE AS A FUNCTION OF CALCIUM CONCENTRATION

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SUMMARY

1. By applying the X-ray diffraction method to chemically skinned papillary muscles of the rat, the transfer of myosin heads from the thick to the thin filaments was studied as a function of Ca^{2+} concentration.

2. No significant transfer of the heads occurred when the Ca^{2+} concentration was below the threshold of contraction (pCa 6·2).

3. During the maximum isometric contraction at pCa 4·4, 80% of the myosin heads were transferred to the thin filament.

4. When the muscle was activated isometrically at low Ca^{2+} concentrations (pCa 6·2–5·8), where the average tension was less than 20% of the maximum, a disproportionately large number of myosin heads were transferred to the thin filament.

5. It was concluded that a significant fraction of the heads transferred at the low Ca^{2+} concentrations does not produce tension.

INTRODUCTION

The contractile proteins of cardiac muscle are believed to work in essentially the same manner as those of skeletal muscle (Katz, 1970; Matsubara & Millman, 1974). Namely, the myosin heads interact with actin to produce force when the intracellular Ca^{2+} concentration is elevated. However, the details of such a cross-bridge mechanism differ between the two types of muscle. One important difference exists in the molecular events during relaxation. In skeletal muscle at physiological ionic strengths, most myosin heads are detached from actin during the resting state (Huxley, 1968; Thomas & Cooke, 1980). In contrast, in cyclically contracting cardiac muscle a significant number of myosin heads appear to remain attached to actin without producing force during the diastolic phase (Matsubara, Suga & Yagi, 1977; Matsubara, 1980). To clarify the cause of this difference, we have studied the

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behaviour of myosin heads in skinned cardiac muscle at various grades of activation using the X-ray diffraction method. The results suggest that the movement of myosin heads in skinned cardiac muscle as a function of Ca^{2+} concentration is, as in intact muscle, different from that reported for skinned skeletal muscle (Matsubara, Umazume & Yagi, 1985).

METHODS

Specimen technique

A 6- to 7-week-old Wistar rat weighing 180–200 g was killed by an overdose of sodium pentobarbitone (70 mg/kg). The heart was isolated and immersed in an oxygenated Tyrode solution (37 °C) of the following composition (mM): NaCl, 138·6; KCl, 2·68; $CaCl_2$, 1·8; $MgCl_2$, 0·49; glucose, 5·6; Tris HCl, 5·4 (pH 7·2). A papillary muscle of less than 0·3 mm diameter was removed from the right ventricle; the insertion of the muscle into the ventricular wall was ligated with thin silk thread and the wall muscle was cut at least 0·8 mm away from the knot. The isolated papillary muscle was washed for 2 min with a Ca^{2+}-free Tyrode solution, which had the same composition as above except for the absence of $CaCl_2$, to reduce extracellar Ca^{2+}. The muscle was then treated for 1 h at room temperature (18–21 °C) with saponin (250 μg/ml), dissolved in the relaxing solution (Table 1), to render the sarcolemma permeable to the solutes of the bathing medium.

The muscle, thus chemically skinned, was held isometrically in a specimen chamber by connecting one end to a force transducer (Aksjeselskapet Mikro-Elektronikk AE-801) and the other end to a hook mounted on a micrometer. The chamber had a capacity of 1·2 ml and was connected to a peristaltic pump (Ayela MP3) to perfuse bathing solutions (20 °C) at a rate of 4·2 ml/min. The chamber had Mylar windows to allow passage of light and X-rays through the middle of the muscle. The sarcomere length was adjusted to 2·1–2·2 μm using the laser diffraction method (Rome, 1967). The cross-sectional area of the muscle at this length was calculated from the thickness and width of the muscle in its middle.

Bathing solutions

The compositions of the bathing solutions are given in Table 1. All solutions except the rigor solution contained an ATP regenerating system consisting of phosphocreatine and creatine phosphokinase. All solutions had an ionic strength of 0·20 M.

The relaxing solution, which was used to put the muscle into the resting state, had a Mg-ATP concentration of 10 mM and a free Mg^{2+} concentration of 1 mM assuming affinity constants given by Taqui-Khan & Martell (1966). Before each activation, the muscle was bathed for 10 min in another relaxing solution which had a lower EGTA concentration. This was to facilitate the tension development on the subsequent application of an activating solution (Moisescu, 1976).

Different degrees of activation were obtained by using the solutions of different Ca^{2+} concentrations (pCa 6·4–4·4). All the activating solutions had a Mg-ATP concentration of 10 mM and a free Mg^{2+} concentration of 1 mM. To put the muscle into rigor a solution containing no ATP was used. This solution also had a free Mg^{2+} concentration of 1 mM.

Some experiments were carried out without the ATP-regenerating system. In such experiments the ionic strengths of relaxing and activating solutions were adjusted to 0·20 M by increasing potassium methanesulfonate (K(Ms)).

TABLE 1. Composition of relaxing and activating solutions

Solution	Composition K(Ms) (mM)	$Mg(Ms)_2$ (mM)	$Ca(Ms)_2$ (mM)	ATP (mM)	EGTA (mM)	CP (mM)	CPK (units/ml)
Relaxing	29·0	11·40	—	10·9	10	14	24
Low EGTA	58·0	11·05	—	10·9	0·5	14	24
pCa 6·4	24·3	11·30	2·42	10·9	10	14	24
pCa 6·2	22·5	11·30	3·37	10·9	10	14	24
pCa 6·0	20·4	11·20	4·46	10·9	10	14	24
pCa 5·8	18·2	11·20	5·61	10·9	10	14	24
pCa 5·6	16·1	11·10	6·70	10·9	10	14	24
pCa 5·4	14·3	11·10	7·63	10·9	10	14	24
pCa 5·2	12·8	11·10	8·37	10·9	10	14	24
pCa 5·0	11·7	11·00	8·93	10·9	10	14	24
pCa 4·4	9·8	11·00	9·83	10·9	10	14	24
Rigor	126·5	1·22	—	—	10	—	—

All solutions contained 20 mM-piperazine-*N*,*N*′-bis (2-ethanesulphonic acid) buffer (pH = 6·8 at 20 °C). (Ms): methanesulphonic acid; EGTA: ethyleneglycol-bis-(*β*-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CP: phosphocreatine disodium salt (Sigma); CPK: creatine phosphokinase (Sigma); pCa: negative logarithm of the free Ca^{2+} concentration. An apparent binding constant of $8{\cdot}4 \times 10^5$ M^{-1} for Ca-EGTA (Harafuji & Ogawa, 1980; their eqns 2 and 3) was used to calculate the free Ca^{2+} concentration.

X-ray diffraction

The equatorial X-ray patterns were recorded using a double-mirror focusing camera (Franks, 1955; Elliott & Worthington, 1963). The X-ray source was a rotating-anode generator (Rigaku FR) with a line focus (1 mm × 0·1 mm, viewed at an angle of 6 deg) on a copper target. This was operated at 50 kV with a tube current of 70 mA. The muscles were exposed at 20 °C for 5–10 min to record the diffraction patterns on X-ray film (Kodak) with a specimen-to-film distance of 22 cm. In some experiments a position-sensitive counter of the triangular-cathode type (Allemand & Thomas, 1974; Matsubara & Yagi, 1978) was used to record the diffraction pattern. The specimen-to-counter distance was 40 cm and the required exposure time was 0·5–1 min.

The positions and the integrated intensities of the 1,0 and 1,1 equatorial reflections from the hexagonal myofilament lattice were measured with a densitometer (Joyce–Loeble, Scandig 3). Using the intensity ratio of the two reflections, the electron-density distribution in the transverse section of the myofilament lattice was calculated by Fourier synthesis on the assumption that the phases of the two reflections are 0 deg. From the density distribution, the mass associated with the thin filaments, relative to that associated with the thick filaments, was estimated following the method described by Huxley (1968) and Haselgrove & Huxley (1973). From this, the fraction of myosin heads which were transferred to the vicinity of the thin filaments during contraction was calculated. The calculation was based on an assumption that all myosin heads are transferred to the thin filaments when a relaxed muscle is put into rigor (Huxley, 1968; Thomas & Cooke, 1980).

Experimental protocol

At the beginning of each experiment X-ray diffraction patterns were recorded from the resting skinned muscle in the relaxing solution and in the low-EGTA solution. The muscle was then exposed during contractions at two different Ca^{2+} concentrations; each diffraction pattern was recorded while the muscle was producing a steady active tension (Fig. 1). Between the two contractions the resting pattern was recorded in the relaxing solution. If the effect of the first contraction on the pattern was later found to be irreversible, the second active pattern was discarded. When the two contractions did not include the maximum contraction at pCa 4·4, the muscle was activated at this pCa for 2 min to provide the value to which the tension data were normalized. Then the muscle was put into rigor and the diffraction pattern was recorded while a steady rigor tension was produced. In some experiments the rigor pattern was recorded before the contractions.

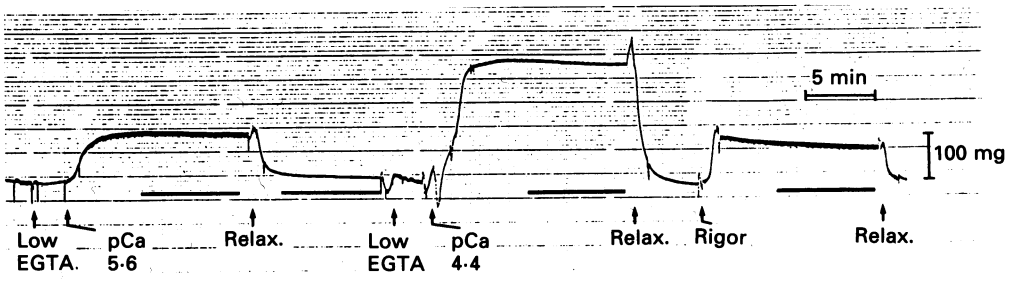

Fig. 1. A tension record showing a typical experimental protocol. A papillary muscle with a cross-sectional area of $4{\cdot}9 \times 10^{-4}$ cm^2 at its middle was used. The rapid oscillation which resulted in thickening of the tension record was caused mainly by the pulsatile flow of bathing solutions through the specimen chamber. The occasional slow oscillations of larger amplitudes were due to the passage of air bubbles through the chamber on exchange of solutions. The names of the perfusing solutions are given below the tension record. The horizontal bars represent the periods of X-ray exposure. Exposure of the intact preparation (before skinning) in the quiescent state in the Tyrode solution (20 °C) and exposure of the skinned preparation in the relaxing and the low-EGTA solutions preceded the recordings shown in this chart.

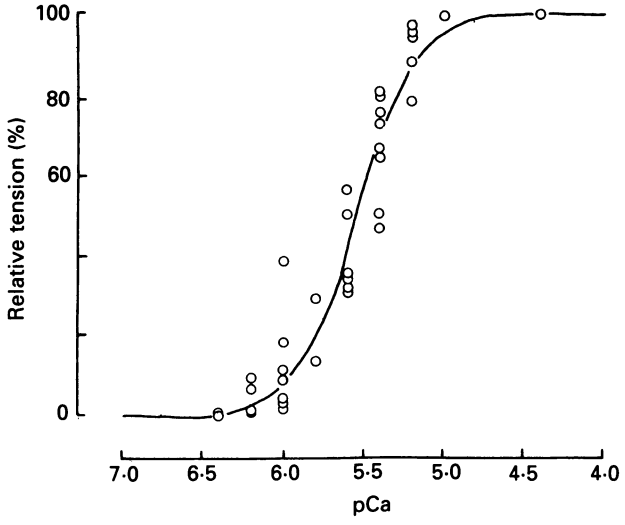

Fig. 2. Isometric tension plotted against pCa. The tension is expressed as a percentage of the maximum tension measured at pCa 4·4. The continuous line represents the Hill equation fitting the data: $\log\{R/(100-R)\} = 13{\cdot}3 - 2{\cdot}4 \times \mathrm{pCa}$, where R represents the relative tension (%).

RESULTS

Tension production

The isometric tension relative to the tension produced at pCa 4·4 is plotted against Ca^{2+} concentration in Fig. 2. The threshold concentration for tension production was around pCa 6·2, and the maximum tension was reached at pCa 5·0. The maximum

tension measured at pCa 4·4 was 5·15 ± 0·53 N cm^{-2} (mean ± S.E. of the mean, $n = 12$). The isometric contraction could be maintained for 10 min with little decrease in tension. The data in Fig. 2 could best be fitted by the Hill equation with a Hill constant of 2·4:

$$\log\{R/(100-R)\} = \log K - 2{\cdot}4 \times \mathrm{pCa},$$

where R represents the relative tension (%) and $\log K = 13{\cdot}3$.

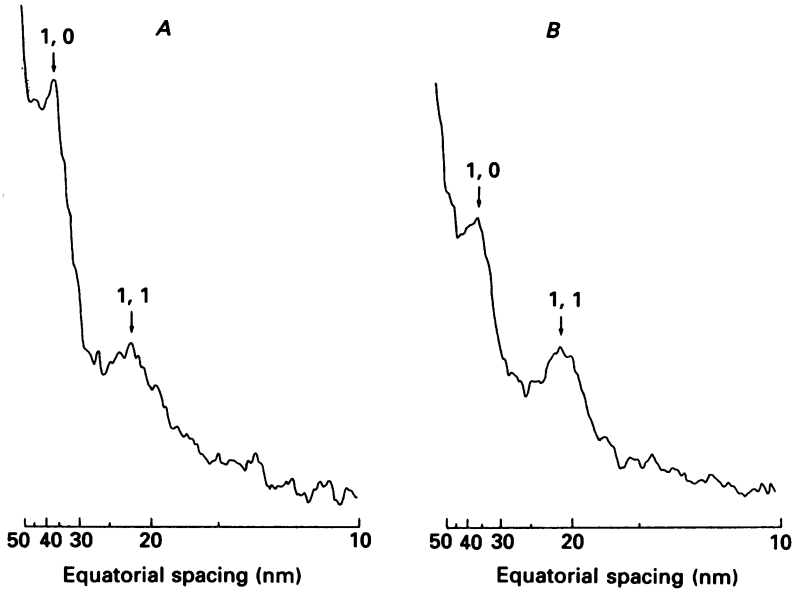

Fig. 3. The equatorial intensity distributions of X-rays scattered by a chemically skinned papillary muscle in the relaxing (*A*) and the maximally activating (*B*) solutions. Only the right halves of the distributions are shown. Two diffraction peaks, corresponding to the 1, 0 and the 1, 1 reflections from the myofilament lattice, are seen in each diagram. The horizontal scale represents the spacing in real space. Recorded with X-ray film.

The equatorial X-ray diffraction pattern

Figure 3*A* shows a typical diffraction pattern recorded in the relaxing solution. The 1,0 and the 1,1 reflections from the hexagonal myofilament lattice are clearly seen. The intensity ratio (I_{10}/I_{11}) was 2·24 ± 0·18 ($n = 10$), not significantly different ($P > 0{\cdot}05$) from the ratio for intact heart muscle in the quiescent state without cyclic contractions (2·36 ± 0·13, $n = 10$). When the muscle was contracted, the integrated intensity of the 1,0 reflection (I_{10}) decreased while that of the 1,1 reflection (I_{11}) increased (Fig. 3*B*), leading to a marked decrease in the intensity ratio. The diffraction pattern recorded with the position-sensitive counter gave approximately the same intensity ratio as that obtained with X-ray film both during relaxation and contraction.

The 1,0 spacing in the relaxing solution was 38·4 ± 0·3 nm ($n = 10$), 8% greater than that before skinning (35·6 ± 0·4 nm, $n = 10$); the change was statistically significant ($P < 0{\cdot}05$). When the skinned muscle was contracted at pCa 4·4, the

spacing was 37·6±0·3 nm ($n = 4$). Thus the spacing did not change appreciably ($P > 0·05$) on activation. When the skinned muscle was put into rigor, the spacing became 37·7±0·3 nm ($n = 10$); this was not significantly different ($P > 0·05$) from that in the relaxing solution.

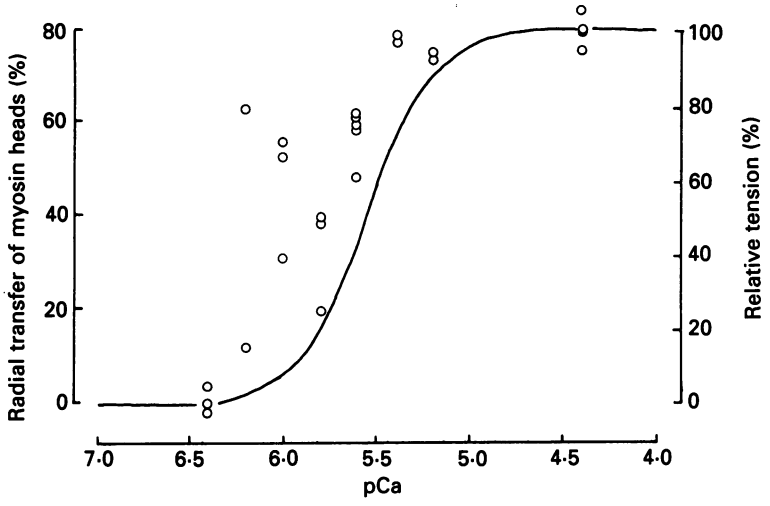

Fig. 4. The radial transfer of myosin heads plotted against pCa. Each circle represents a relative value to the radial transfer occurring in rigor; all myosin heads were assumed to be transferred in rigor (see Methods). The continuous line represents the Hill equation fitting the tension data in Fig. 1. The scale for the relative tension is given on the right.

The radial transfer of myosin heads as a function of calcium concentration

In Fig. 4 the fraction of myosin heads transferred radially to the vicinity of the thin filaments is plotted against pCa. The curve represents the Hill equation fitting the tension data of Fig. 2.

When Ca^{2+} concentration was below the threshold of contraction, there was no radial transfer. When the muscle was producing the maximum isometric tension at pCa 4·4, about 80 % of the myosin heads were associated with the thin filaments. This number was approximately the same as the value obtained previously (Matsubara, Yagi & Hashizume, 1975) for frog skeletal muscle during isometric tetanus (80–90 %).

An unexpectedly large number of myosin heads were transferred to the thin filaments at pCa values ranging from 6·2 to 5·8, where the average tension was less than 20 % of the maximum. The average percentage of heads transferred in this pCa range amounted to 39 %, almost half of that during the maximum contraction. Similar results were obtained in relaxing and activating solutions which did not contain the ATP-regenerating system.

The radial transfer of myosin heads as a function of isometric tension

The percentage of myosin heads transferred at various Ca^{2+} concentrations is plotted against the relative tension (Fig. 5). During contractions at low tension levels

(less than 20% of the maximum tension), a large fraction of myosin heads was transferred, making the relation strongly convex upward.

DISCUSSION

In a similar study using chemically skinned skeletal muscle of the mouse, Matsubara *et al.* (1985) observed that the mass transferred radially to the thin

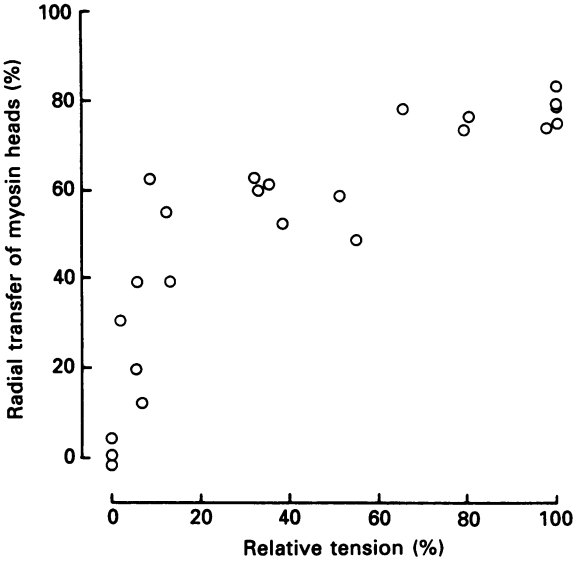

Fig. 5. The radial transfer of myosin heads plotted against the relative tension. The same data as shown in Fig. 4 were used.

filaments was roughly proportional to the relative tension. This suggested that nearly all, or a fixed fraction, of the myosin heads transferred were force producing. In contrast, the present result with a skinned papillary muscle shows a pronounced convex relation between radial transfer of mass and relative tension (Fig. 5). This suggests the possibility that in cardiac muscle at low intracellular free Ca^{2+} concentrations a significant number of myosin heads are attached to actin without producing tension. However, one cannot exclude the possibility that these heads are merely transferred to the vicinity of actin without attaching.

It is also possible that during the course of contraction the fibre may become depleted of ATP, thereby forming rigor links which would change the equatorial intensities. This, however, is unlikely to be the case for the following reasons: first, with 10 mM-Mg-ATP in the bathing solution, the equatorial intensity ratio during contraction in the absence of the ATP- regenerating system was similar to that in its presence. If rigor links had formed during contraction, more would have been evident in the absence of the ATP-regenerating system than in its presence. Secondly, the results obtained with the position-sensitive counter were similar to those obtained with X-ray film. The former required a contraction duration of only one-tenth of the

latter, so again, if rigor links had formed during contraction, more would have been evident with X-ray film. Since this was not the case, it is unlikely that rigor links formed or contributed significantly to the equatorial patterns during Ca^{2+} activation.

An alternative explanation is that internal shortening of sarcomeres during

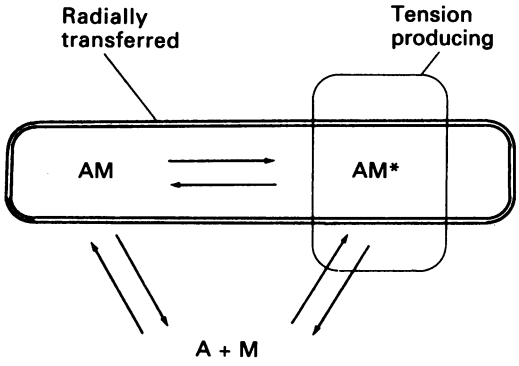

Fig. 6. A simplified model accounting for the convex relation between the radial mass transfer and the relative tension (Fig. 5). The symbols A and M represent an actin molecule and a myosin head respectively. The symbol A + M represents a fully dissociated (relaxed) state, while AM and AM* represent attached states.

contraction may have caused the discrepancy between radial transfer and tension in their responses to pCa (Fig. 4), and hence the curved relation between the two (Fig. 5). According to Krueger & Pollack (1975) the sarcomeres in the centre of muscle shorten by 7% during contraction at the optimum muscle length. Shortening of a similar magnitude was expected in the present experiments, and this might have reduced tension without affecting radial transfer, resulting in the discrepancy between the two. The following argument, however, makes such an explanation unlikely. During contraction at pCa 4·4, the internal shortening is supposed to be greatest (Allen & Kentish, 1985) so the reduction of tension should be maximal. When tensions at lower Ca^{2+} concentrations are normalized to the tension at pCa 4·4, the pCa–tension curve is expected to shift to the left, rather than to the right as is the case in Fig. 4. Thus it is unlikely that the observed discrepancy was due to internal shortening of sarcomeres. However, one cannot exclude the possibility that internal shortening reduces the Ca^{2+} sensitivity of the myofilaments, shifting the pCa–tension curve to the right (Hibberd & Jewell, 1982; Kentish, ter Keurs, Ricciardi, Bucx & Noble, 1986), without affecting the radial transfer of myosin heads.

Figure 6 represents one possible model that can account for the observed convex relation between the radial mass transfer and tension. The following were assumed. (1) There are two types of attached states, AM and AM*, and only AM* produces tension. However, both AM and AM* are counted as 'radially transferred' in our X-ray analysis. (2) During steady isometric contraction, an equilibrium is reached between the two attached states. (3) This equilibrium depends on Ca^{2+} concentration and shifts toward AM* at high concentrations.

According to this model, a skinned cardiac muscle responds to a gradual increase of Ca^{2+} concentration in the following manner. When the concentration is below the

threshold of contraction, all myosin heads are in the detached state. When the concentration exceeds the threshold, the equilibrium between detached and attached states shifts toward attachment, increasing the number of radially transferred heads. However, a significant fraction of the attached heads is in the AM state, so the muscle produces only a small tension. As the Ca^{2+} concentration increases further, the equilibrium between AM and AM* shifts toward AM*, increasing the tension. This leads to a convex relation between the radial mass transfer and tension, as observed. It should be noted that the model postulates two Ca^{2+}-dependent steps with different Ca^{2+} sensitivity.

By modifying the third assumption above, one can explain the linear relation reported for skeletal muscle (Matsubara *et al.* 1985). Namely, if most attached heads in skeletal muscle are in the AM* state at all Ca^{2+} concentrations above the threshold of contraction, then the relation between radial mass transfer and tension will be approximately linear.

Matsubara *et al.* (1977) found, in cyclically contracting heart muscle, that a significant fraction of myosin heads remains attached to actin during the diastolic phase without producing tension. Such attachments may correspond to AM in the above model. On the other hand, the intracellular Ca^{2+} concentration during the diastolic phase is known to be about 0·2 μM (Yue, Marban & Wier, 1986), at which no significant attachment was found (Fig. 4). However, these findings do not contradict each other, since the present observation on skinned heart muscle is concerned with a steady-state property of myosin heads rather than their dynamic property in cyclically contracting muscle. When the cyclic contractions are interrupted, myosin heads which remain attached after the last contraction are expected to dissociate from actin at a limited rate, reducing the radial transfer to a steady-state value close to zero. The present result that the equatorial intensity ratio for quiescent intact muscle agreed approximately with that for relaxed skinned muscle supports this view.

The AM* state in the model (Fig. 6) may correspond to the strongly bound state proposed by Eisenberg & Hill (1985) for skeletal muscle. The AM state, on the other hand, may correspond to the weakly bound state which is considered to be in rapid equilibrium with the dissociated (i.e. A + M) state. If this is the case, the AM state in cardiac muscle is unlikely to be reflected in muscle stiffness unless very rapid length changes are used in the stiffness measurements. Sinusoidal length oscillations of 50–100 Hz do not indicate the presence of the AM state in skinned heart muscle (Y. Saeki & I. Matsubara, unpublished observation); the stiffness is roughly proportional to active tension, in agreement with the previous report on glycerol-extracted heart muscle (Herzig & Rüegg, 1980). Stiffness measurements with much faster length changes would be required to detect the AM state, as is the case with skeletal muscle at low ionic strengths (Brenner, Schoenberg, Chalovich, Greene & Eisenberg, 1982).

In conclusion, a significant number of cardiac myosin heads appear to be attached to (or in the immediate vicinity of) actin without producing tension at low Ca^{2+} concentrations. In this respect the contractile proteins in cardiac muscle must differ from those in skeletal muscle.

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