RADIAL STIFFNESS OF FROG SKINNED MUSCLE FIBERS IN RELAXED AND RIGOR CONDITIONS

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ABSTRACT Radial stiffness in various conditions of mechanically skinned fibers of semitendinosus muscle of *Rana catesbeiana* was determined by compressing the fiber with polyvinylpyrrolidone (PVP K-30, $M_r = 40{,}000$) in incubating solution. The change in width (D) of fibers with increasing and decreasing PVP concentrations was highly reproducible at a range 0–6% PVP. Radial stiffness of relaxed fibers was almost independent of the sarcomere length. On the other hand, radial stiffness of rigor fibers showed a linear relation against the sarcomere length. These results indicate that cross-bridge attachment would be a major factor in the increase of the radial stiffness. Radial stiffness of relaxed and rigor fibers was $(2.14 \pm 0.52) \times 10^4$ N/m^2 (mean ± SD) and $(8.76 \pm 2.04) \times 10^4$ N/m^2, respectively, at the relative fiber width (D/D_0) of 0.92, where D_0 denotes the fiber width in the rigor solution at 0% PVP. Radial stiffness of a fiber in a rigor solution containing pyrophosphate (PPi) was between those of relaxed and rigor fibers, i.e., $(4.76 \pm 0.86) \times 10^4$ N/m^2 at D/D_0 of 0.92. In PPi and rigor solutions, radial stiffness reversibly increased to around 150 and 130%, respectively, in the presence of 10^{-6} M Ca^{2+}. To explain these results, especially the Ca^{2+}-induced change in the radial stiffness, some factor in addition to the number of attached cross-bridges has to be taken into account. The variation of radial stiffness under various conditions will be discussed in relation to the possible manner of cross-bridge attachment.

INTRODUCTION

Single skeletal muscle fibers swell markedly after removal of the sarcolemma in a relaxing solution (1). The swelling of the filament lattice was also observed in skinned crayfish fibers (2, 3), glycerinated rabbit psoas fibers (4, 5), and frog skinned fibers (6). The swelling is considered to be due to several factors, which include the elastic structure of sarcoplasm (M-lines, Z-bands, and "connectin") and various interaction energies between filaments (electrostatic repulsive energy, the van der Waals attractive energy, and hydration energy) (7). When long chain polymers in incubating solution are added, the skinned fiber shrinks in width (1) and in lattice spacing (8, 9). These phenomena indicate that skinned fibers act as an elastic body in the radial direction. Cross-bridge attachment might also contribute to the radial elasticity as well as a longitudinal one.

From the relation between the osmotic compressing force exhibited by long chain polymers and the diameter of the fiber, we can determine the radial stiffness that would reflect the number of attached bridges and the manner of attachment. We investigated the radial stiffness of relaxed and rigor skinned fibers including the effects of calcium ions.

METHODS

Fibers from semitendinosus muscle of frog (*Rana catesbeiana*) were mechanically skinned (10) in a relaxing solution. A segment of skinned fiber, 5 to 10 mm long, was mounted horizontally in a trough ($3 \times 3 \times 20$ mm^3) so as to change the length under an ordinary microscope with a 20× objective. In exchanging the bathing medium, the old solution was sucked up at one end of, and at the same time a new solution was poured at the other end of, the trough. Every time the solution was changed, we carefully washed the fiber by sufficient irrigation of a new solution. Before the experiment, the fiber was incubated for 15 min in relaxing solution containing 0.5% Brij-58. The following solutions were used (*a*) rigor solution: 100 mM KCl, 10 mM morpholinopropanesulfonic acid (MOPS), 4 mM EGTA, and 5 mM $MgCl_2$. (*b*) PPi solution: composition of rigor solution plus 4 mM K-pyrophosphate; (*c*) relaxing solution: composition of rigor solution plus 4 mM Na_2-ATP. In PPi and rigor solutions, we also used ($+Ca^{2+}$) solutions. Assuming the stability constant of Ca^{2+}-EGTA complex to be 5×10^6 M^{-1}, the Ca^{2+} concentration was calculated. Both ($+Ca^{2+}$) solutions contained 7.9×10^{-7} M free Ca^{2+}. The pH of all solutions was fixed at 7.0 at 20°C. Precipitate appeared in PPi solution 3 h after the preparation. For this reason, all experiments in PPi solution were performed within 2 h after the preparation. PVP solution, polyvinylpyrrolidone (PVP K-30, $M_r = 40{,}000$, Tokyo Kasei), was added to the above relaxing, PPi, and rigor solutions. The pressure due to added PVP was calculated by using the empirical equation of Vink (11); $\pi = 0.878\,C + 17.25\,C^2 + 144.1\,C^3$, where π is the osmotic pressure in atmospheres at 23°C and C is PVP concentration in grams per milliliters. Although the present experiments were performed at 19–21°C, the osmotic pressure was not corrected to this temperature range.

An ocular micrometer was used for measuring the sarcomere length and the fiber width. These dimensions could be determined within a precision of 0.5 μm. Before each experiment, we selected part of the fiber where the boundary between the fiber and solution could be clearly recognized at the same focal level. At three places in the field of view, total lengths of ten neighboring sarcomeres were measured. The sarcomere length was estimated by averaging these measured numbers. Because of the noncircular shape of the cross section of a fiber, the

apparent width will be affected by rolling and/or twisting of the fiber. By observing landmarks, the fiber rotation was always monitored. Except for the relaxed to rigor process, whenever it occurred, we stopped the experiment with this fiber. Throughout the experiment, we ignored a possible change in the cross-sectional shape.

RESULTS

Reproducibility of Changes in Fiber Width

We observed the fiber width on stepwise increasing and decreasing the osmotic pressure. The fiber width reached its apparent final value within 1 min after the change of the condition. So the width was measured 3 min after changing the solution. Below 6% PVP, the width changed in the same manner for both processes. Above 7% PVP, however, the width change showed hysteresis and did not return to the original value. For example, after incubating the fiber in 10% PVP solution, the width returned to 95% of the original value in 0% PVP. This tendency was observed in a similar manner in both relaxing and rigor conditions. For this reason, all the following experiments were performed below 6% PVP.

Variation of Fiber Width Under Various Conditions

We observed width-pressure relations of fibers at the sarcomere length L = 2.5–2.7 μm under various conditions. Fig. 1*A* summarized the width-pressure relations of the fibers in relaxing, PPi, and rigor solutions. In this plot, the fiber width (D) was normalized by the fiber width (D_0) in rigor solution at 0% PVP. Actually, at 0% PVP, no difference in the fiber width was observed in any solutions except for the relaxing one. Because of the movement of the fiber during the relaxed to rigor process and also during its reverse one, it was impossible to directly compare the fiber widths in relaxing and rigor solutions. The fiber width in the relaxing solution was plotted by assuming that the width (D_0) of the relaxed fiber was 105% of the rigor one. This assumption was derived from x-ray diffraction of mechanically skinned fibers (8); the plot in that paper showed that 1,0 spacing was 40 to 41 nm and 37 to 38 nm for relaxed and rigor fibers, respectively, at L = 2.5–2.7 μm.

Fig. 1*A* shows qualitatively that the radial stiffness, or the degree of resistance to the width change against the compressing force, increases in the following order: relaxing, PPi($-Ca^{2+}$), PPi($+Ca^{2+}$), rigor($-Ca^{2+}$), and rigor($+Ca^{2+}$) conditions. We show below that under PPi and rigor conditions, the increased stiffness of the fiber, as compared with the relaxed fiber, mainly comes from cross-bridge attachment. As possible factors (other than cross-bridges) that may increase the stiffness of the fiber with the addition of Ca^{2+}, we considered, for example, the elastic structure of sarcoplasm and/or the flexibility of thin filaments (12). We performed an experiment in the rigor solution using the fiber stretched to L = 5 μm. This

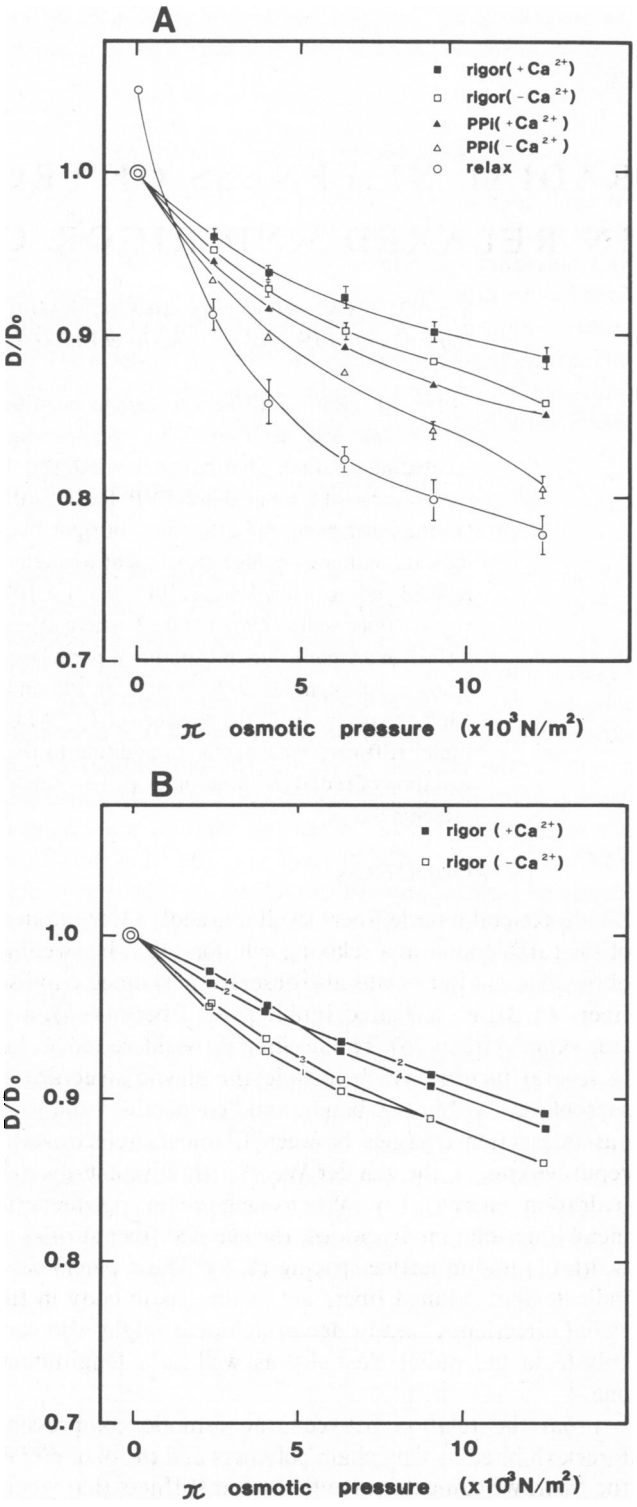

FIGURE 1 Relation between fiber width and osmotic pressure. D and D_0 denote the widths of the fiber at the osmotic pressure at π and zero, respectively. (*A*) Data obtained from fibers in various solutions. Points and vertical bars indicate the mean values and standard errors, respectively. Each solid line shows the least-squares fitted curve to Eq. 2, where mean values were used. (*B*) Data obtained from a fiber in rigor ($-Ca^{2+}$) and ($+Ca^{2+}$) solutions. The experimental points were connected simply by solid lines. Numbers attached to lines mean the order of the experiments.

overstretched fiber, however, showed no observable difference in stiffness between the presence and absence of Ca^{2+}.

Reversible Response to Calcium Ions

To confirm the reversibility of the effect of Ca^{2+} to the radial stiffness in rigor and PPi solutions, we examined the width-pressure relation in $(-Ca^{2+})$ and $(+Ca^{2+})$ solutions for the same fibers. Fig. 1 *B* shows an example of the results obtained for a fiber in rigor solution. The radial stiffness increased reversibly by 7.9×10^{-7} M Ca^{2+}. The reversible increase of the radial stiffness by Ca^{2+} was also observed for fibers in the PPi solution.

On Residual ATP in Fibers Under Rigor Conditions

We observed the reversible Ca^{2+} effect on the radial stiffness of the fibers in the rigor and PPi solutions. As far as we know, there is no report on the effect of Ca^{2+} on physical properties of the fibers or myofibrils in rigor solutions. So, we have to carefully examine whether our experiments were carried out for fibers in a true rigor state or not. For this purpose, we studied the longitudinal tension development in the rigor condition. Fig. 2 demonstrates one of the typical data. When the solution was changed from relaxing to $(-Ca^{2+})$ rigor one, the fiber developed tension in association with the release of substrate inhibition and then fell into a rigor state. After the application of a small decrease in the fiber length, no redevelopment of the tension was observed even after 6 min. Irrigation of the $(-Ca^{2+})$ rigor solution containing intentionally added 3 μM ATP caused instant but gradual redevelopment of tension. In six fibers, the threshold concentration of added ATP for the redevelopment of tension was in the range from 3 to 5 μM. The variation of the threshold concentrations would be due to the variation of the residual amount of ATP at a $(-Ca^{2+})$ rigor condition. In addition, the fibers in a $(-Ca^{2+})$ rigor solution did not develop tension on replacement of the solution with a $(+Ca^{2+})$ rigor one.

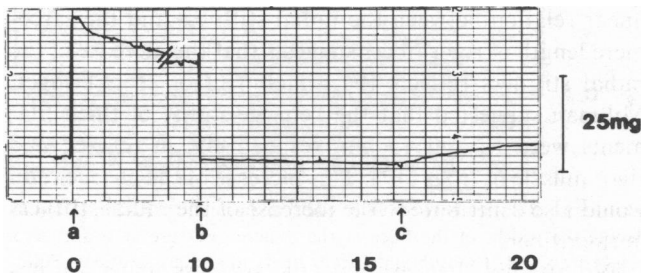

FIGURE 2 Tension development in rigor solution. Numbers indicate the time (minutes) after changing the solution from relaxing to rigor one. The solution was changed at point *a* from relaxing to rigor one, the fiber was released at point *b* to 98% of the original length, and 3 μM ATP was added at point *c* (see text).

These results indicate that the fiber was in a true rigor state.

Radial Stiffness

For quantitative comparison, we attempted to calculate the radial stiffness. The elastic property of the fiber can be expressed in terms of the radial stiffness, K, which is defined by

$$K = \left(\frac{1}{D} \frac{dD}{d\pi} \right)^{-1}, \quad (1)$$

where D is the width of the fiber as a function of pressure π. Following Maughan and Godt (13), we first fitted the data in Fig. 1 with their empirical expression $D/D^* = b \times \ln(\pi/\pi^*) + 1$, where D^* is the fiber width in 4% PVP solution, π^* is the pressure exerted by 4% PVP, and b is a constant. The data in the relaxing solution fitted the above empirical equation. The data in the rigor solution, however, did not fit it. So, the relative fiber width, D/D_0, against the osmotic pressure (of all the present data) was least-squares fitted to a polynomial expression

$$D/D_0 = k_0 + k_1\pi + k_2\pi^2 + k_3\pi^3 + k_4\pi^4, \quad (2)$$

where k_i's are constants independent of π (solid lines in Fig. 1 *A*). Then Eq. 1 is rewritten as

$$K = \frac{D/D_0}{k_1 + 2k_2\pi + 3k_3\pi^2 + 4k_4\pi^3}. \quad (3)$$

Throughout this paper, the term "radial stiffness" is used as a synonym of "bulk modulus" defined by Maughan and Godt (13). Because of no observable change in the sarcomere length at varied pressure, radial stiffness, $(dD/D d\pi)^{-1}$, is different from the bulk modulus only by the factor of two. The relation between the fiber width and radial stiffness at $L = 2.5$–2.7 μm was computed. Radial stiffness at various fiber widths is quantitatively summarized in Table I.

Variation of Radial Stiffness with Sarcomere Length

If cross-bridge attachment contributed largely to the stiffness, the radial stiffness would depend strongly on the sarcomere length. The radial stiffness at a particular osmotic pressure was plotted against the sarcomere lengths from 2.00 to 4.05 μm (Fig. 3). The radial stiffness, K at $\pi = 3 \times 10^3$ N/m^2, of rigor fibers appeared to be smaller at longer sarcomere lengths. The least-squares regression line in Fig. 3 shows $K = -3.17\ L + 15.9$ in units of K in 10^4 N/m^2 and L in microns, with the correlation coefficient $r = 0.87$. Similar linear relations were also obtained at $\pi = 1$, 2, 4, and 5×10^4 N/m^2 with $r = 0.85$, 0.81, 0.87, and 0.89, respectively. On the other hand, the radial stiffness of relaxed fibers was almost independent of sarcomere lengths. These results suggested that the cross-bridge

TABLE I
VARIATION OF RADIAL STIFFNESS (MEAN ± SD) WITH RELATIVE FIBER WIDTH (D/D_0) UNDER VARIOUS CONDITIONS (SARCOMERE LENGTH 2.5–2.7 μm)

D/D_0	Relax 5§	PPi($-Ca^{2+}$) 12§	PPi($+Ca^{2+}$) 6§	Rigor($-Ca^{2+}$) 12§	Rigor($+Ca^{2+}$) 6§
—	× 10^4N/m^2	× 10^4N/m^2	× 10^4N/m^2	× 10^4N/m^2	× 10^4N/m^2
1.00	1.58 ± 0.48	2.48 ± 0.28	*3.04 ± 0.66	3.90 ± 1.46	4.82 ± 1.88
0.96	1.78 ± 0.68	3.16 ± 0.22	*4.18 ± 0.66	5.36 ± 1.54	*7.08 ± 2.46
0.92	2.14 ± 0.52	4.76 ± 0.86	*7.22 ± 1.74	8.76 ± 2.04	*11.66 ± 3.56
0.88	3.02 ± 0.80	7.04 ± 1.82	*11.78 ± 4.56	11.18 ± 3.56	‡

Mean and SD were obtained from computed values of K based on Eq. 3 in the text, where the coefficient k_i's for individual fibers had been determined by the least-squares fitting to Eq. 2.
*Increase of radial stiffness by Ca^{2+} is statistically significant.
‡Fibers could not be compressed to $D/D_0 = 0.88$ up to 6% PVP.
§Numbers of fibers measured.

attachment would be a major factor contributing to the excess stiffness in all other conditions over that in the relaxing one.

When the solution was changed from relaxing to rigor one, the movement of the fiber and disorder of the striation pattern were observed. This tendency was much clearer at shorter sarcomere lengths than longer ones. Above $L = 3.6$ μm, say 3.9 μm, where no overlap between thick and thin filaments may be expected, the movement of the fibers was still observed on reducing ATP. Although the fiber at $L = 3.9$ μm in the relaxing solution showed clear striation (and optical diffraction) patterns over its whole length, slight overlap between both filaments would exist in some of the sarcomeres. To avoid this problem, we observed width-pressure relation for fibers at $L = 5$ μm in both relaxing and rigor solutions in which the fibers showed clear striation patterns. At this sarcomere length, no movement was observed on reducing ATP and the width-pressure relation showed no observable difference between relaxing and rigor solutions.

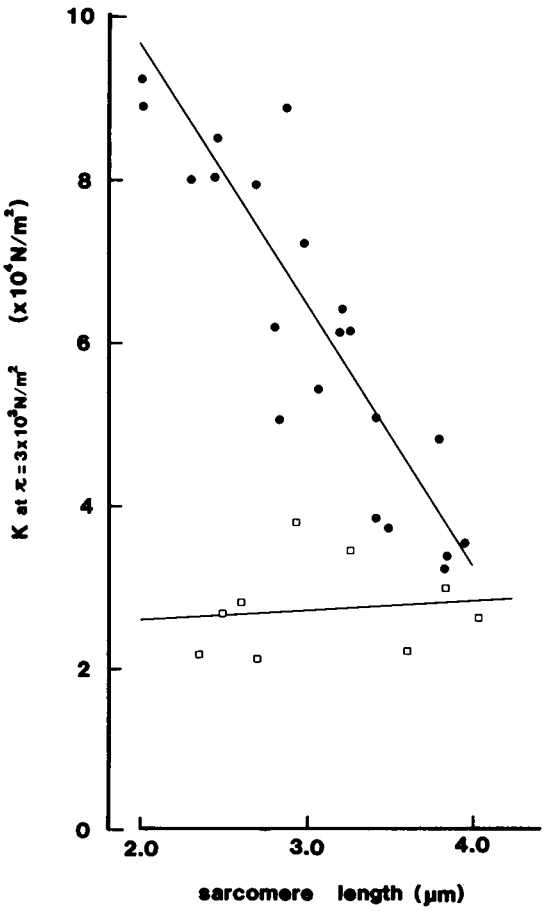

FIGURE 3 Relation between radial stiffness at the osmotic pressure of 3×10^3N/m^2 and sarcomere lengths. ●, fibers in rigor solution and □, fibers in relaxing solution. Solid lines show the least-squares linear regression lines. The relatively large scatter of experimental points probably came from the fact that one fiber gave one point in this plot.

DISCUSSION

The radial stiffness of rigor fibers strongly depended on the sarcomere length, i.e., the stiffness was less at longer sarcomere lengths. This is analogous to a linear relation between tetanic tension and the sarcomere length in living muscle (14). Huxley (15) interpreted his own x-ray diffraction results and the electron microscopic studies of Reedy (16) as indicating that, in rigor, myosin heads permanently attach to the thin filaments. The present linear relation between the radial stiffness and the sarcomere length of rigor fibers suggests that the increase of the radial stiffness reflects the attachment of cross-bridges. Millman suggested that the "charge radii" of thick filaments were 12 and 15 nm, respectively, in relaxed and rigor muscle of frogs (17). This increase of the charge radii would also contribute to the increase of the radial stiffness in rigor fibers.

We attempted to examine the stiffness under various conditions that would affect the cross-bridge attachment. The radial stiffness of rigor fibers at $L = 2.5$–2.7 μm was two- to four-times greater than that of relaxed fibers (Table I). Maughan and Godt also observed the increase of the radial stiffness in rigor fibers and indicated that this

increase is much smaller than the increase in longitudinal stiffness, which is some two orders of magnitude greater than that of relaxed fibers (18).

In the present study, the osmotic pressure was calculated by assuming that no PVP entered the inside of the fiber. There is, however, a possibility that a small amount of PVP will enter the inside of the fiber (I. Matsubara, personal communication). If the entry exists, our present values of the radial stiffness should be regarded as the upper bound. Moreover, there is a possibility that the amount of entered PVP is greater in the relaxed fiber than in the rigor one because of a looser packing of the filament lattice of the former. If so, the ratio of the radial stiffness of rigor to relaxed fibers was underestimated.

Pyrophosphate (PPi) is often classed as a muscle relaxant or ATP analogue. The results presented here showed that the effect of PPi upon the radial stiffness of skinned fibers was different from that of ATP. The radial stiffness of the fiber in PPi solution was between that in relaxed and rigor ones. White investigated the effect of PPi on glycerinated flight muscle from giant waterbugs (19). He observed that (*a*) PPi caused a reduction of the tension of muscles that had developed rigor in an isometric condition, (*b*) the longitudinal stiffness, when measured at frequencies between 1 and 100 Hz, remained indistinguishable from that of rigor muscle, and (*c*) the longitudinal stiffness when measured by applying slow length changes was similar to that of relaxed fiber or intermediate between those of rigor and relaxed fibers (Fig. 11 in reference 19). The effect of PPi on the radial stiffness presented here would be static and correspond to the third observation of White. The present observation on the effect of PPi is also consistent with the results of the study of the rotational motion of cross-bridges in myofibrils by a saturation transfer EPR method (20, 21). These papers reported that in the presence of 5 mM PPi, the saturation transfer EPR spectra were intermediate between those of relaxed and rigor myofibrils. Furthermore, they described that the spectra were sensitive to Ca^{2+} in a micromolar range and interpreted by assuming a mixture of cross-bridges whose motion was determined by whether they were attached or detached. The spectra were consistent with an increase in the fraction of attached cross-bridges with increasing Ca^{2+} concentrations. The present result showed that, in the presence of PPi, the radial stiffness is significantly greater in the presence of 10^{-6} M Ca^{2+} than in its absence. This Ca^{2+} effect would reflect partly the increase of the number of the attached cross-bridges and/or partly the changes of the manner of cross-bridge attachment.

Kawai and Brandt investigated the rigor stiffness in two states of rigor fibers and showed that those rigor states were insensitive to Ca^{2+} (22). Saturation transfer EPR spectra were also insensitive to Ca^{2+} in rigor myofibrils (21). From these studies, we would not have expected the present significant increase of radial stiffness by Ca^{2+} in the rigor condition. A related description to the present Ca^{2+} effect appeared in the study of the longitudinal rigor stiffness of frog whole skeletal muscle poisoned with iodoacetic acid (23); it was observed that withdrawing Ca^{2+} from Ca-rigor muscle (the muscle poisoned in the presence of Ca^{2+}) reduced slow and fast rigor resistances and readdition of Ca^{2+} reversed the effect. Because of the whole muscle system, there is a possibility that the Ca^{2+} effect was observed in the presence of a residual amount of ATP (see Addendum in reference 22). In our present condition, ATP was sufficiently reduced from the filament lattice space and the fiber was in a true rigor state.

Only the number of attached cross-bridges can account for changes in the longitudinal stiffness (18, 19, 22) and EPR spectra (20, 21) under various conditions so far studied. Probably due to this fact, no Ca^{2+}-induced changes were observed for both longitudinal stiffness (22) and EPR spectra (20) in the rigor solution. On the other hand, the Ca^{2+}-induced changes were observed for the radial stiffness in both rigor and PPi solutions. To explain our results, especially in the Ca^{2+}-effect in the rigor solution, we have to consider some factor in addition to the number of attached cross-bridges.

Haselgrove has shown that Ca^{2+} affects the position of tropomyosin in the grooves of the thin filament (24). Most at present suggests that in rigor, tropomyosin is already in the "Ca-activated" position and does not change its position depending upon the presence or absence of Ca^{2+}. However, we could expect a possibility that the position on actin surface of, and/or the manner of, cross-bridge attachment will change associated with the movement of tropomyosin. The cross-bridge in rigor would respond reversibly to Ca^{2+}, directly and/or indirectly, in a manner to increase the radial stiffness but not the longitudinal one.

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