Thin Filament Cooperativity as a Major Determinant of Shortening Velocity in Skeletal Muscle Fibers

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ABSTRACT The mechanism underlying the calcium sensitivity of the velocity of shortening of skeletal muscle fibers was investigated using a multiple shortening protocol: within a single contraction, skinned rabbit psoas fibers were made to shorten repetitively under a light load by briefly stretching back to their initial length at regular intervals. At saturating [Ca^{2+}], the initial fast shortening pattern was repeated reproducibly. At submaximal [Ca^{2+}], the first shortening consisted of fast and slow phases, but only the slow phase was observed in later shortenings. When the fibers were held isometric after the first shortening, the velocity of the second shortening recovered with time. The recovery paralleled tension redevelopment, implying a close relationship between the velocity and the number of the preexisting force-producing cross-bridges. However, this parallelism was lost as [Ca^{2+}] was increased. Thus, the velocity was modified in a manner consistent with the cooperative thin filament activation by strong binding cross-bridges and its modulation by calcium. The present results therefore provide evidence that the thin filament cooperativity is primarily responsible for the calcium sensitivity of velocity. The effect of inorganic phosphate to accelerate the slow phase of shortening is also explained in terms of the cooperative activation.

INTRODUCTION

Contraction and relaxation of muscle is regulated by intracellular [Ca^{2+}]. In skeletal muscle, this regulation is achieved through the association of calcium to troponin C, a subunit of a regulatory protein troponin on the thin filament (e.g., Ebashi and Endo, 1968). Binding of calcium to troponin C is believed to relieve the inhibitory action of troponin I, thereby initiating the interaction between myosin and actin.

Two hypotheses have been proposed to account for calcium activation of the contractile machinery of skeletal muscle fibers: one assumes that calcium activates the fibers in an all-or-none fashion, i.e., calcium increases the population of fully activated myosin heads (cross-bridges) without changing their kinetic properties; the other assumes that calcium also affects the kinetics of the attached cross-bridges. These hypotheses were tested by measuring the unloaded velocity of shortening (V_{us}), which is generally considered to be independent of the number of attached cross-bridges and thus directly reflect their kinetics. Testing of these hypotheses led to a major controversy when Podolsky and Teichholtz (1970) reported that V_{us} is not affected by [Ca^{2+}], while Julian (1971) observed a substantial decrease of V_{us} at lowered [Ca^{2+}].

Although the conclusions from later studies are equally divided (reviewed by Moss, 1992; Podolin and Ford, 1983), observations seem to concur in that the velocity of shortening does depend on [Ca^{2+}]. Nevertheless, the dependence of the velocity on [Ca^{2+}] is not simple. When an isometrically contracting muscle fiber is subjected to unloaded or lightly loaded shortening, the velocity of the initial part of shortening is reduced only modestly as [Ca^{2+}] is lowered. In the later part of shortening, however, the calcium sensitivity becomes much more pronounced (Brenner, 1980; Farrow et al., 1988). The transition from the faster, relatively calcium-insensitive part of shortening to the slower, more calcium-sensitive part is often discrete (Martyn et al., 1994; Moss, 1986), giving rise to a characteristic biphasic shortening pattern in a slack-test plot.

The present paper focuses on the time-dependent increase of calcium sensitivity. Previously suggested explanations for this time dependence include a passive internal load (Brenner, 1980; Gulati and Babu, 1985; Wise et al., 1971) and the decreased rate of cross-bridge detachment from actin (Moss, 1986; Martyn et al. 1994; Metzger, 1996). However, the actual mechanism for the time-dependence may be more complex, since recent studies show that the calcium sensitivity of shortening is affected by various factors, including partial extraction of C-protein (Hofmann et al., 1991a), extraction of troponin C (Moss, 1986) and addition of inorganic phosphate (P_i) or ADP (Metzger, 1996). The effect of P_i is notable since at submaximal [Ca^{2+}] it reduces the time dependence of calcium sensitivity by accelerating the later phase of shortening.

Another complicating factor that should be taken into consideration is the cooperative activation of the thin filament by strong binding cross-bridges. It is known that calcium alone is not sufficient to turn on a seven-actin unit (seven consecutive actin monomers in contact with a tropomyosin molecule) of the thin filament, but the binding of strong binding myosin heads is also needed (reviewed by Lehrer, 1994). The cooperative activation is ascribed to the

Received for publication 22 May 1997 and in final form 19 November 1997.

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0006-3495/98/03/1452/13 $2.00

tendency that actin monomers in a seven-actin unit are turned on in concert (Bremel and Weber, 1982), and the tendency that a seven-actin unit in the "on" state turns on the neighboring seven-actin units (Hill et al., 1980). The cooperativity is more pronounced in the absence of Ca^{2+} than in the presence of saturating Ca^{2+} (e.g., Greene and Eisenberg, 1980). Since active shortening affects the number of strong binding cross-bridges (e.g., Ford et al., 1985; Julian and Sollins, 1975), it would be natural to expect that the cooperative activation of the thin filament plays some role in determining the velocity of shortening at submaximal [Ca^{2+}]. However, its role in the time-dependent calcium sensitivity has not been thoroughly investigated.

The present paper shows evidence that the time-dependent increase of calcium sensitivity is a direct manifestation of the cooperative activation of the thin filament. Although the present study does not exclude the possibility of calcium-dependent modulation of cross-bridge kinetics, the time dependence of calcium sensitivity can be explained without taking it into consideration. The effect of P_i to reduce the time dependence is also understood on the basis of the thin filament cooperativity. A brief account of a part of the present study has appeared elsewhere (Iwamoto and Wakabayashi, 1996).

MATERIALS AND METHODS

Preparation

Bundles (diameter, ~2 mm) of fibers were excised from rabbit psoas muscle, skinned in a relaxing solution containing 0.5% (w/v) Triton X-100, and stored in the 50% mixture of glycerol and relaxing solution. Single fibers or small bundle of fibers (containing 2–3 fibers) were isolated and mounted to the experimental chamber with aluminum T-clips at both ends, as described previously (Iwamoto, 1995a).

Solutions

Bathing solutions used were based on those described previously (Iwamoto, 1995a,b): The composition of the solution was (in mM): potassium propionate, 80; EGTA, 10; $MgCl_2$, 5; Na_2ATP, 4; phosphocreatine, 20; creatine phosphokinase, 125 units/ml; Dextran T-500, 4% (w/v); imidazole, 20 (pH = 7.2; ionic strength ~200 mM). When 20 mM inorganic phosphate (P_i) was added, the concentration of potassium propionate was reduced to 40 mM to keep the ionic strength constant. In the preactivating solution, the EGTA concentration was reduced to 0.1 mM. The contracting solution contained up to 10.1 mM $CaCl_2$ in addition to the components in the relaxing solution. Experiments were done at 3–5°C.

Apparatus and procedure for mechanical measurements

The length of the specimens was controlled by a servo-motor (G-120D, General Scanning Inc., Watertown, MA), the tension was recorded with a semiconductor strain gauge (AE801, SensoNor, Horten, Norway), and the sarcomere length was monitored by He-Ne laser beam diffraction by a position sensitive photodiode (Iwamoto, 1995a). The fibers were activated and relaxed as described earlier (Iwamoto, 1995a). The solution exchange was achieved by exchanging solution chambers made of anodized aluminum blocs.

For the measurement of the velocity of shortening, a digital isotonic controller (Iwamoto, 1995b) was used. This controller consisted of a quartz clock-operated 12-bit digital up/down counter connected to a digital-to-analog converter whose output was fed to the length-control input of the servo-motor amplifier. Whether the counter counted up or down was determined by the sign of the output of a difference comparator, which compared the tension and the command signals.

The multiple shortening protocol was similar to that used by Stehle et al. (1994). When the isometric tension reached a plateau, the isotonic controller was set in the tension-control mode, e.g., for 494 ms, during which reset pulses (duration, 2 ms) were input at a regular intervals, e.g., every 62 ms. These pulses reset the counter so that the fibers were suddenly returned to their initial length. Thus, isotonic releases were repeated eight times in a single contraction, and the period of each shortening was 60 ms.

The redevelopment of tension after shortening was fitted to a double exponential association by using the Prizm software package (GraphPad Software Inc., San Diego, CA). Fiber stiffness was measured by applying 500 Hz sinusoidal oscillation (amplitude, 0.1–0.2% of fiber length). The tension signal was band-pass filtered and rectified to obtain the stiffness signal.

RESULTS

Force-pCa relationship

Before measurement of shortening velocities the relation between isometric tension and [Ca^{2+}] (force-pCa relation) was determined. Fig. 1 shows a Hill plot in which the isometric tension is plotted as a function of pCa (= $-\log[Ca^{2+}]$). The values of pCa_{50} and Hill coefficient were 6.22 and 3.17, respectively. Isometric tension started to rise at pCa ~ 6.7 and nearly saturated at pCa ~ 5.5.

Dependence of the velocity of shortening on [Ca^{2+}] in the multiple shortening protocol

First the muscle fibers were made to shorten eight times under a light load (<0.1 × isometric tension P_o, mostly around 0.05 P_o). The duration of each shortening was fixed at 60 ms, and the duration of the reset pulse (during which the fibers were stretched back to and held at their initial length) was 2 ms.

Fig. 2, *A* and *C* show a typical record of overall fiber length (output of the position sensor built into the servo-motor), sarcomere length, and tension during such multiple shortening experiments at saturating [Ca^{2+}] (pCa = 4.49). Although the number of shortenings was limited to eight, repeating more shortenings did not damage the fibers (not

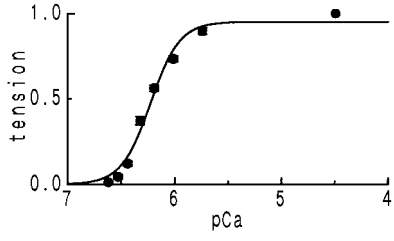

FIGURE 1 Relation between [Ca^{2+}] and isometric tension of skinned rabbit skeletal muscle fibers, fitted to Hill equation. Data are expressed as mean ± S.D. (n = 6).

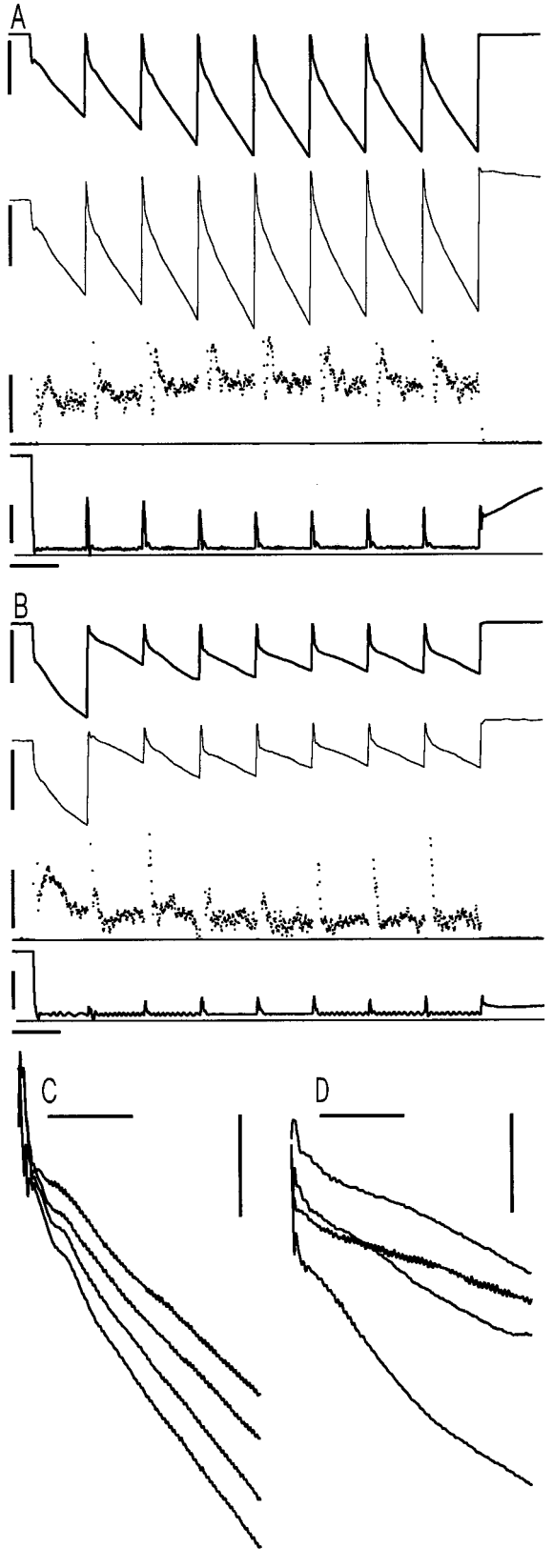

FIGURE 2 Overall fiber length, sarcomere length, velocity of shortening, and tension (from above) during eight consecutive shortenings under a finite load of $<0.1\ P_o$, at saturating and submaximal $[Ca^{2+}]$. The velocity is the derivative of the overall fiber length. Each shortening was 60 ms in duration and followed by a 2-ms period of restretch to the initial fiber length. (*A*) pCa = 4.49. (*B*) pCa = 6.19. The vertical scale bars indicate 2% L_o, 50 nm, 50% L_o/s and 0.2 mN from above. The horizontal scale bar indicates 50 ms. Records in (*A*) and (*B*) are from different specimens. (*C*) and (*D*), magnified view of the overall fiber length in (*A*) and (*B*), respectively. At the right end of the records, the traces represent the 1st, 2nd, 3rd, and 5th shortenings from above in (*C*) and the 2nd, 5th, 3rd, and 1st shortenings from above in (*D*). The vertical and horizontal bars indicate 1% L_o and 20 ms, respectively.

shown). The velocity of shortening, measured in the period between 10 and 30 ms after the start of each shortening, gradually increased to up to ~140% of the initial value in the first three or four shortenings, and was then decreased slightly (see Fig. 3). The mechanism for this acceleration will be discussed later, and we will tentatively call this stretch-induced augmentation of contractility "superactivation."

Fig. 2, *B* and *D* show a record of the same experiment done at submaximal $[Ca^{2+}]$ (pCa = 6.19). In the first of eight shortenings the time course of shortening was biphasic, as has been reported, i.e., an initial fast phase of shortening was followed by a slower phase. The fast phase lasted for ~30 ms from the onset of shortening. Conspicuous were the velocities of the second shortening and later: the fast phase seen in the first shortening was completely absent or very brief if it existed. Since a large and rapid restretch to the fiber's initial length would forcibly detach the existing force-producing cross-bridges, it is likely that the velocities of the second shortening and later reflect the properties of the force-producing cross-bridges newly formed after the preceding stretch. If the state of the fibers was unchanged throughout a contraction at submaximal $[Ca^{2+}]$, the combination of the fast and slow phases should be observed in every shortening. Therefore, the observation is more readily explained if the slow phase of shortening is a consequence of some alteration induced by the first shortening (which is believed to be the thin filament deactivation, as will be discussed later), and the alteration persists for the rest of the period of multiple shortenings.

The velocity of shortening, measured in the period between 10 and 30 ms after the start of each shortening, showed a great drop between the first and the second shortenings. In the later shortenings, the velocity tended to recover slightly, and was stabilized at the sixth shortening and later (Fig. 3). Thus, the mechanism of the "superactivation" seen at saturating $[Ca^{2+}]$ also seems to operate at submaximal $[Ca^{2+}]$.

The loss of the fast phase at submaximal $[Ca^{2+}]$ is not due to the smaller amount of restretch compared with that at saturating $[Ca^{2+}]$; when the period of later shortenings was prolonged to 120 or 180 ms to ensure a sufficient amount of restretch, the fast phase of shortening was still missing (not shown).

The velocities of the eight consecutive shortenings at various $[Ca^{2+}]$ are summarized in Fig. 3. The velocity of the first shortening was not strongly dependent on $[Ca^{2+}]$ unless $[Ca^{2+}]$ was very low. The velocities of the second

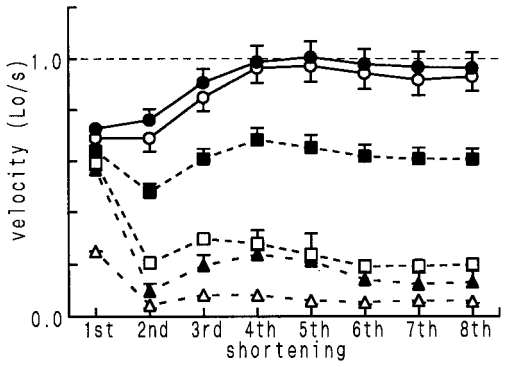

FIGURE 3 Summary of the velocities of eight consecutive shortenings under a load of $<0.1\ P_o$ as shown in Fig. 2, measured at various $[Ca^{2+}]$. The velocity was measured in the period between 10 and 30 ms after the start of each shortening. Data are expressed as mean ± S.D. ($n = 11$). Filled circles, pCa = 4.49; open circles, pCa = 5.74; filled squares, pCa = 6.01; open squares, pCa = 6.19; filled triangles, pCa = 6.32; open triangles, pCa = 6.44.

shortening and later were strongly dependent on $[Ca^{2+}]$. This result is another demonstration of the time-dependent increase of calcium sensitivity of the velocity of shortening at submaximal $[Ca^{2+}]$ (see Introduction). Since the velocity is stabilized after a few shortenings, the reduced shortening velocity must represent a shift of the contractile machinery from one stable state to another. The phenomenon of "superactivation" was observed at all $[Ca^{2+}]$.

If the slow phase of shortening was caused by a passive internal load independent of cross-bridges, its influence on the shortening velocity would be greatest during unloaded shortening, but would be diminished with increasing load. To test this possibility, the velocity of shortening was measured in the fibers subjected to multiple shortening procedure under a greater load, i.e., 0.3 P_o or 0.5 P_o. There was a greater tendency for the stretch to tear the fibers which had shortened under these loads. The results coincided with those at a load of $<0.1\ P_o$: at saturating $[Ca^{2+}]$ (Fig. 4, *A* and *B*), the pattern of the first shortening was repeated many times. In this range of loads, the velocity transient as observed by Civan and Podolsky (1966) was clearly recognized before the fibers started to shorten at a constant velocity. The velocity transient was less conspicuous in the second shortening and later, giving an impression that the fibers started to shorten with a more constant velocity. At submaximal $[Ca^{2+}]$ (Fig. 4, *C* and *D*), the first shortening

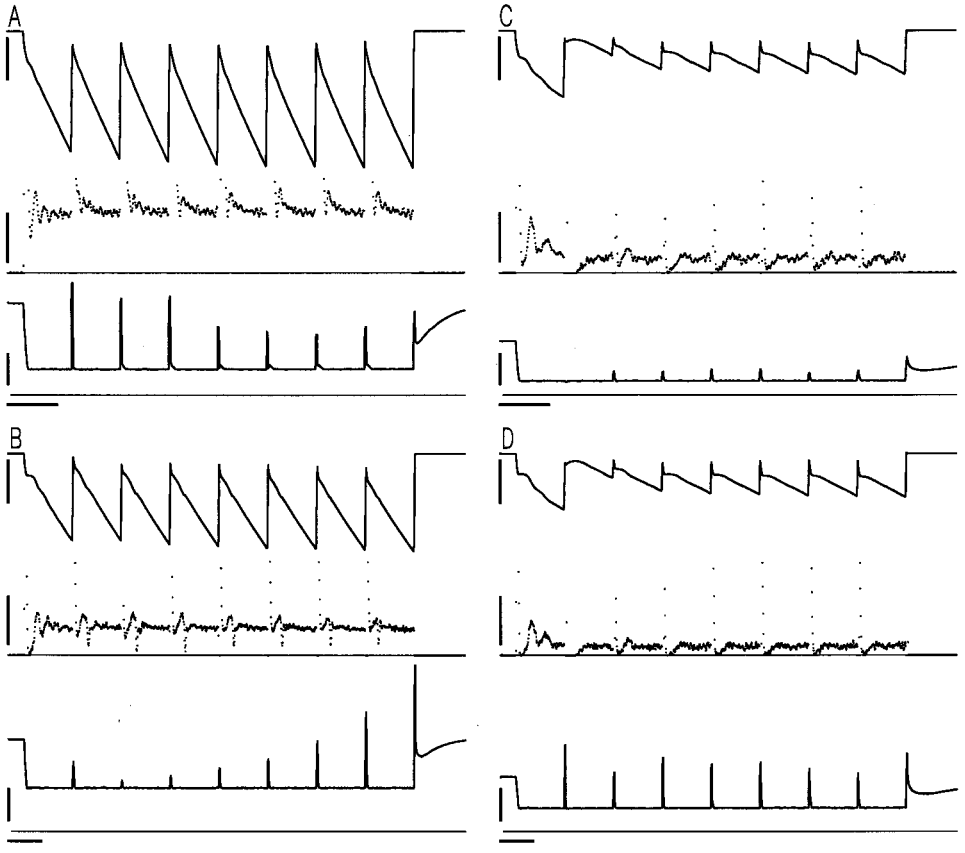

FIGURE 4 Overall fiber length, velocity of shortening, and tension during eight consecutive shortenings under higher loads at saturating and submaximal $[Ca^{2+}]$. Upper traces, length; lower traces, tension. (*A*) pCa = 4.49, 0.3 P_o; (*B*) pCa = 4.49, 0.5 P_o; (*C*) pCa = 6.32, 0.3 P_o; (*D*) pCa = 6.32, 0.5 P_o. Each shortening was 200 ms (*A, C*) or 300 ms (*B, D*) in duration and followed by a 2-ms period of restretch to the initial fiber length. The vertical scale bars indicate 2% L_o, 20% L_o/s, and 0.2 mN from above. The horizontal scale bars indicate 200 ms. Note the difference in time scales in (*A, C*) and (*B, D*). From the same specimen.

seems to consist of fast and slow phases as in the case of light load, but the transition is obscured by the velocity transient. The shortening distance needed for the fast-to-slow transition seemed to be smaller than that for light load (~2% vs. >3% of fiber length). Again, the fast phase was missing in later shortenings. It is therefore concluded that the loss of the fast phase of shortening is not caused by a passive internal load independent of cross-bridges. Interestingly, a clear lengthening of the fibers was observed in the second shortening, i.e., the fibers became temporarily unable to even isometrically sustain the load of 0.3 P_o or 0.5 P_o.

Repriming of the fast phase of shortening in fibers held isometric after an isotonic release

The preceding experiment suggested that, at submaximal [Ca^{2+}], the alteration caused by the first shortening persists for the rest of the period of repetitive shortenings. The time between one shortening and the next, during which the fibers were stretched back to their initial length and held isometric, was short (2 ms). If this isometric period is prolonged, it is expected that the condition of the fibers before the first shortening will be restored eventually, and as a result, so will be the fast phase of shortening. To examine the time course of the restoration (repriming) of the fast phase, the number of shortenings was limited to two and the interval between them was varied. The load was again a finite value of <0.1 P_o.

Fig. 5 *A* shows the records of overall fiber length and tension during a control experiment done at saturating [Ca^{2+}]. Three sets of records are shown in which the interval between the two 60-ms shortenings are 62 (the same as in the preceding experiment), 100, and 300 ms. In the isometric period between the two shortenings, tension redeveloped approximately in an exponential manner. Regardless of the amount of tension redeveloped, the second shortening (*solid line* in the magnified records on the *right*) was faster than the first shortening (*dotted line*). In Fig. 5 *B*, both the velocity of the second shortening (measured between 10 and 30 ms after the onset of the release) and the amount of tension redeveloped in the isometric period are plotted against the time elapsed from the onset of the first shortening. The velocity of the second shortening was always high and seemed to be independent of the amount of tension redeveloped.

When the same experiment was done at submaximal [Ca^{2+}] (pCa = 6.32), the situation was very different (Fig. 6). When the interval between the two shortenings was 62 ms, the second shortening was visibly slower than the first shortening (the first lane in Fig. 6 *A*). As the interval was prolonged, however, the velocity of the second shortening approached that of the first shortening, and at an interval of 5 s, the recovery was complete (the second and third lanes in Fig. 6 *A*).

In the isometric period between the two shortenings, the redevelopment of tension was much slower than at saturating [Ca^{2+}], in accord with the observation made by Brenner (1988). If enough recovery time was allowed, the final tension reached in the isometric period very often exceeded the isometric tension before the first shortening, and this could be related to the "superactivation" mentioned before. Clear parallelism is recognized between the velocity of the second shortening and the amount of tension redeveloped in the isometric period, when they are plotted against the time elapsed from the onset of the first shortening (Fig. 6 *B*).

The relation between the velocity of the second shortening and the amount of isometric tension redeveloped is summarized for various [Ca^{2+}] in Fig. 7. At the lowest level of [Ca^{2+}] tested (pCa = 6.32), strong positive correlation was observed between the two parameters. The correlation was gradually lost as [Ca^{2+}] was increased, and at saturating [Ca^{2+}] (pCa = 4.49), the velocity was almost independent of the amount of tension redeveloped. The apparent correlation seen in the left half of the plot for saturating

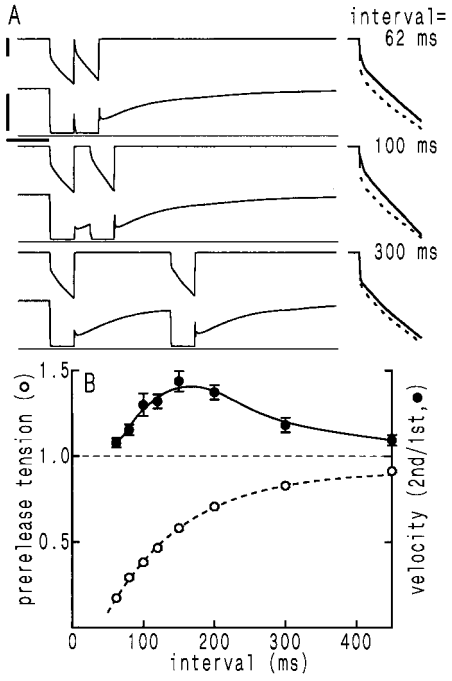

FIGURE 5 Repriming of the velocity of the second shortening with tension redevelopment at saturating [Ca^{2+}] (pCa = 4.49). Shortening is 60 ms in duration and under a light load (<0.1 P_o). (*A*) records of length (*upper traces*) and tension (*lower traces*). Three sets of records are shown with different intervals between the onsets of two shortenings (62, 100, and 300 ms). The magnified view of the length records during shortening are shown on the right. Dotted line, first; solid line, second shortening. (*B*) The velocity of the second shortening (*filled circles*) relative to the first, and the amount of tension redeveloped during the isometric period during the two shortenings (normalized to P_o, *open circles*), plotted against the interval between the onsets of the two shortenings. The amount of tension at an interval of 62 ms was not determined accurately, so that the point was extrapolated by assuming that the tension redevelopment was a single exponential association. Data are expressed as mean ± S.D. (n = 10). The error bars for the tension redevelopment are almost hidden by the circles. The vertical scale bars indicate 2% L_o and 1 mN from above. The horizontal scale bar indicates 100 ms.

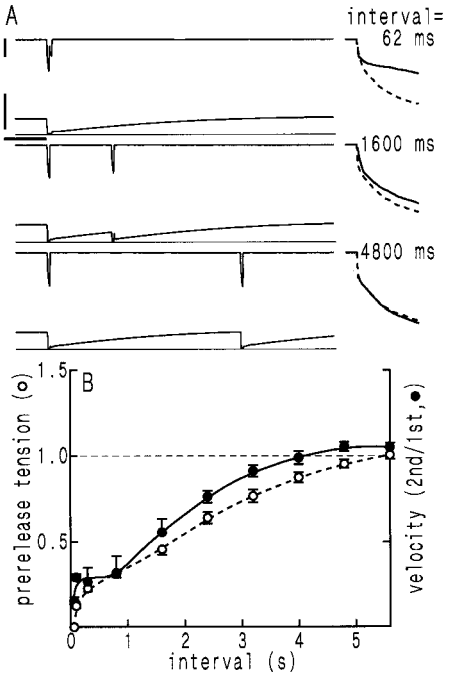

FIGURE 6 Repriming of the velocity of the second shortening with tension redevelopment at submaximal [Ca^{2+}] (pCa = 6.32), presented as in Fig. 5. Shortening is 60 ms in duration and under a light load (<0.1 P_o). (*A*) records of length (*upper traces*) and tension (*lower traces*). The magnified view of the length records during shortening are shown on the right. Dotted line, first; solid line, second shortening. The intervals between the onsets of two shortenings are 62, 1600, and 4800 ms. (*B*) The velocity of the second shortening relative to the first, and the amount of tension redeveloped during the isometric period during the two shortenings are plotted against the interval between the onsets of the two shortenings. Data are expressed as mean ± S.D. (n = 8). The vertical scale bars indicate 2% L_o and 1 mN from above. The horizontal scale bar indicates 1 s.

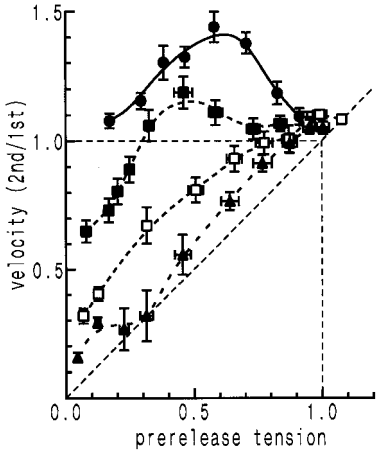

FIGURE 7 Relationship between the velocity of the second shortening and the amount of tension redeveloped at various [Ca^{2+}]. Filled circles, pCa = 4.49 (n = 10); filled squares, pCa = 6.01 (n = 9); open squares, pCa = 6.19 (n = 8); filled triangles, pCa = 6.32 (n = 8). Data are expressed as mean ± S.D. Data for pCa = 4.49 and pCa = 6.32 are replotted from Figs. 5 *B* and 6 *B*.

[Ca^{2+}] is rather considered to reflect the "superactivation," since the increased velocity of the second shortening was seen while the redeveloped tension was still lower than the isometric tension level.

These observations suggest that, at submaximal [Ca^{2+}], the ability of fibers to shorten at a high velocity is maintained by the presence of attached, force-producing (strong binding) cross-bridges. The slow phase of shortening is then explained by the shortening-induced loss of the force-producing cross-bridges and the subsequent deactivation of fibers. The strong dependence of the fiber activity on the force-producing cross-bridges, along with its loss at higher [Ca^{2+}], is in accord with the cooperative activation of the thin filament by strong binding myosin heads (discussed later in detail). Therefore, it is believed that the shortening-induced alteration responsible for the slow phase of shortening is the deactivation of the thin filament.

Effect of phosphate on the velocity of shortening in multiple shortening experiments

It has been reported that the addition of inorganic phosphate (P_i) reduces the biphasic feature of shortening at submaximal [Ca^{2+}] by accelerating the slow phase of shortening (Metzger, 1996). To examine the effect of P_i on the velocities of repetitive shortenings, eight shortenings under a light load of <0.1 P_o (a protocol identical to that in Fig. 2) were applied in the presence of 20 mM P_i. In the presence of Dextran T-500, 20 mM P_i reduced isometric tension by ~50%.

The pattern of shortening at saturating [Ca^{2+}] (Fig. 8 *A*) was very similar to that in the absence of P_i, i.e., the velocities of the second shortening and later were higher than that of the first shortening, indicating that the mechanism of "superactivation" also operates in the presence of P_i. At submaximal [Ca^{2+}], the deceleration of the later shortenings was observed, but it was apparent at lower [Ca^{2+}]. Fig. 8 *B* shows the record taken at pCa = 6.38.

The velocities of shortening in the presence of P_i at various [Ca^{2+}] are summarized in Fig. 9. The velocities of the second shortening and later were much less sensitive to calcium than in the absence of P_i. The first clear sign of slowed shortening was observed at pCa = 6.19, at which the second shortening was slower than the first, but this tendency was reversed in the later shortenings because of the "superactivation."

To summarize, the present results confirmed the observation made by Metzger (1996) that P_i reduces the biphasic feature of shortening. P_i reduces the time dependence of calcium sensitivity of shortening, but does not completely eliminate it.

Rate of tension redevelopment after shortening in the presence and absence of P_i

From the repriming experiment (Figs. 5–7), the slow phase of shortening is suggested to be the consequence of the thin filament deactivation induced by the cross-bridge detach-

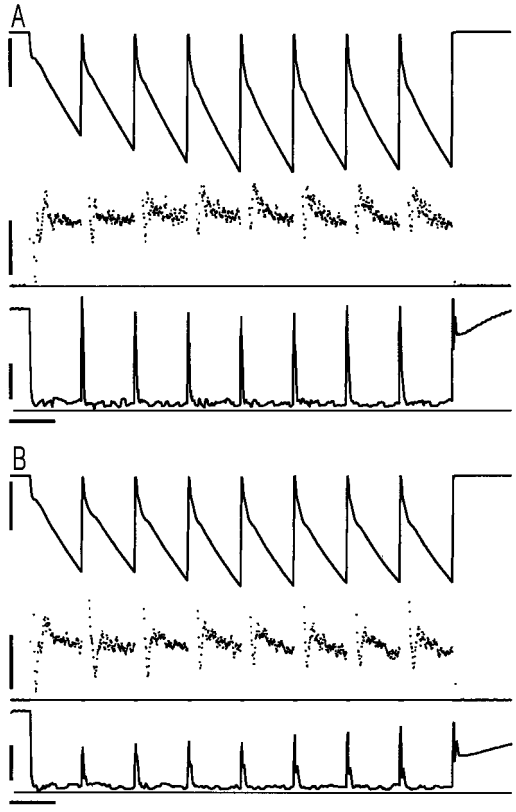

FIGURE 8 Overall fiber length, velocity of shortening, and tension (from above) during eight consecutive shortenings under a load of $<0.1\ P_o$ in the presence of 20 mM P_i, at saturating and submaximal [Ca^{2+}]. The same experiment in Fig. 2 except for the inclusion of P_i. (*A*) pCa = 4.49. (*B*) pCa = 6.19. The vertical scale bars indicate 2% L_o, 50% L_o/s, and 0.2 mN from above. The horizontal scale bar indicates 50 ms.

ment. The preceding effect of P_i to reduce the biphasic feature of shortening would be explained if P_i reduces the cooperativity of thin filament activation, i.e., the detachment of force-producing cross-bridges is less effective in

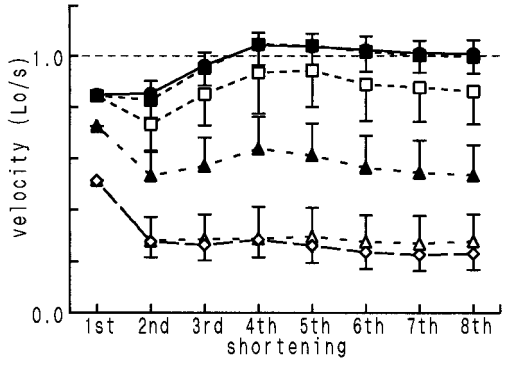

FIGURE 9 Summary of the velocities of eight consecutive shortenings under a load of $<0.1\ P_o$ in the presence of 20 mM P_i, measured at various [Ca^{2+}]. The velocity was measured in the period between 10 and 30 ms after the start of each shortening. Data are expressed as mean ± S.D. (n = 6). Filled circles, pCa = 4.49; filled squares, pCa = 6.01; open squares, pCa = 6.19; filled triangles, pCa = 6.32; open diamonds, pCa = 6.38; open triangles, pCa = 6.44.

deactivating the thin filament. If this is correct, the presence of P_i is expected to affect the time course and final extent of thin filament deactivation after shortening. We estimated the level of the thin filament activation by measuring the rate constants for tension redevelopment after shortening. The rationale for this method will be given in the Discussion. We did not restretch the fibers to their initial length since the restretch could alter cross-bridge kinetics (see Iwamoto, 1995a,b; Iwamoto et al., 1995).

Figure 10 shows the time course of tension redevelopment after shortening under a light load ($<0.1\ P_o$) for 10, 40, and 80 ms and after a repetition of three 60-ms shortenings. The experiments were done under four combinations of conditions [at saturating [Ca^{2+}] or submaximal [Ca^{2+}] (pCa = 6.38), and in the presence or absence of P_i]. At saturating [Ca^{2+}] or in the presence of P_i, the time course of the tension redevelopment was adequately fitted to a process of double exponential association with a constant, equated as follows:

$$Y = A_1[1 - \exp(-k_1 t)] + A_2[1 - \exp(-k_2 t)] + C \quad (1)$$

where A_1 and A_2 are the amplitudes, and k_1 and k_2 are the rate constants, of fast and slow exponential components, respectively. C is a constant and t is time in seconds. At submaximal [Ca^{2+}] in the absence of P_i, the fit was less satisfactory.

The rise of tension consisting of two exponential components has also been reported for caged calcium photolysis experiments (Barsotti et al., 1994; Szczesna et al., 1996; Wahr and Rall, 1997), and the fast component is considered physiologically more relevant in the last two references. At saturating [Ca^{2+}], the rate constant of the fast component (k_1) was always greater in the presence of P_i (Fig. 11 *B*), consistent with the biochemical scheme in which P_i is expected to reverse the force-producing step. On the other hand, it is not the case for the slow component (Fig. 11 *D*). Therefore, the slow component could be rate-limited by a slow process other than cross-bridge kinetics.

At saturating [Ca^{2+}], the amplitude of the fast component (A_1) in the absence of P_i decayed as the preceding shortening was prolonged (Fig. 11 *A, filled circle*), and the values after shortening longer than 20 ms can be fitted to a single exponential decay with a constant [$Y = 0.47 \exp(-29t) + 0.59$; normalized to prerelease isometric tension, t is in seconds]. This tendency seemed to extend after a repetition of three 60-ms shortenings (*triangles* in Fig. 11). In the presence of P_i, A_1 was almost independent of the duration of the preceding shortening (Fig. 11 *A, open circle*), but after a repetition of three 60-ms shortenings, it was reduced to a smaller value. The rate constant of the fast component tended to increase with increasing duration of preceding shortening, regardless of whether P_i was present or not.

Striking is the observation that the fast component was also observed at submaximal [Ca^{2+}] (Fig. 10, *C* and *D*), and this was *faster* than at saturating [Ca^{2+}] (Fig. 12 *B*). The presence of the *faster* component has not been reported for

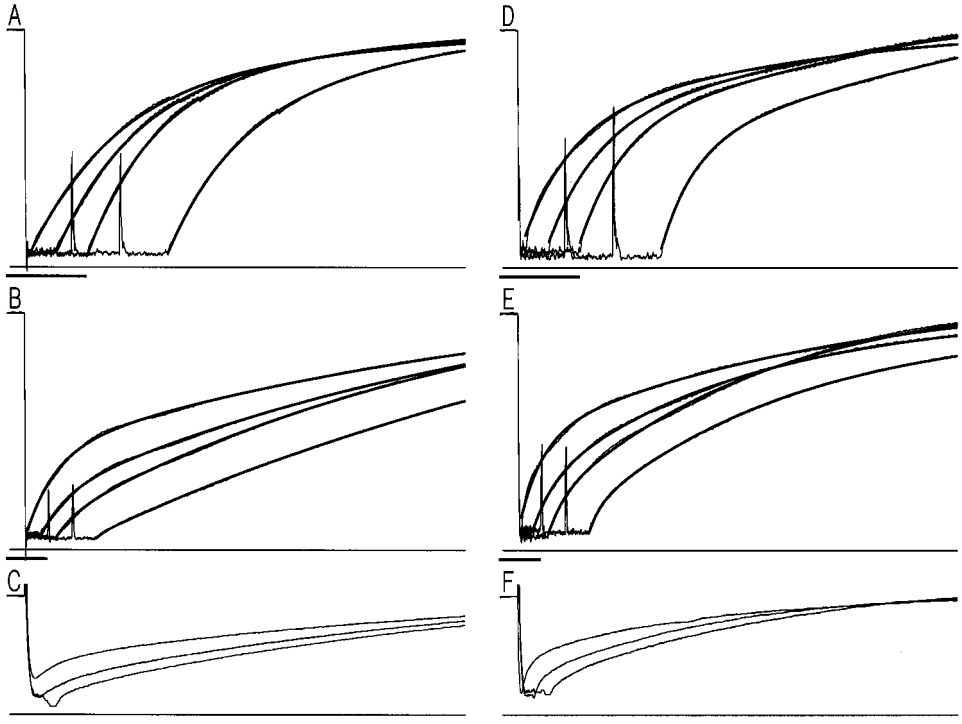

FIGURE 10 Time course of the redevelopment of tension and stiffness after shortening under a light load (<0.1 P_o) for 10, 40, and 80 ms and after a repetition of three 60-ms shortenings. (*A*)–(*C*), recorded in the absence of added P_i. (*A*) tension, pCa = 4.49. (*B*) tension, pCa = 6.38. (*C*) stiffness, pCa = 6.38 (only the records for 10, 40, and 80 ms shortenings are shown). (*D*)–(*F*), same as in (*A*)–(*C*) but recorded in the presence of 20 mM P_i. Data are normalized to the preshortening value of tension or stiffness. Thin lines represent the real data, thick lines are the fit to a process of double exponential association (Eq. 1). Scale bar, 100 ms. Data taken from the same specimen. Note the concomitant rise of tension and stiffness.

the tension redevelopment after a shortening and restretch (Brenner, 1988) or caged calcium photolysis experiments (Araujo and Walker, 1994). Especially in the absence of P_i, the tension rise was not a simple sum of the two exponential components. A plateau, or even a temporal decay, was often observed at the transition from the fast to slow components. Nevertheless, the rise was fitted to a double exponential association for analytical purposes.

To test whether the fast redevelopment of tension observed at submaximal [Ca^{2+}] represents the fast reattachment of cross-bridges to actin or the detachment of negatively strained cross-bridges (bearing negative force), fiber stiffness was measured by applying 500 Hz sinusoidal oscillation. The traces for stiffness are also shown in Fig. 10, *C* and *F*. The stiffness rose with tension and the rise also consisted of fast and slow components. It is concluded, therefore, that the fast component of tension rise at submaximal [Ca^{2+}] represents the *attachment* of cross-bridges to actin.

In the absence of P_i, the amplitude of the fast component (A_1) decayed as the preceding shortening was prolonged, with a rate constant similar to that at saturating [Ca^{2+}] but decayed to a much smaller fraction of prerelease isometric tension level [*filled circles* and *solid line* in Fig. 12 *A*; $Y = 0.36 \exp(-27t) + 0.05$]. After a repetition of three 60-ms shortening periods, the faster component was reduced to a very small value (*filled triangle* in Fig. 12 *A*) so that the entire time course of tension redevelopment may be regarded as a single exponential association. If the fast component is to represent a higher level of the thin filament activation, this result is taken to indicate that the shortening-induced deactivation of the thin filament follows an exponential time course.

In the presence of P_i, the amplitude of the fast component (A_1) also decayed, but with a greater rate constant, leaving a greater fraction of prerelease isometric tension level [*open circles* and *broken line* in Fig. 12 *A*; $Y = 0.24 \exp(-37t) + 0.12$]. The fast component was still clearly observed after a repetition of three 60-ms shortenings (the lowest trace of Fig. 10 *D* and the rightmost open triangle in Fig. 12 *A*). This result is taken to indicate that, after the loss of the force-producing cross-bridges, the thin filament remains more activated than in the absence of P_i. The greater rate constant for the thin filament deactivation is consistent with the observation that, in the presence of P_i, the velocity of the first of the eight consecutive shortenings was more sensitive to [Ca^{2+}] (Fig. 9).

The behavior of the amplitude of the slow component (A_2) was more or less reciprocal with respect to the fast component (Figs. 11 *C* and 12 *C*). The reason for the prominent rise of A_2 observed at saturating [Ca^{2+}] in the presence of P_i (Fig. 11 *C, open circle*) is unknown but related to the distance shortened. This is not due to increasing overlap between thick and thin filaments since this

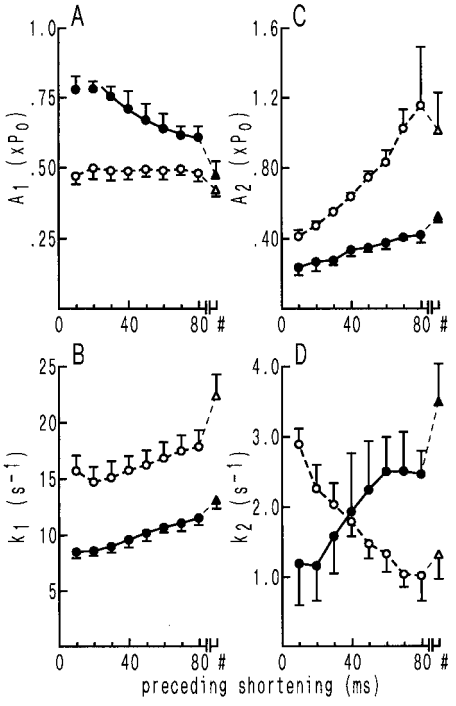

FIGURE 11 Summary of amplitudes and rate constants of the fast and slow exponential components of tension redevelopment at saturating [Ca^{2+}] (pCa = 4.49). In (*A*)–(*D*), the constants A_1 (amplitude of the fast component), k_1 (rate constant of the fast component), A_2 (amplitude of the slow component), and k_2 (rate constant of the slow component), respectively, in Eq. 1 (see text) are plotted against the duration of preceding shortening under a light load (<0.1 P_o). Filled symbols, in the absence of added P_i; open symbols, in the presence of 20 mM P_i. The data taken after a repetition of three 60-ms shortenings (#) are represented as triangles. Data are expressed as mean ± S.D. (n = 6). The curve in (*A*) is a fit to a single exponential decay [$Y = 0.47 \exp(-29t) + 0.59$].

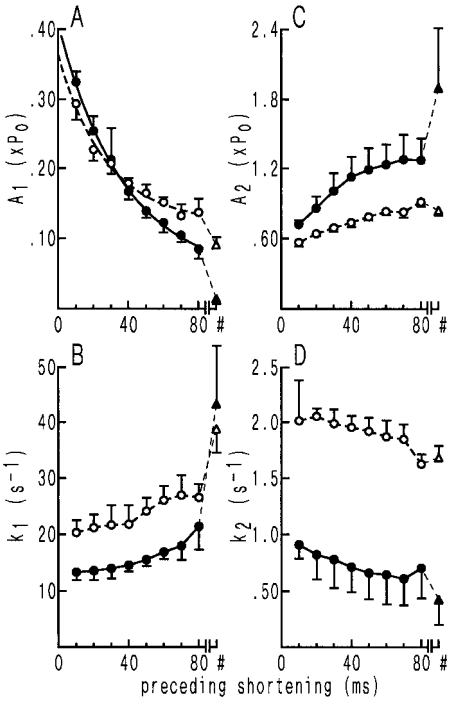

FIGURE 12 Summary of amplitudes and rate constants of the fast and slow exponential components of tension rise at submaximal [Ca^{2+}] (pCa = 6.38). The assignments of figure subsets and symbols are the same as in Fig. 11. Data are expressed as mean ± S.D. (n = 6). The two curves in (*A*) are fits to a single exponential decay [*solid line*, without added P_i, $Y = 0.36 \exp(-27t) + 0.05$; *broken line*, with 20 mM P_i, $Y = 0.24 \exp(-37t) + 0.12$].

phenomenon was also observed when the shortening was started at a shorter sarcomere length (not shown). Unlike at saturating [Ca^{2+}], the rate constant of the slow component (k_2) at submaximal [Ca^{2+}] was always greater when P_i was present (Fig. 12 *D*). The slow component at submaximal [Ca^{2+}] may reflect more directly the cross-bridge kinetics than at saturating [Ca^{2+}].

DISCUSSION

Use of the multiple shortening protocol

In the present study, the multiple shortening protocol was used to investigate the mechanism by which the velocity of shortening is controlled by calcium. At saturating [Ca^{2+}], isotonic shortenings could be repeated many times within a single contraction while keeping the fiber's fast shortening pattern. The amount of restretch imposed at the end of each shortening was ~7% of fiber length, or ~90 nm per half sarcomere. Since this amount is much greater than the size of the myosin head (<20 nm, e.g., Rayment et al., 1993), it is probable that the force-producing cross-bridges are forcibly detached by the restretch. The reproducible pattern of steady shortening suggests that the detached cross-bridges can quickly reattach to actin and resume their sliding motion. The velocity transient, prominently observed after the onset of the first shortening, was less conspicuous in the second shortening and later (Fig. 4). Since the velocity transient is considered to represent the transition of the cross-bridges from one stable distribution to another, this observation implies that the cross-bridge distribution is not disturbed by the restretch in any significant manner.

To summarize, the multiple shortening protocol can be regarded to create a situation analogous to a continuous steady-state sliding along a limited stretch of filaments.

Mechanism of the biphasic shortening pattern observed at submaximal [Ca^{2+}]

Using this protocol, we confirmed the time-dependent increase of calcium sensitivity of the velocity of shortening at submaximal [Ca^{2+}], i.e., the initial fast, relatively calcium-insensitive phase of shortening is followed by a slower, more calcium-sensitive phase. This biphasic shortening pattern is clearly seen in the record of the first isotonic shortening (Fig. 2 *B*). Significant is the observation that the fast phase of shortening is almost or completely missing in the second shortening and later. This observation applies to the shortenings under loads of 0.3 and 0.5 P_o as well as light

($<0.1\ P_o$) loads. This observation poses some restrictions upon the possible mechanisms giving rise to the biphasic shortening pattern.

First, a progressive increase of a passive internal load independent of cross-bridges is clearly excluded, since its effect on the velocity of shortening is expected to decline as the externally imposed load is increased. Therefore, it is likely that the biphasic shortening pattern reflects the behavior of cross-bridges and the thin filament.

The second possibility is an altered kinetics of cross-bridges at submaximal [Ca^{2+}], specifically the kinetic steps later than the force generation. In fact, there is evidence that altered [Ca^{2+}] modulates the kinetics of force-producing cross-bridges (e.g., Martyn and Chase, 1995; Walker et al., 1992). However, the biphasic feature of shortening can be explained without modulation of the kinetics of force-producing cross-bridges per se, if the cooperative activation of the thin filament is taken into consideration (see below). Long-lived cross-bridges, which would exert a resistive force after sliding for a fixed amount of distance, are not a likely source of the slow phase, since the slow phase starts at longer fiber lengths in the second shortening and later. It would be more natural to consider that the alteration produced by the first shortening persists for the rest of the period of repetitive shortenings.

The information about the nature of the shortening-induced alteration responsible for the slow phase of shortening comes from the results of the second experiment, in which the interval between the first and the second shortenings was varied. After the first restretch, the isometric tension started to redevelop from a low value in an approximately exponential manner. At submaximal [Ca^{2+}], the recovery of the velocity of the second shortening showed strong parallelism with the redevelopment of tension, i.e., the regeneration of the force-producing cross-bridges (Fig. 6 *B*). This parallelism, along with its gradual loss with increasing [Ca^{2+}], is in accord with the biochemical studies showing cooperative activation of the thin filament by the attachment of strong binding myosin heads. Therefore, the present results strongly suggest that the biphasic shortening pattern originates from thin filament cooperativity.

The cooperative binding of strong binding myosin to regulated actin and the effect of calcium on it were first demonstrated by Weber's group (e.g., Bremel and Weber, 1972). Since then, numerous studies compared the extent of cooperativity in the presence and absence of calcium using soluble proteins (Greene and Eisenberg, 1980, Lehrer and Morris, 1982; McKillop and Geeves, 1993; Schaertl et al., 1995; Trybus and Taylor, 1980; Williams et al., 1988) and myofibrils (Swartz et al., 1996). The results concur in that the S-1 (subfragment-1 of myosin) binding to regulated actin occurs in a highly cooperative fashion in the absence of calcium, but the cooperativity is greatly reduced at saturating [Ca^{2+}].

The cooperativity and calcium effect on it was systematically treated by the model of Hill et al. (1980). In that model, the seven-actin unit is regarded to be either in the on state or in the off state, and the equilibrium constant L = [off-state]/[on-state] is much greater than 1 in the absence of calcium, i.e., very few units are in the on state. The S-1 binding is cooperative because once a seven-actin unit is turned on by an attached S-1, other S-1's preferentially bind to that seven-actin unit, turning on more seven-actin units due to unit-unit interactions and attracting more S-1's. This preferential binding to the seven-actin unit in the on state is unique to the strong binding form of S-1. At low [Ca^{2+}], therefore, the average level of the thin filament activation is expected to be proportional to the S-1 binding. As [Ca^{2+}] increases, the equilibrium constant L decreases to a much smaller value. As a result, the activation of the thin filament will become less cooperative. At the same time, the proportionality between the level of activation and S-1 binding will be gradually disrupted, since the thin filament activation will be saturated earlier than the S-1 binding (see, e.g., Schaertl et al., 1995).

From this standpoint, the effect of shortening on the activation level of the thin filament is understandable. Shortening is expected to accelerate the detachment of force-producing (strong binding) cross-bridges from actin, reducing the number of attached force-producing cross-bridges. Under unloaded conditions, the number of force-producing cross-bridges could be reduced to a very small fraction of the total myosin (Brenner, 1993; Iwamoto, 1995b; see also Homsher et al., 1981). At saturating [Ca^{2+}], the thin filament is expected to stay activated in spite of the reduced number of force-producing cross-bridges. On the other hand, at submaximal [Ca^{2+}], cross-bridge attachment is now a prerequisite for activation. Thus, the loss of the attached force-producing cross-bridges is expected to reduce inevitably the level of the thin filament activation.

How does reduction of the thin filament activation affect the velocity of shortening? If [Ca^{2+}] affects only the number of attached force-producing cross-bridges but not their mechanical properties, then the velocity of isotonic shortening is solely determined by the load relative to the isometric tension that would be achieved if shortening did not affect the thin filament activation. The present results suggest that, at submaximal [Ca^{2+}], the level of the thin filament activation decays as the shortening proceeds. Since the load is held constant throughout the period of the repetitive shortenings, the load relative to the isometric tension is expected to increase, causing the velocity to decrease to a new steady value. Although the relationship between load and velocity is nonlinear, to a first approximation the velocity of shortening under a given light load can be regarded as a linear function of the level of the thin filament activation.

The biphasic shortening pattern has been previously reported mostly under unloaded conditions, using the slack test method (Edman, 1979). Even in this case, the presence of a small amount of internal load would give rise to the slow phase. Alternatively, there may be too few force-producing cross-bridges to ensure continuous sliding of each filament (see discussion by Gordon et al., 1997 and Homsher et al., 1996 for in vitro motility assay).

In summary, the present results imply that the time-dependent increase of calcium sensitivity at submaximal [Ca^{2+}] is caused by the thin filament deactivation due to the shortening-induced loss of force-producing cross-bridges. To account for this effect, it is not necessary to take into consideration the calcium-dependent modification of cross-bridge properties.

Effect of P_i on the biphasic shortening pattern

The idea that the time-dependent increase of calcium sensitivity is a manifestation of the cooperative thin filament activation is further supported by the measurement of the rate of tension redevelopment after shortening in the presence and absence of P_i.

The process controlling the rate of tension redevelopment is unresolved. If the level of the thin filament activation is unaffected by the shortening (and restretch), it would be determined solely by the steady-state cross-bridge kinetics. If the level of the thin filament activation varies with time, both the activation process and the cross-bridge kinetics would contribute to the rate of tension redevelopment. At least it would be right to say that the rate of tension redevelopment reflects the level of the thin filament activation at the start of redevelopment, regardless of which mechanism is correct.

At submaximal [Ca^{2+}], the tension redevelopment after shortening under a light load consisted of two exponential components (fast and slow). The fast component is not apparent if a restretch is included in the protocol (e.g., Brenner, 1988).

At submaximal [Ca^{2+}], the amplitude of the fast component (A_1) decayed as the shortening was prolonged (Fig. 12 *A*). In the presence of P_i the decay occurred less thoroughly, and a greater fraction (~34% vs. ~11% in the absence of P_i) of the initial amplitude remained as a time-independent component. From the considerations described above, the decay of the faster component is considered to reflect the rate and extent of the thin filament deactivation induced by the shortening. The rate of deactivation may be limited by either the deactivation process per se or the detachment of force-producing cross-bridges, but the stiffness measurements (Fig. 12, *C* and *F*) suggests that the latter is a fast process. If the temperature difference is taken into consideration, the rate constant observed in the present study (27–37 s^{-1} at 3–5°C) can be regarded comparable to the rate constant for the turning off of the thin filament regulatory complex (63 s^{-1} at 25°C) as monitored by the excimer fluorescence of pyrene probe on tropomyosin (Ishii and Lehrer, 1993). The less complete decay in the presence of P_i should then indicate that the shortening-induced loss of force-producing cross-bridges is less effective in deactivating the thin filament than in the absence of P_i. This means a reduced extent of cooperativity in thin filament activation. Reduced cooperativity in the presence of P_i has also been reported for the tension/stiffness − pCa curves, in which the Hill coefficient as an index for cooperativity is significantly smaller than in the absence of P_i (Brozovich et al., 1988).

In the light of Hill et al.'s (1980) model, the tendency of P_i to reduce the thin filament cooperativity is consistent with the idea that the generation of the low force quaternary complex of actin, myosin, ADP, and P_i ($A \cdot M \cdot ADP \cdot P_i$) is the primary target of calcium regulation (Iwamoto, 1995a, 1996; Regnier et al., 1995). To make calcium regulation possible, the myosin heads in this low force state should have a higher affinity for the seven-actin unit in the on state. The model predicts that, because of the difference in the affinities, the low force $A \cdot M \cdot ADP \cdot P_i$ complex cooperatively activates the thin filament. Since the population of this complex is increased in the presence of P_i but it does not produce force, it will be observed as if P_i reduced the ability of force-producing cross-bridges to cooperatively activate the thin filament. If the above consideration is correct, it would be appropriate to say that the low force $A \cdot M \cdot ADP \cdot P_i$ complex has a trait of strong binding states, not just because it supports fiber stiffness (Brozovich et al., 1988; Regnier et al., 1995) but also because of its ability to cooperatively activate the thin filament. Possibly the formation of the low force $A \cdot M \cdot ADP \cdot P_i$ complex involves some of the myosin-binding sites on actin not accessible for myosin in the off state.

The population of the low force $A \cdot M \cdot ADP \cdot P_i$ complex is also expected to increase during shortening and at saturating [Ca^{2+}]; this complex is considered to account for a substantial part of fiber stiffness during lightly loaded shortening (Iwamoto, 1995b). The low force $A \cdot M \cdot ADP \cdot P_i$ complex may help keep the thin filament activated during high-speed shortening, in spite of the paucity of force-producing cross-bridges.

From the above consideration about the effect of P_i, it is predicted that any intervention that reduces the thin filament cooperativity will reduce the biphasic feature of shortening at submaximal [Ca^{2+}]. In this context, the effect of C-protein extraction is worth noting. The partial extraction of C-protein has been reported to accelerate the velocity in the slow phase of shortening (Hofmann et al., 1991a). They explained the slow phase of shortening as a result of the straining of the S-2 portion of myosin by C-protein. However, the present study provides an alternative explanation, i.e., partial loss of the thin filament cooperativity. In fact, they have also reported that the C-protein extraction substantially reduces the Hill coefficient of the tension-pCa curve (Hofmann et al., 1991b).

The observation that tension redevelopment after shortening at submaximal [Ca^{2+}] was faster than at saturating [Ca^{2+}] (Figs. 10–12) is at a glance surprising, but it can be explained if the decaying level of thin filament activation is taken into account. If the number of actin monomers available for the myosin heads is decreasing with time, the rise of tension would be clipped, resulting in a premature peaking and a spuriously high rate constant. Therefore, the observation does not prove that cross-bridges are activated and inactivated in an all-or-none fashion. However, the

observation does provide further support for the view that, at submaximal [Ca^{2+}], the thin filament in isometrically contracting fibers is more activated than immediately after shortening (and a restretch). Now it is clear that the cooperative activation of the thin filament has a substantial contribution in determining the rate of tension redevelopment at submaximal [Ca^{2+}] (see Ashley et al., 1991; Campbell, 1997; Landesberg and Sideman, 1994; Millar and Homsher, 1990). How much room is left for the cross-bridge kinetics to contribute to the calcium-dependent rate of tension redevelopment depends on the kinetics of the on-off transition of the thin filament. If the on-off transition is much faster than the cross-bridge kinetics, the attachment of the cross-bridges will be governed by a second-order rate constant as regards the concentrations of cross-bridges and actin sites available for cross-bridges. If the rate constant is taken as first-order, it will change with [Ca^{2+}] in a graded manner. If the on-off kinetics is slow, the cross-bridge kinetics will be regulated in an all-or-none fashion and therefore it will have little contribution to the calcium sensitivity of the rate of tension redevelopment. Further studies are needed to decide which possibility is correct.

Possible effects of fiber length on the velocity of shortening

The length-dependent activation of the thin filament is prominently observed in cardiac muscles, but is also observed in skeletal muscles (Endo, 1972; Gulati et al., 1990; Wang and Fuchs, 1995). This effect, proposed to be a result of the altered spacing of the filament lattice (Fuchs and Wang, 1996), may also participate in the process of inducing the slow phase of shortening. However, this mechanism is unlikely to have a major contribution to the process, since the slow phase of the second shortening and later started at sarcomere lengths longer than that in the first shortening.

Mechanism of the "superactivation"

During the course of repetitive shortenings, it was commonly observed that the velocity of later shortenings was up to ~40% higher than that of the first shortening. The reason for this modest increase is not clear, but it may reflect the stretch-induced shift of the cross-bridge populations from low force to force-producing states (Iwamoto et al., 1995; Iwamoto, 1995a). This shift would result in an increased number of force-producing cross-bridges, which would enable the fibers to shorten at a higher velocity. The same mechanism that gives rise to the superactivation may underlie the accelerated shortening observed after stretch or isotonic lengthening (Edman et al., 1978; Sugi and Tsuchiya, 1981).

CONCLUSION

Present results showed that the biphasic shortening pattern (or time-dependent increase of calcium sensitivity) of rabbit skeletal muscle fibers at submaximal [Ca^{2+}] is most readily explained in terms of the cooperative activation of the thin filament. This idea is supported by the observation that the emergence of the slow phase of shortening and the decay of the fast component of tension redevelopment (A_1) occur with comparable time scales.

Although the properties of the attached cross-bridges may be to some extent modified by [Ca^{2+}], the present results can be explained without taking it into consideration. Therefore, the present results are consistent with the idea that the primary target of calcium regulation in the actomyosin ATPase cycle is at the entrance to mechanical events (Iwamoto, 1995a), specifically the generation of the low force $A \cdot M \cdot ADP \cdot P_i$ complex (Iwamoto, 1995a, 1996; Regnier et al., 1995).

The author expresses his hearty thanks to professor H. Sugi for his support and encouragement.

This work was supported by Ministry of Education Grant 08640783.

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