The off rate of Ca\(^{2+}\) from troponin C is regulated by force-generating cross bridges in skeletal muscle

YING WANG AND W. GLENN L. KERRICK
Department of Physiology and Biophysics, University of Miami School of Medicine, Miami, Florida 33101
Received 20 April 2001; accepted in final form 1 February 2002

Wang, Ying, and W. Glenn L. Kerrick. The off rate of Ca\(^{2+}\) from troponin C is regulated by force-generating cross bridges in skeletal muscle. J Appl Physiol 92: 2409–2418, 2002. First published February 8, 2002; 10.1152/japplphysiol.00376.2001.—The effects of dissociation of force-generating cross bridges on intracellular Ca\(^{2+}\) binding to troponin C (TnC) in intact skeletal muscle. Mechanical length perturbations were used to dissociate force-generating cross bridges in either intact or skinned fibers. In intact muscle, an impulse stretch or release, a continuous length vibration, a nonoverlap stretch, or an unloaded shortening during a twitch caused a transient increase in intracellular Ca\(^{2+}\) compared with that in isometric controls and resulted in deactivation of the muscle. In skinned fibers, sinusoidal length vibrations shifted pCa-force, and pCa-actomyosin ATPase rate relationships to higher Ca\(^{2+}\) concentrations and caused actomyosin ATPase rate to increase at submaximal Ca\(^{2+}\) and increase at maximal Ca\(^{2+}\) activation. These results suggest that dissociation of force-generating cross bridges across a twitch causes the off rate of Ca\(^{2+}\) from troponin C to increase (a decrease in the Ca\(^{2+}\) affinity of troponin C), thus decreasing the Ca\(^{2+}\) sensitivity and resulting in the deactivation of the muscle. The results also suggest that the Fenn effect only exists at maximal but not submaximal force-activating Ca\(^{2+}\) concentrations.

Skeletal muscle contraction is regulated by sarcomere length and intracellular Ca\(^{2+}\) concentration. When a muscle contracts, Myosin ATPase accelerates the breakdown of ATP to ADP and Pi, which produces tension and shortens the sarcomere. The activation of Myosin ATPase is a function of the intracellular Ca\(^{2+}\) concentration. When a muscle relaxes, the Ca\(^{2+}\) concentration is reduced and the muscle returns to its resting length. When a muscle is activated, the intracellular Ca\(^{2+}\) concentration increases, which activates Myosin ATPase and causes the muscle to contract. When a muscle is relaxed, the intracellular Ca\(^{2+}\) concentration decreases, which inactivates Myosin ATPase and causes the muscle to relax. The myofilament regulatory protein troponin C (TnC), when bound to Ca\(^{2+}\), interacts with the calmodulin-calmodulin-dependent protein kinase (CaM kinase) to increase the activity of Myosin ATPase. The Ca\(^{2+}\) activity of TnC is regulated by the affinity of TnC for Ca\(^{2+}\), which is decreased at submaximal Ca\(^{2+}\) concentrations and resulted in deactivation of the muscle. In intact skeletal muscle, most studies that investigated the effect of dissociation of force-generating cross bridges on the Ca\(^{2+}\) binding to TnC have been carried out under conditions of tetanus and have shown variable results. Shortening during a tetanus resulted in either a transient decrease (11), or no change (10), or a biphasic change (60) in intracellular Ca\(^{2+}\).

Gradation of skeletal muscle contraction results from twitches, summation of twitches and tetanus in the individual motor unit, as well as variation in the number of motor units that are recruited. The tetanus represents the highest force that a muscle can develop and results in a sustained high level of intracellular Ca\(^{2+}\), whereas the twitch represents the lowest force level of the muscle response. Neither intracellular Ca\(^{2+}\) nor force ever reach sustained high levels and instead are constantly changing during a twitch. Therefore, the effect of force-generating cross bridges on the Ca\(^{2+}\) affinity of TnC during a twitch may differ from that during tetanus.

The working hypothesis for this study is that the dissociation or increasing dissociation rate of force-generating cross bridges causes an increase in the off rate of Ca\(^{2+}\) from TnC, and this would be expected to make the muscle less sensitive to Ca\(^{2+}\) and deactivate muscle contraction. Two types of experiments were designed in this study. The first protocol was to use intact mouse lumbrical muscles and to investigate the effect of dissociation of force-generating cross bridges by mechanical perturbations (impulse length changes,
a continuous length vibration, a nonoverlap stretch, and an unloaded shortening) on the myoplasmic Ca\(^{2+}\) transient and force during a twitch. In skeletal muscle, the twitch is much faster than in cardiac or barnacle muscle, and thus it has posed special experimental problems for resolving the intracellular Ca\(^{2+}\) measurements. We have overcome the time resolution problem in skeletal muscle by using a fast filter wheel, which allows us to make intracellular Ca\(^{2+}\) measurements with a time resolution of 4 ms. The second part of this study was to test the hypothesis in a completely different manner by using skinned lumbrical muscle fibers. The number of the cross bridges and/or the rate of cross-bridge dissociation was controlled by sinusoidal length vibrations, and the effects on the Ca\(^{2+}\) sensitivity of contraction and actomyosin ATPase rate were investigated.

**METHODS**

**Apparatus.** The experimental apparatus used in this study was the Guth Muscle Research System (Scientific Instruments, Heidelberg, Germany), as described in detail in a previous study (61). Briefly, the mechanical parts of the apparatus consisted of a force transducer for measuring force; a servomotor, a feedback circuit, and power amplifier for making length changes; a ramp generator for making impulse stretch and release length changes; a signal generator for producing sinusoidal length changes; and a constant-load module for doing unloaded shortening. The optics consisted of a microscope photometer unit for monitoring emission light from the muscle fiber. The light was focused by an Olympus Quartz condenser onto the muscle preparation after passing through filters appropriate to Ca\(^{2+}\) transient and ATPase measurements. For intact muscle experiments, a 3-mm-diameter cylindrical cuvette was slipped over the preparation and perfused with 95% O\(_2\)-5% CO\(_2\)-saturated Krebs-Henseleit solution containing 30 mM 2,3-butanedione monoxime (Sigma Chemical, St. Louis, MO), saturated with 95% O\(_2\)-5% CO\(_2\). One tendon end was attached to a linear motor, and the other end was attached to the force transducer by use of stainless steel tweezers. Once the muscle was mounted in the apparatus, its length was adjusted until the maximum active twitch force was obtained. The muscle was stimulated at 1.0 Hz. The preparation was then loaded with 5 μM fura 2-AM (Molecular Probes, Eugene, OR) in an oxygenated Krebs-Henseleit solution containing 30 mM 2,3-butanedione monoxide (Sigma Chemical, St. Louis, MO), saturated with 95% O\(_2\)-5% CO\(_2\). One tendon end of the muscle was attached to a linear motor, and the other end was attached to the force transducer by use of stainless steel tweezers. Once the muscle was mounted in the apparatus, its length was adjusted until the maximum active twitch force was obtained. The muscle was stimulated at 1.0 Hz.

**Force and fura 2 measurements.** Mice anesthetized by CO\(_2\) were killed by rapid neck disarticulation, and the intact lumbrical muscles (1.0–2.0 × 0.2–0.3 mm) of the hindfoot were dissected free in a Krebs-Henseleit solution containing 30 mM 2,3-butanedione monoxide (Sigma Chemical, St. Louis, MO), saturated with 95% O\(_2\)-5% CO\(_2\). One tendon end of the muscle was attached to a linear motor, and the other end was attached to the force transducer by use of stainless steel tweezers. Once the muscle was mounted in the apparatus, its length was adjusted until the maximum active twitch force was obtained. The muscle was stimulated at 1.0 Hz.

The preparation was then loaded with 5 μM fura 2-AM (Molecular Probes, Eugene, OR) in an oxygenated Krebs-Henseleit solution containing 0.5% cremophore for 1 h (61). Cremophore can increase the solubility of fura 2 in the bathing solution. The fura 2 fluorescence signals corresponding to the 340- and 380-nm illumination of the preparation were sampled by a signal sorter and recorded by a computer. The time resolution of the 340-nm-to-380-nm fluorescence ratio (340/380) measurements was 4 ms. Background fluorescence from 340- and 380-nm excitations of unloaded preparations was subtracted before the fluorescence 340/380 was calculated and plotted along with force. The 340/380 ratios were not used to calculate the actual intracellular Ca\(^{2+}\) concentration, because we were only interested in relative changes in intracellular Ca\(^{2+}\). Additionally, fura 2 may bind to the intracellular proteins, and this could alter its affinity for Ca\(^{2+}\) (7, 38).

The reasons for using fura 2 in these experiments include the following: 1) it is a ratiometric indicator, which minimizes movement artifacts; and 2) it has the right affinity for Ca\(^{2+}\) to measure Ca\(^{2+}\) dissociating from TnC easily when force-generating cross bridges are dissociated (see Figs. 2–5). The disadvantage of using fura 2 is that the off rate of Ca\(^{2+}\) from fura 2 is too slow to measure the intracellular Ca\(^{2+}\) transient accurately (7) in skeletal muscle. Because we are only interested in knowing whether Ca\(^{2+}\) is released from the TnC, this is not a problem.

Each data record consisted of one control and one test run. Both the control and the test records consisted of an average of 100 individual records. Control isometric records were taken both before and after each test record, and only test data bracketed by identical control records were used. In each test record, the muscle was subjected to an impulse stretch or release, a continuous sinusoidal length vibration, a nonoverlap stretch, and unloaded shortening. Unloaded shortening in these experiments was accomplished by feeding back the force signal to the constant-load unit that was programmed to hold the force constant at the resting level.

**Force and actomyosin ATPase measurement.** A small bundle of lumbrical fibers (diameter: 60–70 μm) was dissected free in relaxing solution and treated with 1% Triton X-100 for 30 min (61). The skinned fiber was mounted in the Guth Muscle Research System. The sarcomere length was adjusted to 2.2 μm by a laser diffraction pattern. The cross-sectional area was calculated based on the measurement of the fiber width by microscope and assuming that the fiber was circular in diameter. The ATPase rate was measured by the NADH fluorescence method (26). The regeneration of ATP from ADP and phosphoenol pyruvate by the enzyme pyruvate kinase is coupled to the oxidation of NADH (fluorescent) to NAD (nonfluorescent) by lactate dehydrogenase (24, 57). The decrease in NADH concentration was detected by a decrease in the fluorescence signal at 450 nm. The slope of the linear decrease in NADH concentration was used to calculate the ATPase rate.

The fiber was subjected to an increasing Ca\(^{2+}\) gradient (a slow, uniform, stepwise increase in Ca\(^{2+}\) concentrations) by using the gradient maker. The gradient maker consisted of a constantly stirred lower chamber and an upper chamber. A small hole connected the two chambers. The outlet of the lower chamber went to a peristaltic pump, which pumped the solution from the lower chamber to the quartz cuvette enclosing the muscle preparation. The pump was commanded by the computer every 20 s to replenish the solution in the cuvette. With every pump rotation, solution perfusing the fiber from the lower chamber was replaced by solution from the upper chamber (high Ca\(^{2+}\)). In this manner, a continuous, increasing Ca\(^{2+}\) gradient was achieved, and fresh, unoxidized NADH solution was introduced into the cuvette every 20 s. The Ca\(^{2+}\) concentration gradient was calibrated by use of the fluorescent Ca\(^{2+}\) indicator calcium green-2 (Molecular Probes). For a detailed description of the calibration, see Ref. 4. In order for each fiber to serve as its own control, the muscle was alternately subjected to 10% sinusoidal length vibration (20 Hz) and isometric contraction throughout the whole range of Ca\(^{2+}\) activation. An example of such data is shown in Fig. 1.

**Solutions.** During the experiment, the intact muscle was perfused with a Krebs-Henseleit solution containing (in mM) 119 NaCl, 4.6 KCl, 1.8 CaCl\(_2\), 1.2 MgSO\(_4\), 25 NaHCO\(_3\), 1.2 KH\(_2\)PO\(_4\), and 11 glucose. This solution was saturated with...
5% CO$_2$-95% O$_2$. For the skinned muscle fibers, the solution contains 10$^{-6}$ to 10$^{-3.4}$ M Ca$^{2+}$, 85 mM K$^+$, 2 mM MgATP, 1 mM Mg$^{2+}$, 7 mM EGTA, 5 mM phosphoenol pyruvate, 100 U/ml pyruvate kinase, 0.4 mM NADH, 140 U/ml lactate dehydrogenase, and propionate as the major anion. Ionic strength was adjusted to 0.15 M, and pH was maintained at 7.00 ± 0.02 with imidazole propionate. All of the experiments were performed at room temperature (21–24°C).

Statistical analysis. The Ca$^{2+}$ transients of isometric control and length perturbations from both positive and negative time block regions of Ca$^{2+}$ difference curves (see Figs. 2, C and D, 3B, 4B, and 5B) were compared statistically for each experiment by using ANOVA (47). The t-test was used to determine the significance between the two Ca$^{2+}$ transients. SAS Procedure General Linear Regression (SAS, Cary, NC) was used to conduct the analysis. The data difference was considered statistically significant when the $P$ value was <0.05.

RESULTS

Changes in force and Ca$^{2+}$ transient in intact muscle. When a 10% impulse stretch (quick linear stretch followed immediately by a linear shortening to the original length) is applied at the peak of twitch force shown in Fig. 2A, the force is dramatically decreased and cannot redevelop, even after the impulse. This indicates that the force-generating cross bridges are dissociated and that the thin filament is deactivated by this impulse stretch. Figure 2A also shows that the impulse stretch causes an increase in the Ca$^{2+}$ transient compared with an isometric control contraction. After this increase, the Ca$^{2+}$ transient after the impulse decreases more rapidly, falling below the control Ca$^{2+}$ transient, as is shown by the difference between the impulse stretch and the control Ca$^{2+}$ transient curves (Fig. 2C). The impulse stretch applied when the muscle is not actively contracting has no effect on the Ca$^{2+}$ transient.

Figure 2B shows that a 10% impulse release (quick linear shortening followed immediately by a linear stretch) in muscle length, like an impulse stretch, causes a decrease in force and an increase in the Ca$^{2+}$ transient. Similar to the impulse stretch, the Ca$^{2+}$ transient after the impulse decreases more rapidly, dipping slightly below the control isometric Ca$^{2+}$ transient, as is evident from the difference in the fluorescence curve between the Ca$^{2+}$ transients in the impulse release and control records (Fig. 2D). After restretch back to the original length, the muscle is deactivated, because it cannot produce further isometric force even though intracellular Ca$^{2+}$ is still elevated. Figure 2B also shows that the impulse release, like the impulse stretch, does not cause a change in intracellular Ca$^{2+}$ when it is applied in the resting state. When a muscle is stretched to nonoverlap between thin and thick filaments (no cross bridges attached), no change in the Ca$^{2+}$ transients is observed after these impulse length changes compared with those without impulse length changes in the same stretched muscle (data not shown). Therefore, this increase in intracellular Ca$^{2+}$ after the impulse is apparently associated with detachment of force-generating cross bridges. In the skeletal muscle experiments reported here, both an impulse stretch and release are associated with same sign increases in the fura 2 fluorescence ratio (340/380), which would argue against any movement artifacts (Fig. 2). The duration of the Ca$^{2+}$ transient appears to be longer than the force. This results from the fact that fura 2 can measure intracellular Ca$^{2+}$ below threshold for force activation.
When a 5% length vibration (600 Hz) is applied continuously throughout the twitch, very little force can develop, and the intracellular Ca\(^{2+}\) transient is initially higher than the control isometric intracellular Ca\(^{2+}\) but then declines more rapidly than control toward the end of the Ca\(^{2+}\) transient (Fig. 3A). This is more easily seen when the difference between the length vibration and isometric control Ca\(^{2+}\) transient is plotted (Fig. 3B). Thus the prevention of the majority of force-generating cross bridges from attaching by continuous vibration (as evidenced by the lack of force development) increases the myoplasmic Ca\(^{2+}\) concentration.

If the Ca\(^{2+}\) transient change is really associated with reduction of the number of force-generating cross bridges, then stretching the muscle to minimize the number of force-generating cross bridges should cause the intracellular Ca\(^{2+}\) transient to increase. Figure 4 shows that, when the muscle was stretched to 1.5 times the optimal length at which twitch force becomes maximal, where the number of force-generating cross bridges would be expected to be minimal, the intracellular Ca\(^{2+}\) transient change is shown to be similar to what resulted from length vibration (Fig. 4): first increasing and then decreasing more rapidly. However, the duration of the positive difference between the control and stretched Ca\(^{2+}\) transient (0.019 ± 0.004 s, n = 6) is shorter than for the other positive difference curves when there is maximum thick and thin filament overlap (i.e., vibration: 0.034 ± 0.003 s, n = 10; shortening: 0.036 ± 0.003 s, n = 10). This might be expected because there should only be a 10% overlap between the thin and thick filaments (as judged by the force development). The stretched muscle would correspond to a situation in which the least number of cross bridges could interact. Thus the buffering capacity of TnC would be expected to be the least under these conditions, and the Ca\(^{2+}\) transient would be predicted to be the shortest. One interesting point in Fig. 4 is that the stretched isometric twitch lasts longer than the control isometric contraction. The reason for this is not clear, but, in the stretched muscle, less internal shortening would occur because only a few cross bridges can attach and all elastic elements would also be stretched. Perhaps less internal shortening of the muscle would allow a cross bridge to remain attached longer. This phenomenon has been observed before and explained by longitudinal inhomogeneity in the duration of activity, known to occur during relaxation, coupled with the decreased compliance of stretched fibers (11).

Figure 5 shows the results of a muscle allowed to shorten freely near its maximum rate by applying a constant load to near its resting force. In this case, the muscle shortens ~3.5% of the initial muscle length. The results show that, like the length vibration or
stretching, the myoplasmic Ca\(^{2+}\) initially remains higher than for the control isometric contraction and declines more rapidly than the control toward the end of the Ca\(^{2+}\) transient. The resulting myoplasmic Ca\(^{2+}\) transients and the difference between the shortening and control isometric contraction shown in Fig. 5 are very similar to that of the same muscle when the majority of force-generating cross bridges are prevented from forming by length vibration (Fig. 3) or stretching (Fig. 4). Therefore, it appears that, when a muscle is allowed to shorten rapidly during a twitch, few force-generating cross bridges are attached at any one time, resulting in an increase in intracellular Ca\(^{2+}\).

Changes in Ca\(^{2+}\) sensitivities of force and actomyosin ATPase rate in skinned muscle fibers. The average isometric actomyosin ATPase and force at full activation are 4.1 ± 0.62 s\(^{-1}\) per myosin head [assuming a myosin head concentration of 0.154 mM (17)] and 158 ± 3.1 kN·m\(^{2}\), respectively (means ± SE, n = 6). In Fig. 6, the force and actomyosin ATPase rate are plotted as a function of pCa. As shown in Fig. 6, when a 10% sinusoidal length vibration (20 Hz) is applied during Ca\(^{2+}\) activation, the resultant ATPase rate is increased relative to the force compared with isometric ATPase rate and force. In other words, the ratio of ATPase rate and force during vibration is always larger than that during isometric contraction (Fig. 6,
This means that the length vibrations not only reduce the number of force-generating cross bridges (as judged by reduced force) but also increase the rate of dissociation of force-generating cross bridges, because the ratio of ATPase and force is a measure of the rate of dissociation of force-generating cross bridges. This conclusion is consistent with the work of other investigators who have studied different length perturbations in tetanus studies in intact skeletal muscle (9, 10, 36). The transient increase in intracellular Ca^{2+} during tetanus, shown by Vandenboom et al. (60), was interpreted to mean that the increase in intracellular Ca^{2+} was associated with cross-bridge detachment, whereas the decrease in intracellular Ca^{2+} was associated with cross-bridge reattachment (force regeneration). However, the biphasic change in intracellular Ca^{2+} was never seen during a twitch other than during tetanus. The transient increase in intracellular Ca^{2+} could be explained by Ca^{2+} release from TnC into the myoplasm because of reduced affinity of TnC for Ca^{2+}, which is caused by dissociation of force-generating cross bridges. This is consistent with the work of other investigators who have studied different length perturbations in tetanus studies in intact skeletal muscle (9, 10, 36). Stopped-flow experiments with the use of fluorescent probes attached to TnC have shown that the on rate of Ca^{2+} binding to TnC is diffusion limited (32). The affinity of TnC for Ca^{2+} can be affected only by changes in the off rate of Ca^{2+} from TnC, as shown by TnC mutation and drug experiments (32). Therefore, it would be the off rate of Ca^{2+} from TnC that is increased by length perturbations to dissociate force-generating cross bridges and looks at their effects on intracellular Ca^{2+} transient during a twitch other than during tetanus.

**DISCUSSION**

A few investigators have studied the effects of length changes on Ca^{2+} transients in intact skeletal muscle under conditions of tetanus, but, to our knowledge, no one has looked at them during a twitch. In those tetanus studies, the skeletal muscle was subjected to shortening or stretch at the beginning, plateau, or end of the tetanus, and intracellular Ca^{2+} was measured with different fluorescent probes. At the beginning and end of tetanus, shortening showed increases in intracellular Ca^{2+} (9, 36). However, shortening at the plateau produced variable effects: a biphasic change (60), a decrease (1, 11, 58), and no change in intracellular Ca^{2+} (9). The biphasic change in intracellular Ca^{2+} during tetanus, shown by Vandenboom et al. (60), was interpreted to mean that the increase in intracellular Ca^{2+} was associated with cross-bridge detachment, whereas the decrease in intracellular Ca^{2+} was associated with cross-bridge reattachment (force regeneration). Our study is unique in that it uses different length perturbations to dissociate force-generating cross bridges and looks at their effects on intracellular Ca^{2+} transient during a twitch other than during tetanus.

The transient increase in intracellular Ca^{2+} is that they all look very similar. It does not matter whether the number of force-generating cross bridges is reduced by an impulse stretch or release (Fig. 2), or by continuous length vibration (Fig. 3), or by stretching the muscle beyond overlap of the thick and thin filaments (Fig. 4), or by shortening (Fig. 5), the difference between the length perturbation and control Ca^{2+} transients is always biphasic: first increasing and then decreasing more rapidly.

The transient increase in intracellular Ca^{2+} could be explained by Ca^{2+} release from TnC into the myoplasm because of reduced affinity of TnC for Ca^{2+}, which is caused by dissociation of force-generating cross bridges. This is consistent with the work of other investigators who have studied different length perturbations in tetanus studies in intact skeletal muscle (9, 10, 36). Stopped-flow experiments with the use of fluorescent probes attached to TnC have shown that the on rate of Ca^{2+} binding to TnC is diffusion limited (32). Thus the affinity of TnC for Ca^{2+} can be affected only by changes in the off rate of Ca^{2+} from TnC, as shown by TnC mutation and drug experiments (32). Therefore, it would be the off rate of Ca^{2+} from TnC that is increased by length perturbations to dissociate force-generating cross bridges from actin, resulting in an increase in intracellular Ca^{2+}. Other possibilities, which include the changes in other intracellular Ca^{2+} buffer systems such as sarcoplasmic reticulum, parv-
albumin, and myosin light chains, could be involved in increasing intracellular Ca\(^{2+}\) during length perturbations as well. However, these seem unlikely because the muscle would not be deactivated if these mechanisms were involved. Additionally, when there is no length perturbation and the muscle is stretched to near non-actin and myosin length perturbation and the muscle is stretched to near mechanisms were involved. Furthermore, when there is no muscle would not be deactivated if these mechanisms as well. However, these seem unlikely because buffering capacity for Ca\(^{2+}\) (2, 55). For example, force-generating cross bridges are dissociated at the peak of the isometric contraction, the muscle no longer contracts (Fig. 2). This is because intracellular Ca\(^{2+}\) has fallen to such a low level at this time that it is no longer possible for Ca\(^{2+}\) to significantly bind to the thin filament because the off rate of Ca\(^{2+}\) has increased. Evidence for deactivation by length perturbation has previously been reported for both cardiac

After the increase, the Ca\(^{2+}\) transient falls more rapidly than the control at the end of the twitch, because the myoplasmic Ca\(^{2+}\) is less buffered when fewer force-generating cross bridges are attached. This decrease in myoplasm buffering capacity for Ca\(^{2+}\) would allow the sarcoplasmic reticulum to sequester intracellular Ca\(^{2+}\) faster so that, eventually, the fura 2 fluorescence 340/380 would fall faster than in the isometric control, giving rise to the biphasic fluorescence ratio difference curves shown in Figs. 2–5. Another mechanism involving Ca\(^{2+}\) rebinding to TnC, as suggested by Vandenboom et al. (60), cannot be the reason for this Ca\(^{2+}\) transient decrease in our study, because after or during length perturbations the muscle is deactivated (no force regeneration).

In contrast to our finding that the dissociation of force-generating cross bridges causes Ca\(^{2+}\) to be released from TnC, some studies in skinned skeletal fibers that measured total Ca\(^{2+}\) binding to the thin filaments (19, 20, 62), intrafibrillar Ca\(^{2+}\) after flash photolysis of caged Ca\(^{2+}\) (48), and structural changes in skeletal TnC with the use of dichroism (44, 45) did not show evidence that changes in cycling cross bridges or sarcomere length affect the amount of Ca\(^{2+}\) bound to TnC. This could be because those measurements were not sensitive enough to detect changes in the amount of Ca\(^{2+}\) bound to skeletal TnC in skeletal muscle, as acknowledged by some investigators (44, 45).

The implication of our findings is that shortening of the muscle during a twitch will cause both dissociation of cross bridges and release of Ca\(^{2+}\) from TnC during the falling phase of the Ca\(^{2+}\) transient. The Ca\(^{2+}\) transient is falling continuously throughout most of the time course of a twitch, so that, in combination with dissociation of force-generating cross bridges, the deactivation of the thin filament is accelerated. If, for example, force-generating cross bridges are dissociated at the peak of the isometric contraction, the muscle no longer contracts (Fig. 2). This is because intracellular Ca\(^{2+}\) has fallen to such a low level at this time that it is no longer possible for Ca\(^{2+}\) to significantly bind to the thin filament because the off rate of Ca\(^{2+}\) has increased. Evidence for deactivation by length perturbation has previously been reported for both cardiac
(15, 42) and skeletal (12, 14, 31, 33, 56) muscle during a twitch. Edman (12) suggested that this deactivation was based on a structural change in the myofilament system that was caused by active sliding of actin and myosin filaments. Later using fluo 3 to measure the Ca\textsuperscript{2+} transient during a tetanus, he hypothesized that the deactivation by shortening was probably due to a decrease in the affinity of troponin for Ca\textsuperscript{2+} (13). The physiological function of the decrease in the off rate of Ca\textsuperscript{2+} from TnC when force-generating cross bridges are attached is to keep the thin filament activated until either a cross-bridge dissociates because of shortening or intracellular Ca\textsuperscript{2+} falls sufficiently low that it causes Ca\textsuperscript{2+} to be removed from the TnC. In our study, the turnover rate of cross bridges in the lumbrical muscle is 4.1 s\textsuperscript{-1} (Fig. 6), and the twitch duration time is much shorter than 250 ms (Figs. 2–5). Therefore, the primary cause of a cross-bridge dissociation during the isometric twitch is the removal of Ca\textsuperscript{2+} by the sarcoplasmic reticulum. This means that the thin filament of an isometric contracting muscle would be activated for a longer time than that of a shortening muscle during a twitch. During shortening, the dissociation of cross bridges would, as shown in this study (Figs. 2, B and D, and 5), increase the off rate of Ca\textsuperscript{2+} from TnC, causing deactivation of the thin filament.

If the off rate of Ca\textsuperscript{2+} from TnC were changed, the Ca\textsuperscript{2+} sensitivity of muscle contraction would be expected to change. Our data from skinned fiber experiments show that increasing the rate of dissociation and/or reducing the number of force-generating cross bridges by sinusoidal length vibration decreases Ca\textsuperscript{2+} sensitivities of Ca\textsuperscript{2+}-activated ATPase and force. In other words, increasing the dissociation rate and/or lowering the number of cross bridges shifts pCa-ATPase and pCa-force curves to higher Ca\textsuperscript{2+} concentrations. This result further supports the hypothesis obtained from the intact muscle experiment. If the off rate of Ca\textsuperscript{2+} from TnC is accelerated by increasing the dissociation rate and/or reducing the number of cross bridges, the muscle will become less sensitive to Ca\textsuperscript{2+} and need more Ca\textsuperscript{2+} to be activated. Pi has been shown to reduce the number of cross bridges (46) and increase the dissociation rate of force-generating cross bridges (37). This could be, in part, the mechanism by which Pi shifts the pCa-force relationship to higher Ca\textsuperscript{2+} concentrations. This may also account for why 2,3-butanedione monoxime (18, 28) and pH (53) also decrease the Ca\textsuperscript{2+} sensitivity in skeletal muscle. The average iso- metric actomyosin ATPase rate [4.1 ± 0.62 s\textsuperscript{-1} per myosin head, n = 6, assuming a myosin head concentration of 0.154 mM (17)] is comparable to values found by others obtained from mammalian fast-twitch fibers under similar experimental conditions. Lumbrical muscle mostly contains fast-twitch fibers (21, 51). Stephenson et al. (54) found an isometric ATPase rate of 3.80 ± 0.53 s\textsuperscript{-1} per myosin head for rat extensor digitorum longus (fast) fibers at 21–22°C. Kawai et al. (35) found a rate of 3 s\textsuperscript{-1} per myosin head.

Our study also shows that the actomyosin ATPase is increased at maximal Ca\textsuperscript{2+} and decreased at submaximal Ca\textsuperscript{2+} concentrations, whereas the force is decreased at all Ca\textsuperscript{2+} concentrations during length vibration, compared with isometric contraction. In rabbit psoas muscle fiber, Potma et al. (49) found that the muscle fiber ATPase rate increased at high-Ca\textsuperscript{2+} and decreased at low-Ca\textsuperscript{2+} concentrations during square-wave length changes. The actomyosin ATPase rate is determined by the cycling rate of the cross bridges and the number of cycling cross bridges. The faster the cycling rate of the cross bridges or the greater the number of the cycling cross bridges, the faster the actomyosin ATPase rate. The increase in the actomyosin ATPase rate during shortening at high Ca\textsuperscript{2+} is known as the Fenn effect (16). The reason for this increase is that the rate of cycling of the cross bridges increases during shortening compared with isometric contraction, even though fewer numbers of cross bridges are attached (less force). At maximal Ca\textsuperscript{2+} concentration, although shortening decreases the affinity of TnC for Ca\textsuperscript{2+} (an increase in the off rate of Ca\textsuperscript{2+} from TnC), the Ca\textsuperscript{2+} is sufficiently high that near-maximal Ca\textsuperscript{2+} is bound to TnC. Therefore, the activation state of the thin filament would be essentially unchanged. Consequently, during shortening, there would be an increase in cross-bridge cycling rate or ATPase rate and hence the Fenn effect, which is present in intact fibers during tetanus or in skinned fibers during maximal Ca\textsuperscript{2+}-activating conditions. However, at submaximal Ca\textsuperscript{2+}, the number of cross bridges would decrease even more than the increase in the cycling rate of the cross bridges. This is because less Ca\textsuperscript{2+} binds to TnC as a result of an increase in the off rate of Ca\textsuperscript{2+} from TnC. The Ca\textsuperscript{2+} binding to the TnC determines the activation state or the number of cross bridges, which can attach. Our data suggest that shortening decreases the affinity of TnC for Ca\textsuperscript{2+} by increasing the off rate of Ca\textsuperscript{2+} from TnC. This would cause a pronounced deactivation of the thin filament and decrease further the number of cycling cross bridges at submaximal Ca\textsuperscript{2+} concentration. The result during shortening would be a decrease in actomyosin ATPase rate at submaximal Ca\textsuperscript{2+} activation compared with an isometric contraction. Thus no Fenn effect would occur.

If no deactivation of the thin filament at submaximal Ca\textsuperscript{2+} concentrations occurred, there should be a Fenn effect, but, as shown in Fig. 6, this is not the case. The Huxley (30) model predicts that fewer cross bridges would be attached (shown by Julian and Sollins (34)) and actomyosin ATPase rate (energy consumption) would be higher during shortening for both submaximal and maximal Ca\textsuperscript{2+} activation of the muscle, compared with isometric contraction. Thus our findings suggest that, at submaximal Ca\textsuperscript{2+}, skeletal muscle is deactivated during shortening by increasing the off rate of Ca\textsuperscript{2+} from TnC and decreasing further the number of cycling cross bridges, which results in less ATP usage. This would explain why the Fenn effect was not observed during a twitch in cardiac and in skeletal muscle at room temperature (50), because, in both cases, the muscle is not maximally activated.
In summary, this study suggests that dissociation or increasing the rate of dissociation of force-generating cross bridges increases the off rate of Ca\(^{2+}\) from TnC and, consequently, decreases the Ca\(^{2+}\) sensitivity and results in the deactivation of the skeletal muscle contraction. In addition, this study shows that the Fenn effect should exist only at maximal, and not submaximal, Ca\(^{2+}\) activation of force.

We thank Dr. Karl Magelby for helpful comments regarding this manuscript.

This study was supported by National Institute of Arthritis and Musculoskeletal and Skin Diseases Grant AR-90406 and the American Heart Association, Florida/Puerto Rico Affiliate.

REFERENCES


21. Gates HJ, Ridge RM, and Rowersloner A. Motor units of the fourth deep lumbral muscle of the adult rat: isotonic contrac-


