Ca$^{2+}$ Binding to Troponin C in Skinned Skeletal Muscle Fibers Assessed with Caged Ca$^{2+}$ and a Ca$^{2+}$ Fluorophore

INvariance of Ca$^{2+}$ Binding as a Function of Sarcomere Length*

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Ca$^{2+}$ sensitivity of tension varies with sarcomere length in both skeletal and cardiac muscles. One possible explanation for this effect is that the Ca$^{2+}$ affinity of the regulatory protein troponin C decreases when sarcomere length is reduced. To examine length dependence of Ca$^{2+}$ binding to troponin C, we developed a protocol to simultaneously monitor changes in sarcomere length, tension, and Ca$^{2+}$ concentration following flash photolysis of caged Ca$^{2+}$. In this protocol, [Ca$^{2+}$] was rapidly increased by flash photolysis of caged Ca$^{2+}$, and changes in [Ca$^{2+}$] due to photolysis and the subsequent binding to troponin C were assessed using a Ca$^{2+}$ fluorophore. Small bundles of fibers from rabbit skinned psoas muscles were loaded with Ca$^{2+}$ fluorophore (Fluo-3) and caged Ca$^{2+}$ (dimethoxy-nitrophenamine or o-nitrophenyl-EGTA). The bundles were then transferred to silicone oil, where [Ca$^{2+}$]$_{free}$ tension, and sarcomere length were monitored before and after photolysis of caged Ca$^{2+}$. Upon photolysis of caged Ca$^{2+}$, fluorescence increased and then decayed to a new steady-state level within $\approx 1$ s, while tension increased to a new steady-state level within $\approx 1.5$ s. After extracting troponin C, fibers did not generate tension following the flash, but steady-state post-flash fluorescence was significantly greater than when troponin C was present. The difference in [Ca$^{2+}$]$_{free}$ represents the amount of Ca$^{2+}$ bound to troponin C. In fibers that were troponin C-replete, Ca$^{2+}$ binding to troponin C did not differ at short ($\approx 1.97 \mu m$) and long ($\approx 2.51 \mu m$) sarcomere length, yet tension was $\approx 50\%$ greater at the long sarcomere length. These results show that the affinity of troponin C for Ca$^{2+}$ is not altered by changes in sarcomere length, indicating that length-dependent changes in Ca$^{2+}$ sensitivity of tension in skeletal muscle are not related to length-dependent changes in Ca$^{2+}$ binding affinity of troponin C.

The regulation of striated muscle contraction involves Ca$^{2+}$-dependent mechanisms that vary with sarcomere length in both skeletal and cardiac muscles. One possible explanation for this effect is that the Ca$^{2+}$ affinity of the regulatory protein troponin C decreases when sarcomere length is reduced. To examine length dependence of Ca$^{2+}$ binding to troponin C, we developed a protocol to simultaneously monitor changes in sarcomere length, tension, and Ca$^{2+}$ concentration following flash photolysis of caged Ca$^{2+}$. In this protocol, [Ca$^{2+}$] was rapidly increased by flash photolysis of caged Ca$^{2+}$, and changes in [Ca$^{2+}$] due to photolysis and the subsequent binding to troponin C were assessed using a Ca$^{2+}$ fluorophore. Small bundles of fibers from rabbit skinned psoas muscles were loaded with Ca$^{2+}$ fluorophore (Fluo-3) and caged Ca$^{2+}$ (dimethoxy-nitrophenamine or o-nitrophenyl-EGTA). The bundles were then transferred to silicone oil, where [Ca$^{2+}$]$_{free}$ tension, and sarcomere length were monitored before and after photolysis of caged Ca$^{2+}$. Upon photolysis of caged Ca$^{2+}$, fluorescence increased and then decayed to a new steady-state level within $\approx 1$ s, while tension increased to a new steady-state level within $\approx 1.5$ s. After extracting troponin C, fibers did not generate tension following the flash, but steady-state post-flash fluorescence was significantly greater than when troponin C was present. The difference in [Ca$^{2+}$]$_{free}$ represents the amount of Ca$^{2+}$ bound to troponin C. In fibers that were troponin C-replete, Ca$^{2+}$ binding to troponin C did not differ at short ($\approx 1.97 \mu m$) and long ($\approx 2.51 \mu m$) sarcomere length, yet tension was $\approx 50\%$ greater at the long sarcomere length. These results show that the affinity of troponin C for Ca$^{2+}$ is not altered by changes in sarcomere length, indicating that length-dependent changes in Ca$^{2+}$ sensitivity of tension in skeletal muscle are not related to length-dependent changes in Ca$^{2+}$ binding affinity of troponin C.

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oil (see Fig. 1). Light from a halogen lamp used to illuminate the fiber bundle was first passed through a cut-off filter (F1, transmission >620 nm) in order that the emitted wavelength did not interfere with either photolysis or fluorescence. After passing through the fiber bundle and a 40 x objective (Zeiss), the light (>620 nm) was transmitted through a dichroic mirror, another cut-off filter (F2, transmission >520 nm), and then split by a beam splitter (BS1). Filtered light that continued toward the photomultiplier tube was prevented from reaching the photomultiplier tube by a band-pass filter (F3, transmission between 490–530 nm). Filtered light that continued toward the eyepiece was split by a second beam splitter (BS2), so that one-half was directed to a CCD camera and the other half to the eyepiece. Images of the fiber bundle were stored on video tape and used for off-line SL measurements.

Ca\(^{2+}\) fluorophore within the fiber bundle was excited by light (λ = 475 nm) from a fluorometer (SLM Aminco, SLM Instruments, Inc., Urbana, IL) directed to the 80-μl quartz-walled trough via a fiber optic light pipe. This light was first passed through a band-pass filter (F4, transmission between 400–490 nm) and then reflected by a dichroic mirror toward the bundle of fibers in the quartz trough. Emitted fluorescence followed the same path as the filtered light from the halogen lamp, except that it passed through the band-pass filter F3 and reached the photomultiplier tube. The output signal from the photomultiplier tube was recorded and stored using SLM 8000C software package. Photolysis of caged Ca\(^{2+}\) was achieved by exposing the caged Ca\(^{2+}\) loaded fiber bundle to a single flash of UV light (~360 nm) from a flash lamp (Hi-Tech). The extent of photolysis was varied by changing the intensity of the UV light. An electronic shutter (Uni-Blitz), with a built in delay of 15 ms, was placed in front of the photomultiplier tube to protect it from the high intensity UV flashes used for photolyzing caged Ca\(^{2+}\).

**Experimental Protocol**—The SL of fiber bundles was initially set to a slack length (~1.97 μm) in relaxing solution, and maximum tension generating capacity was then determined by activating the muscle in solution of pCa 4.5. Fiber bundles were used only if a clear striation pattern was maintained during maximal activation and SL shortened by less than 10%. To assess Ca\(^{2+}\) binding during active force generation, fiber bundles were incubated for 4 min in pre-activating solution containing 0.05 mM EGTA and then bathed for 5 min in loading solution containing Flu-o-3 and either NP-EGTA or DM-nitrophen to ensure uniform distribution of these compounds within the fiber bundle. Next, fiber bundles were transferred to a quartz chamber containing silicone oil where they were exposed to a 1-ms flash of UV light. The resulting changes in tension, fluorescence intensity, and SL were recorded, and the fiber bundles were then transferred back to relaxing solution. In some experiments, variable amounts of caged Ca\(^{2+}\) were photolyzed using multiple intensities of UV light. Maximum tension at pCa 4.5 was determined following each UV flash, and fiber bundles were discarded if tension fell by more than 10% of its initial value. SL was then increased to a long length (~2.51 μm) while the bundle was in relaxing solution, and the above protocol was repeated.

Following characterization of length dependence of Ca\(^{2+}\) binding, TnC was extracted from the fiber bundle to characterize TnC-independent Ca\(^{2+}\) binding. TnC was extracted by first incubating the fiber bundles for 2 min in a solution containing 195 mM potassium propionate and 10 mM BES (pH 7.0) followed by 2 min in solution containing 165 mM potassium propionate, 10 mM BES, and 10 mM EDTA (pH 7.0) and finally 30 min in TnC extracting solution containing 10 mM BES, 5 mM EDTA and 0.2 mM trifluoperazine (pH 7.0, 2 x solution change). This protocol completely depleted the fibers of TnC as judged by the absence of active tension at pCa 4.5 and the absence of TnC from SDS gels of the fiber bundles. After extraction of TnC, the fiber bundles were washed in relaxing solution for 30 min (2 x solution change), and Ca\(^{2+}\) binding was assayed using flash photolysis of caged Ca\(^{2+}\) as described above. To determine [Ca\(^{2+}\)]\(\text{CALC}\) from fluorescence signals recorded before and after flash photolysis, a calibrated fluorescence-pCa relationship for each preparation was determined at both short and long SL. The protocol for this determination was the same as described above except that loading solution was replaced by a range of Ca\(^{2+}\) standard solutions prepared by mixing stocks of pCa 9.0 and pCa 4.5. At the end of an experiment, the fiber bundle was cut at the points of attachment and placed in SDS-PAGE sample buffer for protein analysis.

**Solutions**—Solutions were made using the computer program of Faibato (11) and the stability constants listed by Godt and Lindley (12). The stability constants were corrected to pH 7 and 15°C. In addition, all the solutions listed in Table I contained 100 mM BES, 15 mM creatine phosphate, and 5 mM dithiothreitol, and ionic strength was adjusted to 180 mM with potassium propionate. Loading solution also contained either 0.92 mM DM-nitrophen (Calbiochem) or 0.92 mM NP-EGTA (Molecular Probes, OR), 0.025 mM Flu-o-3 (Calbiochem), and 100 units/ml creatine kinase (Calbiochem). In some experiments, loading solution also contained 0.05 mM calmodulin that was purified from bull testes using procedures described by Dedman et al. (13).

**RESULTS**—The effect of SL on Ca\(^{2+}\) sensitivity of isometric tension in fast skeletal muscle fibers is shown in Fig. 2. The concentration of Ca\(^{2+}\) required to produce half-maximal tension (i.e. pCa\(_{50}\)) was 5.77 ± 0.02 at short SL (1.94 ± 0.01 μM) in the presence of 1

**Table I**

| Solution | EGTA | Total ATP | Total Mg\(^{2+}\) | Total CaCl\(_2\) |
|----------|------|-----------|-----------------|-----------------|
| Relaxing |      |           |                 |                 |
| pCa 9.0  | 5.00 | 13.60     | 5.15            | 0.01            |
| Preactivating | 0.05 | 13.70     | 5.14            |                 |
| Activating  | 5.00 | 14.70     | 5.14            | 5.83            |
| Loading   |      |           |                 |                 |
| pCa 4.5  |      |           |                 |                 |
| Preactivating | 0.05 | 15.46     | 14.30           |                 |
| Activating  | 5.00 | 15.46     | 14.18           | 5.91            |
| Loading   |      |           |                 |                 |

Solutions used in DM-nitrophen experiments

| Relaxing |      |           |                 |                 |
| pCa 9.0  | 5.00 | 15.31     | 14.40           | 0.01            |
| Preactivating | 0.05 | 15.46     | 14.30           |                 |
| Activating  | 5.00 | 15.46     | 14.18           | 5.91            |
| Loading   |      |           |                 |                 |

Solutions used in NP-EGTA experiments

**Fig. 1. Schematic diagram of the recording system.** For details see "Materials and Methods."
mm free Mg\(^{2+}\) (Fig. 1A). At long SL (2.48 ± 0.05 μm) pCa\(_{50}\) shifted to a lower Ca\(^{2+}\) concentration, i.e. pCa\(_{50}\) = 5.95 ± 0.01, indicating an increase in Ca\(^{2+}\) sensitivity of tension. Thus, the Ca\(^{2+}\) sensitivity of tension changes as function of SL in small bundles of psoas fibers, and the magnitude of this effect is quantitatively similar to that previously observed in single psoas fibers (3). In the presence of 0.1 mM free Mg\(^{2+}\) (Fig. 2B), the pCa\(_{50}\) at short SL was 5.95 ± 0.02 and at long SL shifted to 6.27 ± 0.02. Thus, the length-dependent shift in Ca\(^{2+}\) sensitivity of tension over a similar range of SL was much greater when free Mg\(^{2+}\) was lowered from 1 mM to 0.1 mM.

Ca\(^{2+}\) Binding to TnC and Tension Following Flash Photolysis of Caged Ca\(^{2+}\)—Prior to flash photolysis of caged Ca\(^{2+}\), [Ca\(^{2+}\)]\(_{\text{free}}\) within the fiber bundle was pCa 6.8 before flash photolysis of DM-nitrophen, and there was essentially no active tension, even when free Mg\(^{2+}\) was as low as 0.1 mM. Following photolysis, the Ca\(^{2+}\) binding affinity of DM-nitrophen changes from 2 × 10\(^8\) to 3.33 × 10\(^2\) M\(^{-1}\), resulting in the rapid release of Ca\(^{2+}\) (14). Thus, [Ca\(^{2+}\)]\(_{\text{free}}\) rises rapidly following the flash, which is evident in the fluorescence signal in Fig. 3A, and then decays to a new steady-state level that is intermediate between baseline and peak. Coincident with the Ca\(^{2+}\) transient, tension increases and attains a new steady-state level (Fig. 3B). In this example, the steady-state [Ca\(^{2+}\)]\(_{\text{free}}\) increased from pCa 6.80 pre-flash to a final level of pCa 6.27 post-flash, whereas tension increased from −0.05 P\(_0\) to −0.75 P\(_0\). Mean SL was also monitored during changes in free Ca\(^{2+}\) and tension following flash photolysis. Photomicrographs obtained before and after each flash (Fig. 4) indicated that there was minimal sarcomere shortening in the fiber bundle following the flash; in this case, SL shortened from 2.59 to 2.38 μm during the development of tension.

When this protocol was repeated after extracting TnC from the fiber bundle, the fiber bundle failed to generate tension upon photogeneration of Ca\(^{2+}\), and the post-flash steady-state [Ca\(^{2+}\)]\(_{\text{free}}\) was elevated (pCa 5.97) relative to the pre-TnC extraction value. SDS-polyacrylamide gel electrophoresis confirmed that virtually all the TnC was extracted from the fiber bundles (Fig. 5). Based on these results we conclude that part of the Ca\(^{2+}\) released as a result of photolysis of DM-nitrophen...
binds to TnC and induces activation of tension in these fiber bundles.

Similar results were obtained when NP-EGTA was used (Fig. 6) in place of DM-nitrophen. In this case, \([\text{Ca}^{2+}]_{\text{free}}\) increased from \(p\text{Ca} 6.47\) before the flash to \(p\text{Ca} 5.96\) following the flash, consistent with a decrease in the affinity of NP-EGTA for \(\text{Ca}^{2+}\) from \(1.25 \times 10^2\) to \(1.0 \times 10^5\) M\(^{-1}\) (15). After TnC extraction, photolysis increased \([\text{Ca}^{2+}]_{\text{free}}\) from \(p\text{Ca} 6.47\) to 5.14 (Fig. 6A). Corresponding to steady-state \([\text{Ca}^{2+}]_{\text{free}}\) following flash photolysis, the post-flash steady-state tensions were 0.6 \(P_0\) before and 0.05 \(P_0\) after TnC extraction (Fig. 6B).

**Ca\(^{2+}\) Binding to TnC at Short and Long SL**—Figs. 7 and 8 show effects of length on flash-induced changes in \(\text{Ca}^{2+}\) binding to TnC (Fig. 7A) and tension (Fig. 7B). Photomicrographs of the same bundle are shown in Fig. 8. At both short and long SL, \([\text{Ca}^{2+}]_{\text{free}}\) prior to the flash was approximately \(p\text{Ca} 6.67\) and tension was less than 5% of peak. Upon exposing the bundle to a flash of 40% of maximum intensity, steady-state \([\text{Ca}^{2+}]_{\text{free}}\) increased to \(p\text{Ca} 6.13\) at short SL (2.03 \(\mu\text{m}\)) and 6.26 at long SL (2.57 \(\mu\text{m}\)). This increase in \([\text{Ca}^{2+}]_{\text{free}}\) at short SL elicited isometric tension equivalent to 0.55 \(P_0\), and at long SL tension increased to 0.75 \(P_0\). In the same bundle, when flash intensity was reduced to 27% of maximum intensity, steady-state \([\text{Ca}^{2+}]_{\text{free}}\) and fractional tension \((P/P_0)\) were \(p\text{Ca} 6.31\) and 0.25, respectively, at short SL and 6.41 and 0.50, respectively, at long SL (data not shown). When the same fiber bundle was exposed to a flash of 40% maximum intensity following TnC extraction, the fiber bundle did not generate tension (Fig. 7D), and the steady-state \([\text{Ca}^{2+}]_{\text{free}}\) increased from \(p\text{Ca} 6.72\) to 6.05 at both short length and long length. Mean values of results from several experiments are presented in Table II. At both flash intensities, \([\text{Ca}^{2+}]_{\text{free}}\) values were similar at short and long lengths, indicating that \(\text{Ca}^{2+}\) binding by TnC does not vary with SL. In contrast, tension was significantly greater at long SL than at short SL, indicating that the SL dependence of submaximal tension does not involve a change in the extent of \(\text{Ca}^{2+}\) binding to TnC. Also, the similarity of steady-state \([\text{Ca}^{2+}]_{\text{free}}\) values recorded at short and long length after TnC extraction indicates that there was little variation in the amount of \(\text{Ca}^{2+}\) released when DM-nitrophen was photolyzed at the two lengths.

We also used NP-EGTA to investigate length dependence of \(\text{Ca}^{2+}\) binding to TnC. Data from one experiment is shown in Fig. 9, and a summary of the results is presented in Table III. In the example shown, the fiber generated <5% tension both at short and long SL prior to flash photolysis of NP-EGTA. Using flashes with 90% of maximum output intensity, steady-state
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Calculation of the Amount of Ca\(^{2+}\) Bound to TnC—The amounts of Ca\(^{2+}\) bound to TnC before and after flash photolysis of caged Ca\(^{2+}\) were calculated using Fabiato’s solution program (11). We first calculated the extent of photolysis of DM-nitrophen or NP-EGTA, as appropriate. By adjusting our estimate of percent photolysis, we were able to match calculated values of [Ca\(^{2+}\)]\text{free} to those measured in TnC-extracted fibers both before (resting level) and after (steady-state level) photolysis of caged Ca\(^{2+}\) chelators at the two sarcomere lengths (see Tables II and III, respectively). As described under “Materials and Methods,” the measured values of [Ca\(^{2+}\)]\text{free} were calibrated by recording fluorescence intensity as a function of pCa in standard Ca\(^{2+}\) solutions applied to each bundle at both long and short SL. This calculation was done with an iterative process using binding affinities listed in Table IV and assuming 1) that the concentrations of other Ca\(^{2+}\) buffering ligands remained constant and 2) that no TnC remained in the fiber bundles following extraction. The calculated extent of photolysis of DM-nitrophen averaged 28.8 and 34.8% of the total at flashes that were 27 and 40% of maximum intensity, respectively. With respect to NP-EGTA, a flash that was 90% maximum intensity resulted in 25% photolysis of total NP-EGTA.

In the second part of the calculation, TnC was included as a Ca\(^{2+}\) buffering ligand, but all other variables that had been determined in the first part of the calculation were kept constant. The concentrations of the low and high affinity divalent cation binding sites on TnC were each assumed to be 180 \(\mu\)M. This assumption is based on an earlier determination of TnC content (770 pmol/mg myofibril protein) in rabbit muscle (16). By adjusting the affinities of the high and low affinity sites, we matched the calculated [Ca\(^{2+}\)]\text{free} to the mean values of steady-state [Ca\(^{2+}\)]\text{free} recorded before (resting level) and after (steady-state level) photolysis reported in Tables II and III. Table V summarizes the calculated steady-state [Ca\(^{2+}\)]\text{free} together with the mean values of steady-state [Ca\(^{2+}\)]\text{free} determined experimentally and the amount of Ca\(^{2+}\) bound to low affinity sites on TnC. Ca\(^{2+}\) bound to TnC was much lower in the presence of 0.1 mM free Mg\(^{2+}\) (DM-nitrophen data) than in the presence of 1 mM free Mg\(^{2+}\) (NP-EGTA data).

Additional experiments were done to verify that the changes in fluorescence intensity provided accurate reports of Ca\(^{2+}\) binding to TnC. Similar to TnC, calmodulin also has four divalent cation binding sites. From measurements on calmodulin purified from rat testis, Dedman et al. (13) suggested that all four sites on calmodulin are Ca\(^{2+}\)-specific and all four sites have a Ca\(^{2+}\) binding affinity of 2.4 \(\mu\)M (\(K_C = 4.5 \times 10^5 \text{ M}^{-1}\)). Since this binding affinity is similar to that of the low affinity sites on TnC used in the present calculations (\(K_C = 3.00 \times 10^5 \text{ M}^{-1}\)), addition of 45 \(\mu\)M calmodulin (180 \(\mu\)M Ca\(^{2+}\) binding sites) to TnC-extracted fibers should decrease the post-flash fluorescence intensity to the same level as that recorded in TnC-replete fibers. We tested this prediction by recording changes in tension (Fig. 10B) and fluorescence intensity (Fig. 10A) following photolysis of NP-EGTA 1) before TnC extraction without added calmodulin and 2) after TnC extraction in both the absence and presence of 50 \(\mu\)M calmodulin. Before TnC extraction, photolysis of NP-EGTA increased steady-state tension from 0.05 to 0.5 \(P_o\) (0.49 \pm 0.02, \(n = 6\)) and increased steady-state [Ca\(^{2+}\)]\text{free} from pCa 6.74 (6.73 \pm 0.05) to pCa 6.27 (6.27 \pm 0.06). After TnC extraction, photolysis of NP-EGTA had no effect on steady-state tension, whereas the steady-state [Ca\(^{2+}\)]\text{free} increased from pCa 6.69 (6.69 \pm 0.05) to pCa 6.05 (5.99 \pm 0.07) in the absence of calmodulin and from pCa 6.70 (6.70 \pm 0.02) to pCa 6.27 (6.22 \pm 0.06) in the presence of 50 \(\mu\)M calmodulin. Using the calculation method described above, the pre- and post-flash concentrations of Ca\(^{2+}\) bound to the Ca\(^{2+}\)-specific sites on TnC were 10.18 and 27.24 \(\mu\)M, respectively, and to those on calmodulin were 15.07 and 37.82 \(\mu\)M, respectively. This difference can be accounted for by the higher concentration and binding affinity of the Ca\(^{2+}\)-specific sites used in the calculation for calmodulin as compared with those for TnC. These data corroborate our estimated values for concentration and binding affinity of Ca\(^{2+}\)-specific sites on TnC used to calculate the amount of Ca\(^{2+}\) bound to TnC at short and long lengths.

DISCUSSION

A New Technique for Assessing Ca\(^{2+}\) Binding to TnC—This study reports a novel technique for measuring the extent of...
Ca\textsuperscript{2+} binding in skinned muscle fibers, particularly binding to the thin filament regulatory protein TnC. The technique was based on the premise that the Ca\textsuperscript{2+} released following flash photolysis of either DM-nitrophen or NP-EGTA would be buffered to a greater extent in the presence of TnC than in its absence. This idea was confirmed in our studies, since the steady-state fluorescence intensity of Fluo-3 following flash photolysis substantially increased following near-stoichiometric extraction of TnC (Figs. 3 and 6). Additionally, similar differences in steady-state fluorescence were observed when caged Ca\textsuperscript{2+} was photolyzed in the presence and absence of TnC in solution or in rigor muscle fibers before and after extraction of TnC (data not shown). These controls demonstrate that the differences in pre- and post-flash steady-state fluorescence signals in the present study were specifically due to Ca\textsuperscript{2+} binding to TnC and were not artifacts due, for example, to movements of the fiber bundles during tension development.

We found that small changes in [Ca\textsuperscript{2+}] in the range of pCa between 7.0 and 5.3 produced large changes in the fluorescence intensity of Fluo-3 (fluorescence-pCa relationship not shown). Thus, to optimize resolution of small changes in steady-state [Ca\textsuperscript{2+}], the experimental conditions were designed such that steady-state [Ca\textsuperscript{2+}]\textsubscript{free} at rest and following photolysis of DM-nitrophen (0.1 mM free Mg\textsuperscript{2+}) or NP-EGTA (1.0 mM free Mg\textsuperscript{2+}), either before or after TnC extraction, fell within the range of [Ca\textsuperscript{2+}] between pCa 7.0 and 5.3. Even with this constraint, we were able to examine Ca\textsuperscript{2+} binding to TnC over a wide range of tension (~0.20 to ~0.75 P\textsubscript{0}) in the presence of 0.1 mM. However, at 1.0 mM free Mg\textsuperscript{2+}, the range of tensions investigated was smaller because at higher force the steady-state [Ca\textsuperscript{2+}]\textsubscript{free} determined after TnC extraction was calculated to be greater than pCa 5.3 (see Fig. 9). Since even a large change in [Ca\textsuperscript{2+}] in the pCa range between 5.3 and 4.5 produces only a small change in fluorescence intensity of Fluo-3, the [Ca\textsuperscript{2+}]\textsubscript{free} at pCa < 5.3 could not be determined with certainty.

Length Dependence of Ca\textsuperscript{2+} Binding—The variation in Ca\textsuperscript{2+} sensitivity of tension with SL that we observed was similar to that previously reported in single psoas fibers (3). However, there was a marked difference in length dependence of Ca\textsuperscript{2+} sensitivity at the two concentrations of free Mg\textsuperscript{2+} used. The difference in pCa\textsubscript{50} at short and long SL in the presence of 0.1 mM free Mg\textsuperscript{2+} was almost twice the difference recorded in the presence of 1.0 mM free Mg\textsuperscript{2+}, i.e. the difference at 0.1 mM free Mg\textsuperscript{2+} was 0.32 pCa unit, whereas at 1.0 mM free Mg\textsuperscript{2+} the difference was 0.18 pCa unit. Previous studies on frog skinned skeletal fibers (17) and rabbit skinned psoas fibers (18) reported...
that the Ca^{2+} sensitivity of tension increased when [Mg^{2+}]_{free} was reduced. The mechanism underlying the shift in Ca^{2+} sensitivity of tension with changes in [Mg^{2+}]_{free} is not yet known.

The idea that changes in Ca^{2+} binding affinity of TnC might account for length-dependent shifts in Ca^{2+} sensitivity of tension in fast skeletal fibers has been considered previously.

**FIG. 8.** Short and long SL following flash photolysis of DM-nitrophen. Pre- and post-flash video images (scale, 1.0 cm = 20 μm) of the fiber bundle were recorded along with fluorescence intensity and tension as in Fig. 7. The mean pre- and post-flash short SL values were 2.03 and 1.87 μm, respectively, and pre- and post-flash long SL values were 2.57 and 2.44 μm, respectively.

**TABLE II**

| Flash intensity | SL (μm) | Post-flash force | Concentration of free Ca^{2+} (pCa) |
|-----------------|---------|-----------------|-----------------------------------|
|                 | Pre-flash | Post-flash | Pre-flash (0 s) | Post-flash (1 s) |
| **Before TnC extraction** | | | | |
| 27 Short | 1.97 ± 0.02 | 1.85 ± 0.02 | 0.25 ± 0.02 | 6.69 ± 0.01 |
| Long | 2.51 ± 0.02 | 2.36 ± 0.03 | 0.51 ± 0.02^a | 6.73 ± 0.02 |
| 40 Short | 1.98 ± 0.02 | 1.84 ± 0.03 | 0.51 ± 0.03 | 6.69 ± 0.01 |
| Long | 2.48 ± 0.02 | 2.35 ± 0.02 | 0.75 ± 0.03^a | 6.73 ± 0.02 |
| **After TnC extraction** | | | | |
| 27 Short | 1.97 ± 0.02 | 1.95 ± 0.02 | 0.00 | 6.70 ± 0.01 |
| Long | 2.51 ± 0.02 | 2.54 ± 0.03 | 0.00 | 6.71 ± 0.01 |
| 40 Short | 1.98 ± 0.02 | 1.95 ± 0.03 | 0.00 | 6.70 ± 0.01 |
| Long | 2.51 ± 0.02 | 2.54 ± 0.02 | 0.00 | 6.71 ± 0.02 |

^a Statistically significant difference between the post-flash force at long and short SL (p < 0.001).
Using isotopic Ca\(^{2+}\) binding methods, Hofmann and Fuchs (6) reported that Ca\(^{2+}\) binding to TnC changed as a function of SL in cardiac muscle but not in either slow (6) or fast (7) skeletal muscles. Using our new approach, we also found that the extent of Ca\(^{2+}\) buffering by TnC following flash photolysis of caged Ca\(^{2+}\) does not change with SL in bundles of skinned psoas fibers. Despite similar Ca\(^{2+}\) binding at long and short SL, submaximal tension was significantly greater at long SL than at short SL even when the concentration of activating Ca\(^{2+}\) was identical. This result indicates that submaximal tension changes as a function of SL per se and is not a consequence of length-induced changes in the amount of activator Ca\(^{2+}\) bound to TnC. One mechanism that may be involved in length dependence of submaximal tension is the decrease in lateral spacing of thick and thin filaments, i.e., interfilament lattice spacing, as fibers are stretched to longer lengths (3, 19, 20).

**FIG. 9.** Length dependence of fluorescence intensity and tension recorded simultaneously in a bundle of skinned psoas fibers following flash photolysis of NP-EGTA. After incubating the bundle of psoas fibers in loading solution (0.75 mM Ca\(^{2+}\), 0.92 mM NP-EGTA, 0.025 mM Fluo-3, 1.0 mM free Mg\(^{2+}\)) for 5 min, the bundle was transferred to a quartz trough filled with silicone oil. Fluorescence intensity (A before and C after TnC extraction) and tension (B before and D after TnC extraction) were recorded simultaneously before and after exposing the bundle to a maximum intensity flash of UV light (F). Short SL was 1.95 \(\mu\)m, and long SL was 2.54 \(\mu\)m before flash photolysis. The numbers beside the fluorescence intensity traces represent the [Ca\(^{2+}\)]\(_{i}\) in pCa units. Tension is expressed as a fraction of the maximum tension developed by the same bundle at pCa 4.5.

**TABLE III**

| SL, force, and [Ca\(^{2+}\)] in psoas fiber bundles before and after flash photolysis of NP-EGTA at long and short SL |
| Mean \(\pm\) S.E. of the data acquired in four experiments using NP-EGTA. |
| Flash intensity % maximum | SL (\(\mu\)m) | Pre-flash | Post-flash | Concentration of free Ca\(^{2+}\) (pCa) |
| --- | --- | --- | --- | --- |
| Before TnC extraction | | Pre-flash | Post-flash | |
| 90 | Short | 1.99 \(\pm\) 0.02 | 1.83 \(\pm\) 0.02 | 0.30 \(\pm\) 0.03 | 6.45 \(\pm\) 0.04 | 5.92 \(\pm\) 0.02 |
| | Long | 2.54 \(\pm\) 0.04 | 2.40 \(\pm\) 0.05 | 0.62 \(\pm\) 0.04\(^*\) | 6.44 \(\pm\) 0.06 | 5.88 \(\pm\) 0.02 |
| After TnC extraction | | | | |
| 90 | Short | 1.96 \(\pm\) 0.02 | 1.92 \(\pm\) 0.04 | 0.00 | 6.46 \(\pm\) 0.05 | 5.41 \(\pm\) 0.09 |
| | Long | 2.52 \(\pm\) 0.03 | 2.48 \(\pm\) 0.04 | 0.00 | 6.42 \(\pm\) 0.06 | 5.31 \(\pm\) 0.03 |

\(^*\) Statistically significant difference between the post-flash force at long and short SL (\(p < 0.001\)).
Reduced lateral spacing would increase the probability of cross-bridge interaction with actin and thus the amount of tension. Increased numbers of cross-bridges would directly increase tension and also indirectly by cooperatively activating the thin filament. These mechanisms are under investigation using the approach described here.

**Calculated Ca\(^{2+}\) Binding Affinity of Low Affinity Sites on TnC in Skinned Psoas Fibers**—To determine the amount of Ca\(^{2+}\) bound to low affinity site of TnC in the presence of 1 mM free Mg\(^{2+}\), we first assumed a concentration of 0.18 mM for the low affinity sites based on the concentration of TnC (0.09 mM) determined by Yates and Greaser (16). We also assumed a K\(_{Ca}\) of 5.0 \times 10^{-6} \text{ M} for the low affinity sites and K\(_{Mg}\) of 5.0 \times 10^{-8} \text{ M} and 5.0 \times 10^{-4} \text{ M} for the high affinity divalent cation binding sites, as previously reported by Potter and Gergely (1). These constants were also used previously to model the time course of Ca\(^{2+}\) binding to Ca\(^{2+}\) binding proteins in response to trains of transient increases in the free myoplasmic [Ca\(^{2+}\)] (21). Using these values, our calculated concentrations of free Ca\(^{2+}\) at rest (pre-flash) and following 25% photolysis (post-flash) of NP-EGTA were pCa 6.86 and pCa 6.61, respectively, which significantly underestimated the actual [Ca\(^{2+}\)]\(_{\text{free}}\) as determined from our calibration of fluorescence intensity. In order to achieve the measured values of [Ca\(^{2+}\)]\(_{\text{free}}\) using the computer simulation, K\(_{Ca}\) for the low affinity sites on TnC had to be adjusted from 5.0 \times 10^{-6} to 5 \times 10^{-5} \text{ M} and the K\(_{Mg}\) for the high affinity sites from 5 \times 10^{-8} to 1 \times 10^{-6} \text{ M}, while keeping K\(_{Mg}\) and the concentration of TnC constant. Alternatively, the measured and calculated values of steady-state [Ca\(^{2+}\)]\(_{\text{free}}\) could be matched by assuming the binding affinities previously reported by Potter and Gergely (1) but a concentration of TnC of 0.02 mM rather than 0.09 mM. In the present study we were unable to measure the concentration of TnC in our fiber bundles using SDS-PAGE, primarily because we could not precisely determine the diameter of each fiber in the fiber bundles. Even so, it is highly unlikely that the concentration of TnC in the fiber bundles was as low as 0.02 mM, since this is much lower than the concentration of TnC determined accurately by Yates and Greaser (16) and the concentration determined by Fuchs and Black (22) by SDS-PAGE of single fibers. Furthermore, by adding calmodulin to our assay system, we have demonstrated that both the concentration and the Ca\(^{2+}\) binding affinity of low affinity sites on TnC assumed in our calculations are reasonable. Using the Ca\(^{2+}\) binding affinity for the Ca\(^{2+}\)-specific site on calmodulin determined in solution by Dedman et al. (13), we found close agreement between calculated and measured values of pre- and post-flash steady-state [Ca\(^{2+}\)]\(_{\text{free}}\). This finding with calmodulin in solution suggests that the difference in the binding affinity of TnC required in our calculations and that determined in solution by Potter and Gergely (1) is likely due to differences in Ca\(^{2+}\) binding by TnC in solution as compared with TnC in the intact myofilament. Zot and Potter (23) have also suggested that TnC in the regulated thin filament has a lower affinity for Ca\(^{2+}\) than that in whole troponin alone. Such a mechanism would also explain why we were able to use the same constants to calculate the steady-state [Ca\(^{2+}\)]\(_{\text{free}}\) in the presence of 0.1 and 1.0 mM free Mg\(^{2+}\).

When using NP-EGTA, an apparently greater amount of Ca\(^{2+}\) bound to TnC in order to increase tension above 0.5 P\(_{\text{Ca}}\). This can be seen in Table V by comparing the amount of Ca\(^{2+}\) bound post-flash; using DM-nitrophen, a relative tension of 0.75 P\(_{\text{Ca}}\) is achieved when a calculated 26–27 \mu M Ca\(^{2+}\) is bound, but to achieve a relative tension of 0.59 P\(_{\text{Ca}}\) when NP-EGTA is used, 56 \mu M Ca\(^{2+}\) must be bound. Such differences are most likely due to a shift in the tension-pCa relationship to

### Table IV

| Extent of photolysis of DM-nitrophen | Pre-photolysis | Post-photolysis |
|-------------------------------------|---------------|----------------|
| 0.0%                                | 28.8% by 27% and 34.8% by 40% flash intensity | 25% by 90% flash intensity |

### Table V

#### Calculated concentration of Ca\(^{2+}\) bound to low affinity binding site on TnC in skinned psoas fibers

| Flash intensity % maximum | Photolysis | [Ca\(^{2+}\)]\(_{\text{free}}\) (pCa) | [Ca\(^{2+}\)]\(_{\text{bound}}\) (\mu M) | Force (\mu N) |
|---------------------------|------------|------------------------------------|-------------------------------------|--------------|
|                          |            | Pre-flash | Post-flash | Pre-flash | Post-flash |          |
|**DM-nitrophen data (0.1 mM free Mg\(^{2+}\))**|            |            |            |            |            |          |
| 27                        | Exp.       | 6.73      | 6.39      | 9.00      | 21.15      | 0.51     |
| 40                        | Exp.       | 6.73      | 6.22      | 9.00      | 26.53      | 0.75     |
|**NP-EGTA data (1.0 mM free Mg\(^{2+}\))**|            |            |            |            |            |          |
| 90                        | Exp.       | 6.44      | 5.88      | 15.94     | 56.12      | 0.59     |
higher \([Ca^{2+}]\) when \([Mg^{2+}]\) is increased (24). Because of this change in \(Ca^{2+}\) sensitivity of tension, more \(Ca^{2+}\) would be required at 1.0 mM free \(Mg^{2+}\) than at 0.1 mM free \(Mg^{2+}\) to achieve a given level of sub-maximal tension.

It is evident from examination of the data with NP-EGTA (Table V) that disproportionately more \([Ca^{2+}]\) must be bound to achieve a tension of 0.59 \(P_0\) (56 \(\mu M\) \(Ca^{2+}\)) than is required to achieve a tension of 0.49 \(P_0\) (27 \(\mu M\) \(Ca^{2+}\)). Although the mechanism for this effect is not known for certain, activation of tension at low \(Ca^{2+}\) has been shown to involve significant cooperative activation of the thin filament due to strong binding of cross-bridges, but at tensions greater than half-maximal, such cooperativity is much reduced (25). Thus, when the level of sub-maximal activation is relatively high, more \(Ca^{2+}\) would be required to achieve a given increment in isometric tension. Firm conclusions about the mechanisms of effects on bound \(Ca^{2+}\) due to \([Mg^{2+}]\) and level of activation will require additional work.

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