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Central helix role in the contraction-relaxation switching mechanisms of permeabilized skeletal and smooth muscles with genetic manipulation of calmodulin

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A prominent common feature of calmodulin and troponin structures is the unusually long central helix which separates the two lobes, each containing two Ca^{2+}-binding sites. To study the role of certain highly conserved residues in the helix in the contraction-relaxation switching mechanism in muscle, we measured the Ca^{2+}-activated force of permeabilized skeletal and smooth muscles with three genetically manipulated forms of calmodulin. Mutated calmodulin was made to substitute for troponin-C in vertebrate skeletal fiber. The mutants had 1–4 deletions in the conserved cluster (positions 81–84) in the solvent-exposed region of the central helix, which also substantially shortened the helix. The force of the maximally activated fiber was found to be diminished only with the mutant in which the entire cluster Ser-81 to Glu-84 (CaMΔ81–84) was deleted. All such deletions were found to be completely ineffective in blocking the Ca^{2+}-switching process in smooth muscle strips. The results show for the first time that at least a part of the highly conserved four-residue cluster in the central helix is critical for the contraction mechanism of striated muscle. Further, the possibility is raised that the reduced length of the central helix may be a determining factor in the Ca^{2+}-switching mechanism in fast-twitch muscle. These findings combined with the results on smooth muscle indicate diversity in the structure-function specifications for the central helix of calmodulin for different target proteins.

Calcium; Troponin C; Parvalbumin; Oncomodulin

1. INTRODUCTION

TnC and CaM are highly homologous proteins (approx. 75% sequence similarity) and represent a broad class of calcium-modulated proteins with repeated helix-loop-helix motifs for Ca^{2+} binding, first described in parvalbumin [1–4]. TnC is a function-specific protein operating the contraction-relaxation switch in vertebrate striated muscles. CaM can be substituted for TnC under experimental conditions [5,6]. Also, CaM operates the contraction switch in smooth muscle. A great deal is now known about the tertiary structures of CaM and TnC from their sequences and high resolution X-ray studies [7–9]. However, the molecular mechanisms of the Ca^{2+}-switch with either of these calcium-modulated proteins are still not well understood. The recent availability of genetically altered forms of CaM and TnC [10–15], and the ability to substitute them for their native analogs in permeabilized preparations of muscle fibers [15–18], offer remarkable possibilities to advance the understanding of the switching mechanisms in structurally competent milieus [4]. The present study utilizes this strategy with CaM mutants and uses both striated and smooth muscles.

Both CaM and TnC are dumbbell-shaped, and two Ca^{2+}-binding motifs are present in each of the lobes. A prominent feature is the unusually long central helix (approx. 40 Å in CaM, 44.5 Å in TnC) that separates the lobes [7–9]. The solvent-exposed region of the central helix in these proteins contains a highly conserved domain of four residues (Ser-Glu-Glu-Glu). Our aim was to investigate the role of this domain in the contraction-relaxation switching mechanism.

Three genetically manipulated forms of calmodulin were used. These had deletions of Glu-84 (CaMΔ84), Glu-83 and Glu-84 (CaMΔ83–84), or Ser-81 through Glu-84 (CaMΔ81–84) [12]. This particular amino acid cluster has been the subject of several recent studies on calmodulin, but the previous investigations were limited to in vitro studies of isolated proteins. Presently, by substituting the CaM mutants for TnC in fast-twitch fibers, we show that the deletion of this four-

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Abbreviations: CaM, calmodulin; TnC, troponin-C; TnI, troponin I; TnT, troponin T; TM, tropomyosin; LCs, myosin light chains; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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residue cluster, with the associated decrease in central helix length, is critical in the trigger mechanism of muscle contraction. In contrast, the results with smooth muscle strips indicate that its contraction-relaxation switch, operated by the myosin light chain kinase (MLCK) activity under the control of calmodulin, acts independently of this cluster, as in the in vitro results. The possibility is also raised that the reduced length of the central shaft may be a critical factor for the switch in skeletal muscle.

2. MATERIALS AND METHODS

2.1. *Tissue and fiber preparation*

For striated muscle fibers, small bundles (1–2 mm by 4–10 mm) of psoas muscle of adult rabbit were tied to sticks and stored overnight at −20°C for chemical skinning in solution containing (in mM): 150 potassium propionate, 5 Mg-acetate, 5 EGTA, 5 ATP, 1 dithiothreitol, 50% v/v glycerol, and pH 7.00. Single fibers were selected on the basis of sarcomere uniformity and pSr activation, as described previously [6]. The fiber was attached to the force transducer, and transferred to the relaxing solution (typically (in mM): 100 potassium propionate, 20 imidazole, 6.06 $MgCl_2$, 5 ATP, 5 EGTA, 20 phosphocreatine, pH 7.00 and ionic strength 190–200; 1 free Mg^{2+}). In each case, the fiber was briefly treated with 0.5% Triton-X detergent (2 min treatment at 10°C). Activating solutions were made by appropriately varying the Ca^{2+}-EGTA to EGTA ratio. Experiments were carried out at the sarcomere length of 2.5 μm as monitored by laser diffraction. Activations of these fibers were made at 5°C.

For smooth muscle, thin strips (100 μm by 2–3 mm) of *Taenia coli* were used from the guinea pig. The tissue was cleaned of adventitia while bathing it in normal Krebs' solution at room temperature, with vigorous bubbling using a mixture of 5% CO_2 and 95% O_2 [19]. The cleaned samples were transferred to the skinning solution as described above for psoas muscle and the experimental strips were isolated as needed.

2.2. *TnC extraction from striated muscle fiber segment*

To extract TnC from the experimental fiber, the fiber was attached to the force transducer at one end and a servo motor at the other, equilibrated in a Ca^{2+}-free rigor solution (in mM: 165 potassium propionate, 20 imidazole, 2.5 EGTA, 2.5 EDTA, pH 7.0) at 4°C, and transferred to the extracting solution (in mM: 5 EDTA, 10 imidazole, pH 7.2) at 30°C for 5–30 min duration as described previously [6]. The force with maximal activation (pCa4) was checked periodically, and extraction was ended when the force had fallen below 20% P_o. The SDS-PAGE gel runs on such fibers indicated a 70–75% TnC extraction. Further removal of TnC was accompanied with the loss of another as yet unidentified cofactor [20], and the TnC extraction was therefore limited to the level indicated.

To reconstitute the fiber, TnC was recombined with denuded sites with a 10–30 min incubation at 5°C (0.2–1 mg/ml protein in the relaxing solution). Afterwards the free protein was washed out from the fiber with several rinses, unless otherwise indicated. The insertions of tissue CaM and the recombinants were made by loading the fibers with these proteins (200 μg/ml) in the activating solution. Rinsing with protein-free activating solution was necessary to estimate the bound CaM [6]. All fibers were thus rinsed and stored at −70°C for quantitative analysis by gel electrophoresis. These fibers were ultrasonicated in the sample buffer containing 1 mM EGTA. The gels were silver stained to reveal the TnC and CaM bands in these single fiber segments. The detailed protocol for electrophoresis and the staining procedures were similar to those described previously [6,21]. The intensity of the various protein bands were quantified with a laser densitometer of 100-μm beam width.

2.3. *Smooth muscle activation*

There is no established TnC function in smooth muscle, and the contraction of skinned strips requires the presence of exogenous CaM in the activating solution. Accordingly, these solutions were made with a saturating concentration of approximately 1 μM (18 μg/ml) CaM or the same amount of a mutant, unless otherwise indicated. The activations were made at 20°C.

The statistics of the data are given as ± SEM wherever appropriate.

2.4. *Genetic manipulation of CaM*

The cloning strategy and design of recombinants with primer extension mutagenesis of the cDNA encoding control CaM, have been described by Persechini and co-workers [12]. The three bacterially synthesized mutants described previously [12], in which Glu-84 (CaMΔ84), Glu-83–84 (CaMΔ83–84), or Ser-81 through Glu-84 (CaMΔ81–84) have been deleted, were used.

3. RESULTS

3.1. *Fast-twitch fibers*

The Ca^{2+}-dependent mobilities of the bacterially expressed proteins on gels were similar for all three mutants and for the tissue CaM (Fig. 1A). Fig. 1B and C show the traces of original force records of permeabilized fast-twitch fibers subjected to TnC extraction and reconstitution protocols. The calcium-activated force after reinserting TnC in the fiber was 80–100% of the force prior to extraction (Fig. 1B). With brain CaM substituted for TnC, the force recovery was in the 60–80% range among 16 fibers studied. The kinetics of the force rise is seen to be slower with CaM than with TnC (compare the upper trace in Fig. 1C with native), but this is explained by the slow uptake of the Ca-CaM complex by the TnC-denuded sites. Thus, as shown in the lower trace in Fig. 1C, force rise was nearly instantaneous when CaM was preloaded in a rigor solution containing pCa4. Also, the plateau reached in the steady-state was similar in both cases.

CaM uptake by the TnC-extracted fibers was also checked with silver staining, as well as with radiolabelled [^{125}I]CaM [6], and was found to be comparable to the amount of deleted TnC.

The results of force and of uptake for the recombinant mutants in the fast-twitch fiber are summarized in Fig. 2A. The force was unchanged with both CaMΔ84 and CaMΔ83–84 in comparison with the recombinant control, but was sharply inhibited with the additional two deletions in CaMΔ81–84. CaM uptake by the fiber as determined by silver staining was found to be the same for this 4-residue mutant as for non-mutated CaM (Fig. 2A). The results demonstrate a critical role for the mutagenized region 81–84 (Ser-Glu-Glu-Glu) in the regulation of force development in the fiber when CaM was substituted for TnC.

3.2. *Permeabilized smooth muscle*

Fig. 2B shows the results of force on the skinned guinea pig *Taenia coli* strips. It is known that the con-

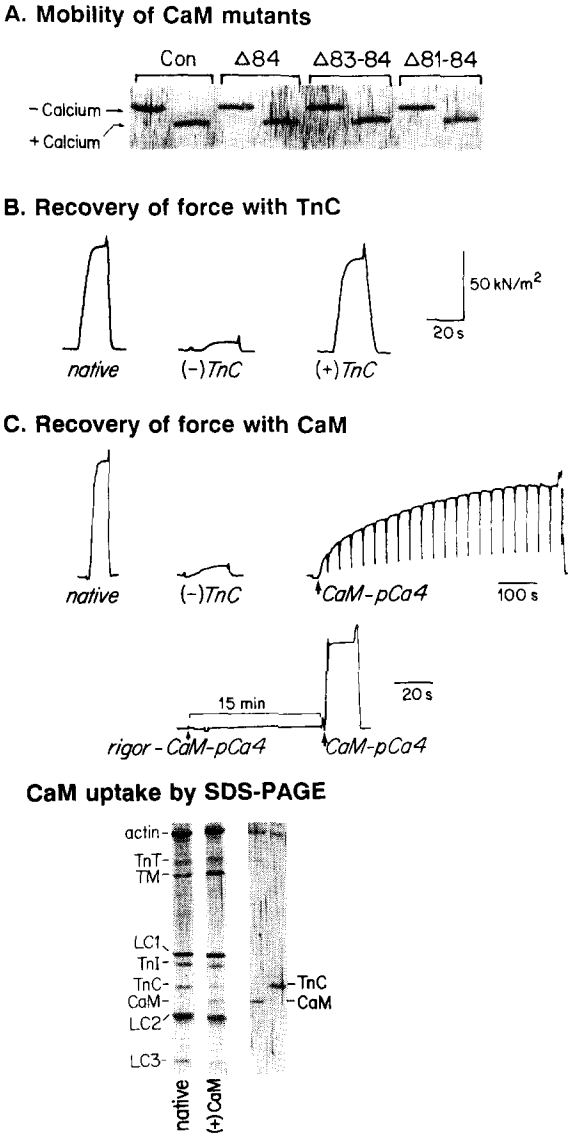

Fig. 1. (A) Electrophoretic mobility of CaM. Purified proteins were resolved by 15% SDS-PAGE. Proteins were solubilized in SDS sample buffer containing either 2 mM EGTA (−Calcium) or 2 mM $CaCl_2$ (+Calcium). No EGTA or $CaCl_2$ was added to the running buffer or the gel polymerizing solution. 40 ng protein was loaded onto each lane. (B) Force response with TnC extraction and reinsertion. A permeabilized fiber was maximally activated with 10^{-4} M Ca^{2+} (pCa4). The force scale (vertical bar) indicates 50 kN/m^2, and time (horizontal bar) indicates 20 s. (C) Insertion of CaM. CaM was loaded in the presence of maximal calcium as described before [6,21]. The time scale for the third trace is indicated as 100 s. The interruptions in the force trace indicate release-stretch length steps applied to the fiber during prolonged activation for CaM uptake. The fiber sarcomere length was monitored with laser diffraction or with a compound microscope. The lower force trace shows the response when both calcium and CaM were included in the loading phase, prior to activation in the presence of ATP. The gels were 12% polyacrylamide. Compare the uptake of CaM by the fiber (lane 2) in relation to TnC in native fiber (lane 1). The third and fourth lanes show purified proteins. The measured intensities of the TnC and CaM bands were normalized to the LC1 band in the same lane to compare the results. The TnC/LC1 fraction was 0.26 in the native, and 0.06 in the (+)CaM lane. CaM/LC1 was 0.19 in lane 2. Similar results were seen in 7 other experiments.

traction switch in smooth muscle involves the activation of MLCK by CaM [22,23], which in turn phosphorylates the light chans to turn on the cross bridges. Since MLCK is also the more common in vitro enzyme assay for the mutant activity, we thought it worthwhile to repeat the tests on mutants in smooth muscle strips to make a direct comparison of the fiber studies with these assays. The experiments with these strips also would serve as controls for the mutant results on skeletal fibers. As seen in Fig. 2B, the wild type CaM (1 μM) and the mutants were all equally ef-

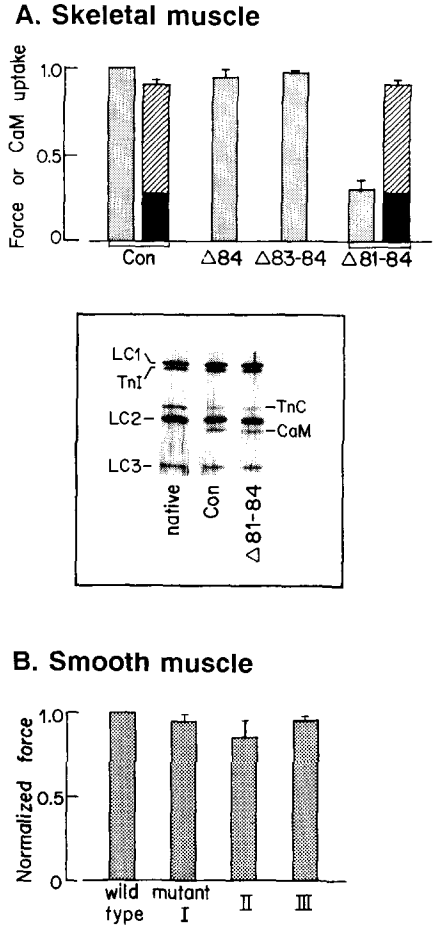

Fig. 2. (A) Force response of the fiber with recombinant CaM. Force (shaded bars) was normalized to the value for the recombinant without deletions. The fractions of residual TnC (black bars) and the uptake of the bacterially synthesized CaM (hatched bars) were determined for CaMΔ81–84. These are shown after normalizing the amounts determined by the densitometer to the original TnC in the native fiber segment [21]. The data shown are on 16 fibers. Activations were made at 5°C, and the force was normalized to that with the wild type CaM. The lower panel shows the uptake of recombinant CaM and of the mutant CaMΔ81–84. The gel was 15%, and the sample buffer contained 1 mM $CaCl_2$. The control lane was with the wild type recombinant. (B) Force response of the skinned smooth muscle strips with recombinant CaM (1 μM). The wild type was bacterially expressed normal CaM, and I to III refer to the mutants CaMΔ84, CaMΔ83–84 and CaMΔ81–84, respectively. Data are on 5 fibers, activation temperature was 20°C, and the normalization was made to the measurements with brain CaM obtained commercially.

fective for force development in the smooth muscle. There was no significant force without exogenous CaM in the activating solution. Also, TnC gave 22% force recovery (± 10%), on 3 preparations, when tested with a 3-fold higher concentration than CaM, indicating that TnC was relatively ineffective in smooth muscle.

4. DISCUSSION

The present study comparing the functional alterations of CaM mutants in skeletal and smooth muscles indicates diversity in the mechanisms of CaM interaction with target proteins. Additionally, since CaM and TnC are so similar in structure, and because CaM substitutes for TnC for the regulation of force in the skeletal fiber [6], the present study has implications for the mechanism underlying the contraction-relaxation switch in striated muscle. Thus, the observation that the activation by Ca^{2+} is inhibited sharply with the shortest mutant CaMΔ81–84 in this study, but not at all with the other mutants, suggests that at least part of the sequence of a highly conserved region in the central helix of the trigger molecule may be critical for force regulation in the fiber.

Furthermore, deletion of a single residue alters the relative orientation between N- and C-terminal lobes by 100°, in addition to shortening the distance by 1.5 Å. Thus the fact that the first two deletions (CaMΔ83–84) were without effect suggests that the angular orientation by itself is not a major factor in the 'on-off' switching mechanism in muscle. The idea that the central helix may compensate for these angular modifications by being able to act as a flexible tether is consistent with our results. The additional possibility that angular orientations between the lobes may selectively alter the Ca^{2+} affinity under partial activations should also be considered. Furthermore, on the basis of our results with CaMΔ81–84, the idea also arises that the global structure of the central helix defined critically by a minimum length (approx. 34 Å) is important for maximal force regulation in striated muscle. The findings on smooth muscle suggest that the minimum length is different for the Ca^{2+}-switch operated via MLCK.

4.1. *Diverse structure-function specifications of CaM*

Trifluoperazine-induced inhibition of CaM has been shown to be similar in the contractions of skeletal and smooth muscles [24]. Trifluoperazine inactivates CaM by binding to the hydrophobic sites in the central helix, and the above results [24] indicate that this hydrophobic domain performs a similar role in the switching mechanisms with TnI and MLCK. On the other hand, the effects of deletion mutants are different in the contraction-relaxation switches in skeletal and smooth muscles. Thus, contrasting with the results of mutants on skeletal fibers which showed diminished activity of CaMΔ81–84, in smooth muscle all mutants were as effective as the wild type CaM. This diversity between smooth and striated muscles provides the first evidence in structurally competent cells that the structure-function specifications of the central helix of CaM vary for different target proteins.

Diversity of factors in the functions of CaM is also seen in other studies. Craig et al. [10] found decreased activation of myosin light chain kinase in solution when the negative charge of the glutamate cluster (positions 82–84) was reversed with positively charged lysines without altering the length, indicating that the electrostatic properties of the cluster may be important for specific functions. The reduced net charge of the central helix in the deletion mutants thus might be a factor in our results too, but this could not be the complete explanation, as the mutants remained active in smooth muscle.

The length of the central helix appears to be a separate additional factor. This was also suggested in an earlier study of MLCK function in vitro, with a set of mutants where 4 additional amino acids were inserted to elongate the central shaft of CaM [11]. In light of the skinned fiber results, the length requirement appears to be different for the contraction-relaxation switches of skeletal and smooth muscles. The CaM-TnI switch in striated muscle was inoperable when the central shaft was close to 34 Å. Fiber studies with similar TnC mutants are needed in the future to find the precise critical length for the TnC-TnI switch. Interestingly, an upper limit (approx. 44 Å) is also suggested for the CaM-MLCK switch from the in vitro results. Such an effect of length would be consistent with the present smooth muscle results, and would also explain the observed ineffectiveness of exogenous TnC in smooth muscle.

The dumbbell shape itself is also critical for force regulation in the fiber. This was suggested on the basis of results with parvalbumin and oncomodulin [25]. Parvalbumin lacks nearly half of the amino acid structure corresponding to the N-terminus lobe. This is associated with a collapsed central helix that folds the protein into an ellipsoidal configuration [1]. Parvalbumin neither binds to TnC-denuded sites in the fiber nor is active in Ca^{2+} regulation [25]. Oncomodulin is an analog of parvalbumin, but is apparently enzymatically active in solution in vitro [26,27]. In the fiber, oncomodulin was inactive like parvalbumin ([25] on fast-twitch fiber; unpublished results of J. Gulati and A. Babu on smooth muscle), suggesting that the molecule in the shape of an ellipsoid would be unable to enter the attachment sites in vivo. This would indicate also that the diminished activity of the CaM mutant CaMΔ81–84 in the present study is unlikely to be due to global folding of the molecule, because interaction of the mutant with the target switching-complex in the fiber was normal.

There is another interesting finding in the in vitro system which is surprising and presently difficult to reconcile with the fiber results. This concerns the AT-Pase activity of the reconstituted actomyosin using a deletion mutant of TnC lacking residues Lys-Gly-Lys in positions 91–93 [13]. In TnC, these residues immediately follow the Ser-Glu-Glu-Glu cluster deleted in the CaM mutants used in the present study. The AT-Pase activity could not be turned off with the mutated TnC. This result is unlikely to be a direct length effect because wild type CaM is the same length (40 Å) as the TnC mutant, lacking amino acids 91–93, and is still able to regulate the contraction of skinned fibers.

4.2. *Conclusions*

The mechanisms of the interactions between the four Ca^{2+}-binding sites in both CaM and TnC are of great interest for the understanding of the Ca^{2+} switch in muscle contraction and other cellular processes. Although specific (regulatory and structural) functions have been previously assigned to the sites in the N- and C-terminal lobes of TnC, the structure of the central shaft connecting the two lobes is also found to be important for function in muscle. Further, the combined approaches of molecular genetics and permeabilized fibers are shown to be increasingly valuable for future studies. The present results for the first time raise the likelihood that a highly shortened central helix may be critical for the contraction-relaxation switch in striated muscle. For TnC, this suggests the possibility of interaction between Ca^{2+}-specific Ca^{2+}-Mg^{2+} sites through the central helix. The distance of separation between the lobes could regulate the CaM interaction with myosin light chain kinase in smooth muscle as well. If so, the findings would suggest that the critical minimum length is different for the interactions of the modulators with TnI than that with MLCK.

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