Inhibition of Cross-Bridge Binding to Actin by Caldesmon Fragments in Skinned Skeletal Muscle Fibers

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ABSTRACT Several regions within the 35-kDa COOH-terminal portion of caldesmon have been implicated in the ability of caldesmon to inhibit actin-activated myosin ATPase activity. To further define the functional regions of caldesmon, we have studied the effects of three chymotryptic fragments, one fragment produced by CNBr digestion and two fragments produced by digestion with submaxillaris arginase C protease, on the relaxed stiffness and active force of rabbit psoas fibers. Each of the regions of caldesmon studied had either direct or indirect effects on single-fiber mechanics. The 35-kDa and 20-kDa fragments of caldesmon, like intact caldesmon, were effective inhibitors of fiber stiffness, a measure of cross-bridge attachment. The 7.3-kDa and 10-kDa fragments, which constitute the NH_2 and COOH halves of the 20-kDa fragment, inhibited both relaxed fiber stiffness and active force production, but with a reduced efficacy compared to the 20-kDa fragment. These results suggest that several regions within the 35-kDa COOH-terminal region of caldesmon are required for optimum function of caldesmon and that function includes inhibition of weak cross-bridge attachment and force production.

INTRODUCTION

The primary switch for contraction of smooth muscle is phosphorylation of the regulatory light chains of smooth-muscle myosin (Itoh et al., 1989). However, there are indications that an actin-based system also participates in the regulation of smooth-muscle contraction (Marston and Smith, 1984; Marston and Lehman, 1985). Caldesmon, a protein that is expressed in smooth-muscle and in non-muscle tissues, has been proposed to be a component of this additional regulatory system. By binding to actin, caldesmon inhibits actomyosin ATPase activity in solution (Chalovich et al., 1987), the motility of actin filaments (Haeberle et al., 1992), and both ATPase activity and force development in single skinned skeletal-muscle (Brenner et al., 1991) and smooth-muscle (Pfitzer et al., 1993) fibers.

Caldesmon is a multifunctional protein with binding sites for both myosin and actin. The NH_2-terminal region of caldesmon binds to myosin (Velaz et al., 1990) and is instrumental in cross-linking myosin to actin in vitro (Hemric and Chalovich, 1988, 1990; Ikebe and Reardon, 1988). There are many possible functions for this interaction, but at present, the physiological role of this interaction has not been determined. The central region of caldesmon is highly α-helical (Wang et al., 1991a) and is flexible enough in parts to allow the caldesmon to bend, so that the NH_2 and COOH regions may come into close contact (Martin et al., 1991; Moody et al., 1990; Mabuchi et al., 1993; Crosbie et al., 1995). The COOH-terminal region, residues 450–756, binds to actin and inhibits the actin-activated ATPase activity of myosin (Szpacenko and Dabrowska, 1986; Fujii et al., 1987; Yazawa et al., 1987; Riseman et al., 1989). This COOH 35-kDa region is thought to contain the entire inhibitory region of caldesmon. Several groups have further dissected the COOH-terminal region of caldesmon to identify its key functional regions. Several fragments have received particular attention because of reports that they are central to the inhibitory activity. A 10-kDa fragment prepared by CNBr digestion of caldesmon (See Fig. 1) has been reported to bind to actin with a stoichiometry of one fragment per seven actin monomers, similar to that of intact caldesmon (Bartegi et al., 1990). This fragment inhibits actin-activated ATPase activity of S1, and this activity is augmented by tropomyosin and attenuated by Ca^{2+}-calmodulin. A similar fragment, 685C, was reported to maximally inhibit ATPase activity when one fragment was bound per 14 actin monomers (Redwood and Marston, 1993). Based on these observations, the 10-kDa fragment was thought to contain most of the regulatory function of intact caldesmon. A 7.3-kDa fragment from the region of caldesmon adjacent to the 10-kDa fragment bound to actin with a 1:1 stoichiometry, but with low affinity (Chalovich et al., 1992). This 7.3-kDa fragment was capable of reducing the actin activation of S1 ATPase activity to low levels. Like that of intact caldesmon, the inhibitory activity was augmented by smooth-muscle tropomyosin and was reversed by Ca^{2+}-calmodulin. The 7.3-kDa fragment overlapped the 10-kDa fragment by seven residues, and these were suggested to be key residues in the function of caldesmon (Chalovich et al., 1992). Zhan et al. (1991) observed that a synthetic peptide analog of the overlap region (Gly^{651}-Ser^{667}) did bind to actin.

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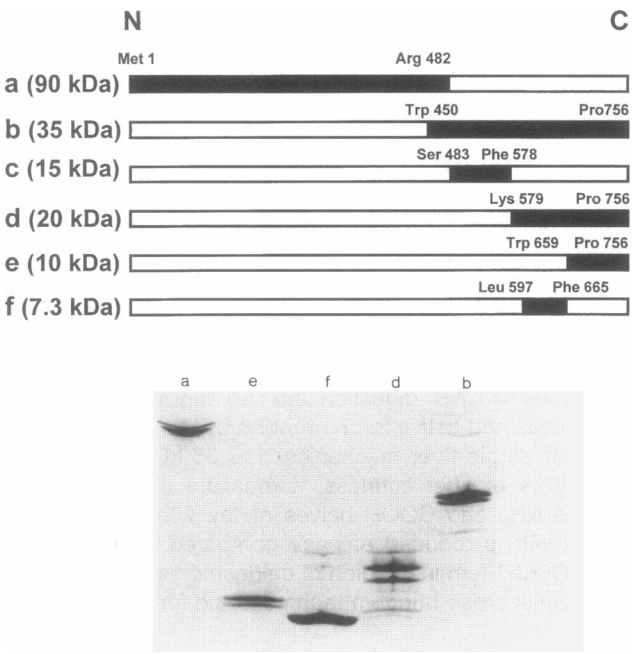

FIGURE 1 Caldesmon fragments used in this study. The top panel shows the relative position of the various fragments in the caldesmon molecule, using the primary structure derived from Bryan et al. (1989) as a reference. The bottom panel shows an SDS gel of the major caldesmon fragments used. The fragments are (*a*) a 90-kDa submaxillaris arginase-C protease fragment, (*b*) a 35-kDa chymotryptic fragment, (*c*) a 15-kDa submaxillaris arginase-C protease fragment, (*d*) a 20-kDa chymotryptic fragment, (*e*) a 10-kDa CNBr fragment, and (*f*) a 7.3-kDa chymotryptic fragment.

Other observations, however, indicate that multiple regions of the 35-kDa region are important in regulation. For example, the stoichiometry of binding to actin decreases with truncation of caldesmon (Chalovich et al., 1992). This indicates that multiple regions of caldesmon are involved in inhibition (see Redwood and Marston, 1993, for another opinion). In addition, the competition of binding of caldesmon and S1 for actin is consistent with a model in which there is a mosaic of binding sites involving multiple regions of caldesmon interacting with different actin monomers (Chen and Chalovich, 1992).

Because of the conflicting evidence for either the entire 35-kDa region or a small part of it having control of ATPase activity and for differences among laboratories in the reported properties of individual regions, we have reexamined the question in a different manner. Several relevant polypeptide regions derived from the 35-kDa region of caldesmon have been compared; these are shown in Fig. 1. The effect of the polypeptides on the mechanics of single fibers was studied. In this way, the function of the fragments could be compared in a highly organized muscle system, and those parameters could be followed that are likely to be important in the regulation of smooth-muscle contraction. Psoas fibers rather than smooth-muscle fiber bundles were used because they lack caldesmon, and the effect of adding caldesmon can be readily seen (Brenner et al., 1991; Kraft et al., 1995a). Skeletal-muscle fibers do contain troponin, which appears to be absent in smooth muscle. However, the presence of troponin has only a minor effect, making the binding of caldesmon to actin slightly stronger in the presence of calcium (Brenner et al., 1991).

Our results show that multiple fragments in the COOH-terminal region of caldesmon inhibit force. Some individual fragments have little effect on the fiber mechanics, although they clearly increase the activity of the intact molecule. All fragments that inhibit force do so largely by inhibiting weak cross-bridge attachment in the skeletal-muscle model. Finally, there is little difference in the behavior of the 7.3-kDa and 10-kDa fragments that comprise the 20-kDa region. It is likely that the 7.3-kDa and 10-kDa fragments are equally important in the inhibitory function of caldesmon.

MATERIALS AND METHODS

Fiber preparation

Single chemically skinned fibers from rabbit psoas muscle were isolated according to a method described earlier (Brenner, 1983; Yu and Brenner, 1989). The skinning solution always contained several protease inhibitors, to prevent degradation of contractile proteins (see Solutions and Kraft et al., 1995a). To provide conditions similar to those in vivo, we replaced propionate by creatine-phosphate as the main anion to adjust the ionic strength. Furthermore, to minimize oxidation of reactive groups within the proteins, we added glutathione (10 mM) to the skinning solution. We also isolated single fibers within a few hours after dissecting the muscle and kept them as isolated fibers to reduce substrate depletion and diffusion problems. These precautions resulted in even better preservation of the mechanical stability of the muscle fibers than we had previously achieved (Brenner, 1983).

Solutions

All solutions were adjusted to pH 7.0 at the appropriate temperature. The chemicals were obtained from Sigma Chemie (Munich, Germany), except where noted. The skinning solution was (μ = 170 mM): 5 mM KH_2PO_4, 3 mM magnesium-acetate, 5 mM EGTA, 1 mM sodium-ATP (Merck), 50 mM sodium creatine phosphate, 5 mM NaN_3, 10 mM glutathione, 1 mM phenylmethylsulfonyl fluoride, and 10 μM each of aprotinin, leupeptin, antipain, E64, and pepstatin A. Glutathione and the protease inhibitors were freshly added before the solution was used. Immediately after dissection, the fiber bundles were incubated for 30 min at 5°C in skinning solution containing 0.5% Triton-X-100. The bundles were then transferred to normal skinning solution for isolation of single fibers. The relaxing solution with MgATP was (μ = 50 mM): 10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM sodium-ATP (Merck), 1 mM dithiothreitol, and 10 mM sodium creatine phosphate. The low-salt relaxing buffer (μ = 50 mM) was: 10 mM imidazole, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, and 35 mM potassium propionate. The preactivating and activating solutions were (μ = 50 mM): 10 mM imidazole, 2 mM $MgCl_2$, 1 mM MgATP, 1 mM dithiothreitol, 10 mM caffeine, 10 mM creatine phosphate, 500 units/ml (Sigma units) of creatine phosphokinase and 1 mM EGTA (preactivating) or 1 mM CaEGTA (activating). The $MgPP_i$ solution was (μ = 170 mM): 10 mM imidazole, 2 mM $MgCl_2$, 1 mM CaEGTA, 4 mM $MgPP_i$, 1 mM dithiothreitol, and 140 mM potassium propionate.

Mechanical apparatus

The mechanical setup, including the system for laser light diffraction to record sarcomere length, was previously described (Brenner, 1980; Brenner and Eisenberg, 1986).

Fiber stiffness and isometric tension

Fiber stiffness was measured during rapid ramp-shaped stretches that were imposed on one end of the fiber. Fiber stiffness was defined as the force change when filament sliding has reached 2 nm per half-sarcomere (chord stiffness). The maximum isometric tension was determined as the drop in force upon changeover to unloaded shortening.

Analysis of stiffness data

Fitting a hyperbolic function to the concentration dependence of the stiffness data enabled us to estimate the maximum inhibition of cross-bridge binding. The association constant of caldesmon binding to actin in the fiber, K_A^{fiber}, was estimated from the reciprocal of the concentration of caldesmon giving half-maximum inhibition. Because the volume of the bath surrounding the fiber was much larger than the fiber volume (>100: 1), we assumed that the free fragment concentration was equal to the total concentration added.

Removal of caldesmon and caldesmon fragments from the fibers

To ensure reversibility of effects on fiber mechanics, caldesmon and caldesmon fragments were removed at the end of each experiment. These proteins were removed by incubation of the fibers in a $MgPP_i$ solution with calcium (pCa = 4.5) containing calmodulin (0.1 mg/ml) for the same length of time that was needed initially to achieve an equilibrium concentration of that fragment in the fiber. Usually 90–110% of the initial fiber stiffness and force were restored.

Proteins

Actin (Eisenberg and Kielley, 1972; Spudich and Watt, 1971), tropomyosin-troponin (Eisenberg and Kielley, 1974), and myosin (Kielley and Harrington, 1960) were prepared from rabbit skeletal muscle. Myosin S1 was prepared as described by Weeds and Taylor (1975). Caldesmon was prepared from turkey gizzards by a modified method of Lynch et al. (1987).

Several caldesmon fragments were used in this study; these are defined in Fig. 1. The 7.3-kDa, 20-kDa, and 35-kDa fragments were obtained by digestion of caldesmon with chymotrypsin under conditions similar to those used by Szpacenko and Dabrowska (1986): 5 min at 25°C with 1:1000 (w/w) ratio of chymotrypsin to caldesmon. The caldesmon was at 5–10 mg/ml in 100 mM NaCl, 10 mM Tris-2-[*N*-morpholino]ethanesulfonic acid (Tris-MES) (pH 7.0), and 2 mM dithiothreitol. The 7.3-kDa and 20-kDa fragments were isolated as described earlier (Chalovich et al., 1992; Velaz et al., 1993). The 35-kDa fragment was isolated using a high-performance liquid chromatography cation exchange column (SP R, Waters) (see figure 1 of Chalovich et al., 1992). Low-molecular-weight components were removed by gel filtration on a 1.5 × 95 cm column of ACA 54 (Spectrum, Los Angeles) equilibrated with 0.5 M KCl, 10 mM imidazole (pH 7.0), and 1 mM EDTA. The first peak contained the 35-kDa fragment.

The 15-kDa and 90-kDa fragments were prepared by a slight modification of the method of Mornet et al. (1988). Caldesmon was diluted to 2 mg/ml with 50 mM Tris-HCl (pH 8.0), 0.5 mM dithiothreitol, and 1 mM EGTA and digested with a 1/500 molar ratio of submaxillaris arginase-C-protease at 37°C for 90 min. The digestion was stopped by the addition of phenyl methyl sulfonyl fluoride, and the mixture was applied to a 1.5 × 100 cm column of ACA 54 equilibrated as above. The first broad peak contained a crude 90-kDa fragment, and the second broad peak contained a crude 15-kDa fragment. The crude 90-kDa and 15-kDa fragments were individually purified by chromatography on a HPLC SP R (Waters) cation exchange column equilibrated with 10 mM Tris-MES, pH 7.0, 60 mM NaCl, 1 mM NaN_3, and eluted with a gradient of NaCl to 1 M.

The 10-kDa fragment was prepared by digestion with CNBr. Caldesmon at 10 mg/ml was mixed with 88% formic acid and CNBr to give 2.5 mg/ml caldesmon, 70% formic acid, and 20 mg/ml CNBr and incubated at room temperature for 24 h. After two cycles of dilution and lyophilization, the pellet was dissolved in 1 M KCl and chromatographed using ACA 54 gel filtration media as described for the 7.3-kDa fragment. The second broad peak contained the 10-kDa fragment. Final purification was done on the SP column as described earlier. The pure 10-kDa fragment was recovered in the sharp second peak.

Binding studies

In those cases in which fragments were without effect on fiber mechanics, the binding of the fragments to actin was estimated by using a cosedimentation assay. A 30-min incubation of 5 μM filamentous actin with 2 μM fragment in low-salt relaxing buffer at 25°C was followed by sedimentation at 160,000 × *g* for 25 min in a 50-Ti rotor. The sediment was dissolved in low-salt relaxing buffer and loaded onto a polyacrylamide gel in the presence of sodium dodecyl sulfate (Laemmli, 1970), together with four known amounts of fragment. After staining with Coomassie blue or silver stain, the amount of bound fragment could be determined by video densitometry. Before estimating the binding constant, $K_A^{solution}$, the data were corrected for the amount of fragment that was present in the sediment in the absence of actin.

ATPase assays

The ATPase activity of S1 was measured at 25°C by the liberation of $^{32}P_i$ from [$^{32}P_i$]ATP as described by Chalovich and Eisenberg (1982). Low-ionic-strength conditions were used in the ATPase assays to promote the interaction of S1 with actin and increase the sensitivity of measurement of inhibitory potential of the caldesmon fragments. The reaction mixture included 10 μM actin, 0.1 μM S1, 10 mM imidazole (pH 7.0), 1 mM MgATP, 1 mM $MgCl_2$, 17 mM potassium propionate, 1 mM dithiothreitol, and either 1 mM EGTA or 0.5 mM CaEGTA.

RESULTS

To study the effect of caldesmon fragments on fiber mechanics, it is critical that the polypeptides be uniformly distributed throughout the fiber. Fig. 2 shows the time course of diffusion of some of the fragments into the fibers, monitored by the effect on fiber stiffness. The rate of diffusion varied with the size and shape of the fragment and is likely to involve affinity of the fragment for actin and other muscle components. The rate of diffusion followed the order 7.3-kDa > 10-kDa (not shown) ≥ 20-kDa > 35-kDa fragment > intact caldesmon. In all subsequent experiments, incubation with fragments was continued for the time that ensured a maximum effect.

The measured inhibition of cross-bridge binding to actin required not only diffusion, but also docking of the caldesmon fragments in the correct orientation relative to the actin filaments. The rate of equilibration measured earlier with a confocal microscope and fluorescently labeled 35-kDa fragment was similar to that shown here (Kraft et al., 1995b). Because there is good correlation between the visually and mechanically measured time courses, we conclude that diffusion is the limiting process, whereas reorientation occurs comparatively quickly.

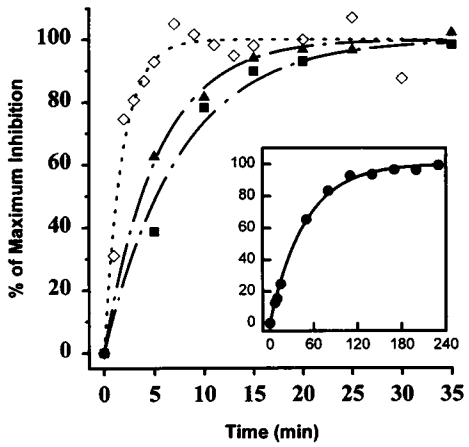

FIGURE 2 Time courses of diffusion into a single fiber for caldesmon (●) and fragments with molecular masses of 7.3 kDa (◇), 20 kDa (▲), and 35 kDa (■). The figure shows data of representative experiments with different fibers. The fibers were incubated with fragment concentrations close to $1/K_A^{fiber}$. The curves shown are normalized to the maximum change in stiffness observed in each case.

To achieve a standard to compare the inhibition caused by caldesmon fragments, we first tested the inhibitory effect of the intact molecule in the fiber (Fig. 3, *filled circles*). From fitting a hyperbolic curve to the data, we obtained an apparent association constant for the binding of caldesmon to native skeletal thin filaments of 3×10^6 M^{-1}. The maximum inhibition of cross-bridge binding to the thin filament was extrapolated to about 87%. This compares to approximately 95% inhibition of ATPase activity in solution. Table 1 contains a summary of the properties of caldesmon and its fragments. The estimate of the affinity of caldesmon for actin in fibers is in good agreement with values obtained by direct binding measurements in solution.

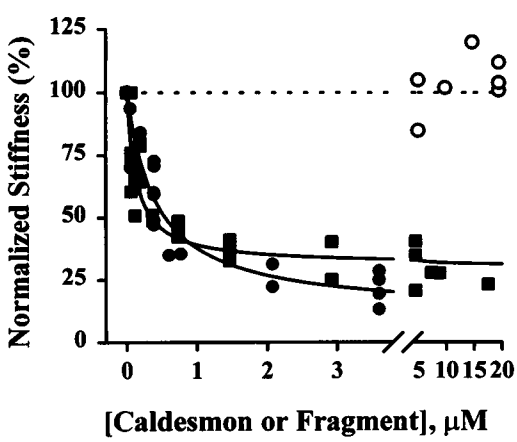

FIGURE 3 Effects of intact caldesmon (●, n = 15), the 90-kDa fragment (○, n = 4), and the 35-kDa fragment (■, n = 16) on fiber stiffness under relaxing conditions. All data were normalized to the value of the fiber stiffness before caldesmon or the fragment was added to the solution. The dotted line marks 100%.

The N-terminal 90-kDa fragment of caldesmon lacks the COOH-terminal actin binding/inhibitory domain. Fig. 4 shows that this fragment did not inhibit the actin-activated ATPase activity of skeletal myosin S1 at low ionic strength. In some cases there was even a small activation (20%) of the ATPase activity. Such an activation would occur if the fragment bound weakly to actin in addition to binding to myosin, thereby increasing the effective concentration of these proteins. The inset to Fig. 4 shows results of cosedimentation of the 90-kDa fragment with actin. The upper limit for the affinity of this fragment is about 7×10^3 M^{-1} or 1/500 that of intact caldesmon. Fig. 3 (*open circles*) shows that this fragment had no significant effect on relaxed fiber stiffness, although this lack of effect could be due to inadequate binding of the 90-kDa fragment.

The 35-kDa fragment contains the C-terminal region of caldesmon and together with the 90-kDa fragment would constitute the entire caldesmon molecule. This fragment was an effective inhibitor of cross-bridge attachment for the concentrations used in our study (Fig. 3, *solid squares*) and had an effect similar to that of intact caldesmon. From the dependence of the inhibition of cross-bridge attachment on the 35-kDa fragment concentration, we have estimated the apparent association constant to actin filaments in fibers to be 7×10^6 M^{-1}. Whereas the apparent affinity was slightly greater than that of intact caldesmon, the maximum inhibition, about 70%, was less than that of intact caldesmon. By comparing the fragments shown in Fig. 3, the 90-kDa fragment contributes little to the potency of inhibition by caldesmon. However, from inspection of Table 1, it appears that the 90-kDa fragment is able to extend the inhibitory effect of caldesmon from four actin monomers to seven.

The 35-kDa fragment may be further dissected to determine which regions are essential for the inhibition of cross-bridge attachment in fibers. The 15-kDa fragment shows sequence homology with troponin T (Bryan et al., 1989) and is reported to contain a weak actin-binding site (Mornet et al., 1988). This fragment had no effect on actin-activated ATPase activity at fragment concentrations up to 20 μM, and a cosedimentation assay indicated that the binding to actin was very weak, $K_A^{fiber} < 2 \times 10^3$ M^{-1} (data not shown). Fig. 5 (*open squares*) shows that the 15-kDa fragment has no apparent effect on relaxed fiber stiffness. Therefore this fragment contains little information, by itself, for binding to actin. In contrast, the other part of the 35-kDa region, a fragment with a molecular mass of 20 kDa (Fig. 5, *filled triangles*), again inhibited cross-bridge binding to actin by 70%, but the apparent affinity was decreased to 1×10^6 M^{-1} in the fiber. This fragment has already been shown to bind to actin and calmodulin, in solution, and to inhibit ATPase activity and the attachment of S1 to actin (Velaz et al., 1993). Although the 20-kDa fragment is a potent inhibitor, it is not as effective in binding or inhibition as the 35-kDa fragment. Thus, at least part of the 15-kDa fragment is important in the function of caldesmon, although by itself the 15-kDa fragment has no activity.

TABLE 1 Effect of caldesmon fragments on fiber mechanics

Species	$n^{*\#}$	$K_a^{solution}$ $(M^{-1})^{\#\S}$	K_a^{fiber} $(M^{-1})^{\P}$	[fragment] for 50% inhibition$^{\|}$	% Inhibition of stiffness	% Inhibition of force
Caldesmon	7 or (5 and 22)	$\approx 10^7$	3×10^6	≈0.8 μM	87	≈90**
90 kDa	—	$<7 \times 10^3$	—	ND$^{\#\#}$	~0	—
35 kDa	4	3.5×10^6	7×10^6	—	70	—
15 kDa	—	$<2 \times 10^3$	—	—	~0	—
20 kDa	2	6×10^5	1×10^6	—	70	85**
7.3 kDa	1	5×10^4	8×10^4	10 μM	~48	>25
10 kDa	7	4×10^3	$<8 \times 10^4$	>30 μM	>30	>45

*n is the number of actin monomers in a single site.

#References: caldesmon, Velaz et al. (1993) and Smith et al. (1987); 35 kDa, Yazawa et al. (1987); 20 kDa, Velaz et al. (1990); 7.3 kDa, Chalovich et al. (1992); 10 kDa, Bartegi et al. (1990). The 90-kDa and 15-kDa values are from the present work.

§Affinity in solution determined by direct binding assays.

¶Estimate of affinity in fibers determined by inhibition of stiffness.

‖The concentration of caldesmon or fragment required to obtain 50% inhibition of ATPase activity in the absence of tropomyosin under the conditions of Fig. 4.

**Brenner et al. (1991).

##ND, No inhibition detected.

The 20-kDa region can be further cleaved to more narrowly define the essential portions of caldesmon. Digestion of the 20-kDa fragment with chymotrypsin yields a 7.3-kDa fragment that is essentially the amino-terminal half of the 20-kDa fragment. This fragment was shown earlier to inhibit the actin-activated ATPase activity by more than 80% (Chalovich et al., 1992). This fragment also inhibited fiber stiffness (Fig. 5, *diamonds*). The data were fitted to a binding constant of 8×10^4 M^{-1}, with a maximum inhibition of 48%.

It was surprising that the 7.3-kDa fragment was more effective in inhibiting ATPase activity in solution than force and stiffness in the fiber. ATPase assays were done to determine if the troponin-tropomyosin present in the psoas fibers limited the activity of the 7.3-kDa fragment. Fig. 6 shows that this was not the case. The 7.3-kDa fragment inhibited the actin activated the ATPase rate of S1, both in the absence of troponin-tropomyosin and in the presence of troponin-tropomyosin and Ca^{2+}. In both cases, the total inhibition of ATPase activity exceeded 90%. Therefore, the reduced efficacy of the 7.3-kDa fragment in fibers cannot be attributed to the presence of troponin.

The 10-kDa actin-binding fragment resulting from a CNBr digest of caldesmon most likely includes amino acid residues 659–756 and is the COOH-terminal half of the 20-kDa fragment (Bartegi et al., 1990). The 10-kDa frag-

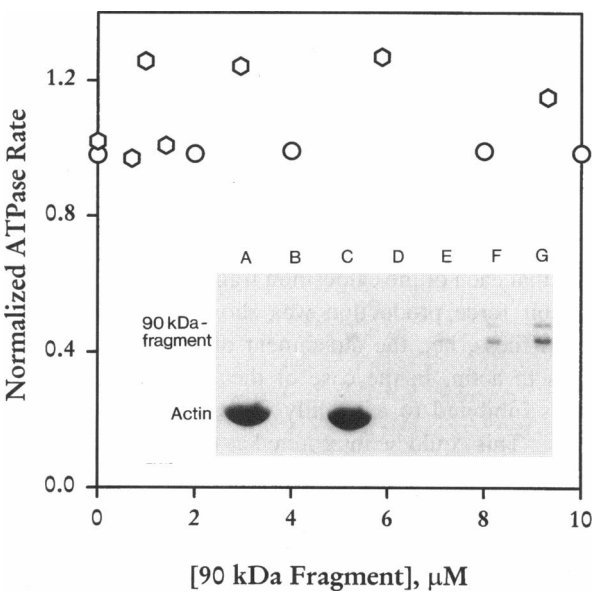

FIGURE 4 Effect of the 90-kDa caldesmon fragment on actin activated ATPase activity and the binding of the 90-kDa fragment to actin (*inset*). The ATPase rates were measured with two preparations of the fragments (○, ◌) at the following conditions: 25°C, 10 μM actin, 0.1 μM S1, 10 mM imidazole, pH 7.0, 1 mM MgATP, 1 mM $MgCl_2$, 17 mM potassium propionate, 1 mM dithiothreitol, and either 1 mM EGTA or 0.5 mM $CaCl_2$ and 0.5 mM EGTA. (*Inset*) The pellets of protein mixtures after high-speed centrifugation were run on SDS-polyacrylamide gels (5–15% gradient). *Lanes*: *A*, actin; *B*, 90-kDa fragment; *C*, actin and the 90-kDa fragment. *Lanes D-F* contain known amounts of 90-kDa fragments: *D*, 0.25 μg; *E*, 0.5 μg; *F*, 1 μg.

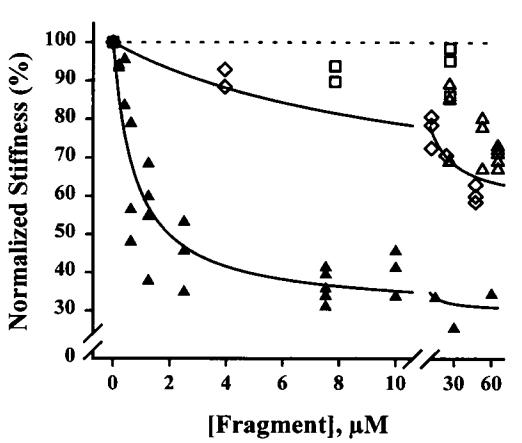

FIGURE 5 Effects of 20-kDa (▲, n = 10), 15-kDa (□, n = 4), and 7.3-kDa (◇, n = 4) and 10-kDa CNBr (△, n = 3) fragments on fiber stiffness under relaxing conditions.

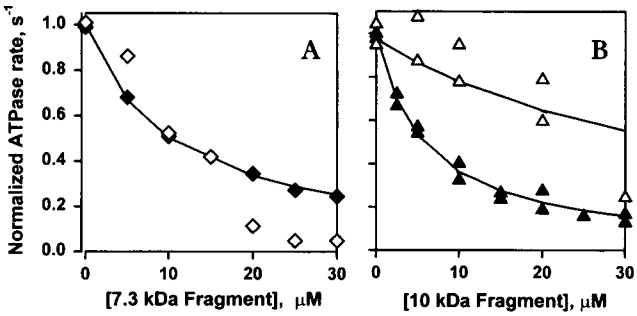

FIGURE 6 Inhibition of actin-activated ATPase activity of S1 by the 7.3- and 10-kDa caldesmon fragments. (*A*) The 7.3-kDa fragment in the absence (◇) and presence (◆) of skeletal muscle troponin-tropomyosin. (*B*) The 10-kDa caldesmon fragment in the absence (△) and presence (▲) of smooth-muscle tropomyosin. The conditions were the same as in Fig. 4.

ment inhibited ATPase activity, as shown in Fig. 6 *B*. As with intact caldesmon, the inhibition was greater in the presence of smooth-muscle tropomyosin. In the absence of tropomyosin the 10-kDa fragment is not as good an inhibitor as the 7.3-kDa fragment; concentrations of the 10-kDa fragment approximately three times higher relative to the 7.3-kDa fragment were required to provide 50% inhibition of ATPase activity. The 10-kDa fragment also inhibited the actin-activated ATPase activity in the presence of troponin-tropomyosin by 60–80% (not shown) and could therefore be studied in single fibers.

Fig. 5 (*open triangles*) shows that the 10-kDa CNBr fragment did inhibit fiber stiffness. However, higher concentrations of the 10-kDa fragment relative to the 7.3-kDa fragment were required to give the same degree of inhibition. Fig. 7 shows that in addition to inhibiting stiffness, both the 10-kDa and 7.3-kDa fragments inhibited the active force produced by single fibers upon activation. Therefore, like the inhibition by intact caldesmon (Brenner et al., 1991) and the 20-kDa fragment (Kraft et al., 1995a), the inhibition of stiffness by the 10-kDa and 7.3-kDa fragments is accompanied by an inhibition of active force.

DISCUSSION

The present results indicate that many regions within the 35-kDa COOH-terminal fragment of caldesmon are instrumental in the inhibition of actin-activated ATPase activity of myosin. The individual regions can be divided into two types, depending on whether they exhibit a direct or indirect effect on ATPase activity. Most of the fragments investigated have a direct effect on the ATPase activity and, as shown in the present work, inhibit force production in muscle. Both the 7.3-kDa and 10-kDa fragments, which comprise the 20-kDa region, have this direct inhibitory activity. These fragments do share a common seven amino acid region, but it is unlikely that this region alone is responsible for the inhibitory activity of both fragments, because the 20-kDa region is much more active than either of these fragments, yet it contains only a single copy of the seven amino acid overlap region. Our results do not support the idea that either the 7.3-kDa or 10-kDa fragment can reproduce all of the functions of caldesmon.

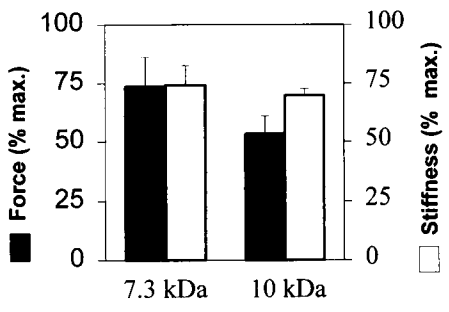

FIGURE 7 Inhibition of force and stiffness caused by 65 μM of either the 7.3-kDa fragment (n = 4) or the 10-kDa fragment (n = 4).

We have suggested earlier that a major component of the inhibition by caldesmon is due to a reduction in the occupancy of actin-binding sites by myosin-ATP complexes (Chalovich et al., 1987; Hemric and Chalovich, 1988; Velaz et al., 1990). Our own earlier work with troponin-tropomyosin indicated that the major effect with this regulatory system was to inhibit the kinetics, presumably the rate of transition from the nonactivating (weak binding) to the activating (strong binding) states (Chalovich et al., 1982; Brenner et al., 1982; Brenner, 1988). The mechanical experiments described here and earlier (Brenner et al., 1991; Kraft et al., 1995a) show that caldesmon functions by inhibiting the attachment of weakly binding cross-bridges to actin. Our earlier studies showed that caldesmon has no significant effect on the cycling kinetics in the skeletal muscle model. Most importantly, those results demonstrated that inhibition of attachment of nonactivating cross-bridges (i.e., myosin-ATP or myosin-ADP-P_i) by caldesmon is by itself capable of inhibiting active force. We have now observed that each of the caldesmon fragments that was shown to inhibit force production was shown to inhibit relaxed fiber stiffness, i.e., the attachment of nonactivating cross-bridges to actin. In the case of the 10-kDa fragment, the force is inhibited to a slightly greater degree than is the stiffness. This could be interpreted as the existence of a dual mechanism of inhibition by this fragment; that is, inhibition of both cross-bridge attachment and the subsequent conversion to the activating state. Even in our hands, caldesmon does have a small effect on the k_{cat} of ATP hydrolysis (Hemric et al., 1993). However, it is more likely that the small disparity between the degree of inhibition of stiffness and force results from the same effect seen earlier with the 20-kDa fragment (Brenner et al., 1991; Kraft et al., 1995a). In that case, the greater inhibition of active force (in the presence of Ca^{2+}) than relaxed stiffness (in the absence of Ca^{2+}) was due to a small increase in the affinity of caldesmon for actin-tropomyosin-troponin in the presence of Ca^{2+}. The stiffness of fibers measured in the presence of ATPγS (an ATP analog that does not support contraction)

and Ca^{2+} did decrease in close parallel to the active force measured in the presence of ATP and Ca^{2+} (Kraft et al., 1995a).

At least one region of caldesmon has an indirect role in the inhibition of contraction. In agreement with Mornet et al. (1988) and Hayashi et al. (1991), we observed that the 15-kDa fragment bound only weakly to actin and was not inhibitory, even at comparably high concentrations. Although the maximum inhibition is similar for the 20-kDa and 35-kDa fragments (Fig. 5), removal of the 15-kDa region from the 35-kDa fragment, to form the 20-kDa fragment, decreased the actin affinity to 14%, and reduced the number of actin monomers bound per fragment from three or four (Yazawa et al., 1987) to two (Velaz et al., 1993). It is conceivable that despite the low actin affinity of the isolated 15-kDa fragment, this region may bind to actin and interfere with cross-bridge attachment when other parts of caldesmon constrain it to a specific location on actin. This is reasonable because NMR studies have implicated residues within the 15-kDa region, as well as in the 20-kDa region, in binding to actin (Mornet et al., 1995).

The competition of binding between S1-AMP-PNP and caldesmon for actin is not purely competitive, but is more readily fit with a model containing a mosaic of binding sites (Chen and Chalovich, 1992). That model assumed that some actin monomers covered by caldesmon were totally excluded from S1 binding, whereas others were partially excluded; a component of regulation through an allosteric effect is also possible in this model. The finding that two small fragments within the 35-kDa inhibitory region, the 7.3-kDa and 10-kDa fragments, inhibit both force and stiffness, whereas the 15-kDa fragment has little effect by itself on either parameter, is consistent with that model. In addition, although caldesmon does not have a repetitive binding sequence for actin, a single caldesmon molecule interacts with at least seven actin monomers. Thus it is likely that different interactions are responsible for the association of each caldesmon region.

An interesting aspect of the present work is that the 7.3-, 10-, 20-, and 35-kDa fragments gave >85% inhibition of ATPase activity in solution, although higher concentrations of the smaller fragments were required to reach this level of inhibition. In the fiber the maximum level of inhibition decreased substantially as the size of the fragment decreased. This difference between solution and fiber studies cannot be attributed to the presence of troponin, because troponin does not affect the maximum degree of inhibition of ATPase activity by the fragments in solution. Among many possible reasons for this difference is the presence of other actin-binding proteins, such as nebulin, which may partially restrict the binding of some of the caldesmon fragments. This difference may provide clues to the function of caldesmon, and it illustrates the importance of testing regulatory proteins both in solution and in a more highly organized system.

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REFERENCES

Bartegi, A., A. Fattoum, J. Derancourt, and R. Kassab. 1990. Characterization of the carboxyl-terminal 10-kDa cyanogen bromide fragment of caldesmon as an actin-calmodulin-binding region. *J. Biol. Chem.* 265: 15231–15238.

Brenner, B. 1980. Effect of free sarcoplasmic Ca^{2+} concentration on maximum unloaded shortening velocity: measurements of single glycerinated rabbit psoas muscle fibers. *J. Muscle Res. Cell Motil.* 1:409–428.

Brenner, B. 1983. Technique for stabilizing the striation pattern in maximally calcium-activated skinned rabbit psoas fibers. *Biophys. J.* 41: 99–102.

Brenner, B. 1988. Effect of Ca^{2+} on cross-bridge turnover kinetics in skinned single rabbit psoas fibers: Implications for regulation of muscle contraction. *Proc. Natl. Acad. Sci. USA.* 85:3265–3269.

Brenner, B., and E. Eisenberg. 1986. Rate of force generation in muscle: correlation with actomyosin ATPase in solution. *Proc. Natl. Acad. Sci. USA.* 83:3542–3546.

Brenner, B., M. Schoenberg, J. M. Chalovich, L. E. Greene, and E. Eisenberg. 1982. Evidence for cross-bridge attachment in relaxed muscle at low ionic strength. *Proc. Natl. Acad. Sci. USA.* 79:7288–7291.

Brenner, B., L. C. Yu, and J. M. Chalovich. 1991. Parallel inhibition of active force and relaxed fiber stiffness in skeletal muscle by caldesmon. *Proc. Natl. Acad. Sci. USA.* 88:5739–5743.

Brenner, B., S. Xu, J. M. Chalovich, and L. C. Yu. 1995. Spatial dimensions of attached cross-bridges in the filament lattice of relaxed, activated and rigor muscle. *Biophys. J.* 68:A9.

Bryan, J., M. Imai, R. Lee, P. Moore, R. G. Cook, and W.-G. Lin. 1989. Cloning and expression of a smooth muscle caldesmon. *J. Biol. Chem.* 264:13873–13879.

Chalovich, J. M., J. Bryan, C. E. Benson, and L. Velaz. 1992. Localization and characterization of a 7.3 kDa region of caldesmon which reversibly inhibits actomyosin ATPase activity. *J. Biol. Chem.* 267:16644–16650.

Chalovich, J. M., P. Cornelius, and C. E. Benson. 1987. Caldesmon inhibits skeletal actomyosin subfragment-1 ATPase activity and the binding of myosin subfragment-1 to actin. *J. Biol. Chem.* 262:5711–5716.

Chalovich, J. M., and E. Eisenberg. 1982. Inhibition of actomyosin ATPase activity by troponin-tropomyosin without blocking the binding of myosin to actin. *J. Biol. Chem.* 257:2432–2437.

Chen, Y., and J. M. Chalovich. 1992. A mosaic-multiple binding model for the binding of caldesmon and myosin subfragment-1 to actin. *Biophys. J.* 63:1063–1070.

Crosbie, R. H., J. M. Chalovich, and E. Reisler. 1995. Flexation of caldesmon: effect of conformation on the properties of caldesmon. *J. Muscle Res. Cell Motil.* 16:509–518.

Dabrowska, R., A. Goch, B. Galazkiewicz, and H. Osinska. 1985. The influence of caldesmon on ATPase activity of the skeletal muscle actomyosin and bundling of actin filaments. *Biochim. Biophys. Acta.* 842:70–75.

Eisenberg, E., and W. W. Kielley. 1972. Evidence for a refractory state of heavy meromyosin and subfragment-1 unable to bind to actin in the presence of ATP. *Cold Spring Harb. Symp. Quant. Biol.* 37:145–152.

Eisenberg, E., and W. W. Kielley. 1974. Troponin-tropomyosin complex. Column chromatographic separation and activity of the three active troponin components with and without tropomyosin present. *J. Biol. Chem.* 249:4742–4748.

Fujii, T., M. Imai, G. C. Rosenfeld, and J. Bryan. 1987. Domain mapping of chicken gizzard caldesmon. *J. Biol. Chem.* 262:2757–2763.

Granzier, H. L. M., and K. Wang. 1993. Passive tension and stiffness of vertebrate skeletal and insect flight muscles: the contribution of weak cross-bridges and elastic filaments. *Biophys. J.* 65:2141–2159.

Haeberle, J. R., K. M. Trybus, M. E. Hemric, and D. M. Warshaw. 1992. The effects of smooth muscle caldesmon on actin filament motility. *J. Biol. Chem.* 267:23001–23006.

Hayashi, K., Y. Fujio, I. Kato, and K. Sobue. 1991. Structural and functional relationships between *h*- and *l*-caldesmons. *J. Biol. Chem.* 266: 355–361.

Hemric, M. E., and J. M. Chalovich. 1988. Effect of caldesmon on the ATPase activity and the binding of smooth and skeletal myosin subfragments to actin. *J. Biol. Chem.* 263:1878–1885.

Hemric, M. E., and J. M. Chalovich. 1990. Characterization of caldesmon binding to myosin. *J. Biol. Chem.* 265:19672–19678.

Hemric, M. E., M. V. Freedman, and J. M. Chalovich. 1993. Inhibition of actin stimulation of skeletal muscle (A1)S-1 ATPase activity by caldesmon. *Arch. Biochem. Biophys.* 306:39–43.

Horiuchi, K. Y., M. Samuel, and S. Chacko. 1991. Mechanism for the inhibition of acto-heavy meromyosin ATPase by the actin/calmodulin binding domain of caldesmon. *Biochemistry.* 30:712–717.

Ikebe, M., and S. Reardon. 1988. Binding of caldesmon to smooth muscle myosin. *J. Biol. Chem.* 263:3055–3058.

Itoh, T., M. Ikebe, G. J. Kargacin, D. J. Hartshorne, B. E. Kemp, F. S. Fay. 1989. Effects of modulators of myosin light-chain kinase activity in single smooth muscle cells. *Nature.* 338:164–167.

Kielley, W. W., and W. F. Harrington. 1960. A model for the myosin molecule. *Biochim. Biophys. Acta.* 41:401–421.

Kraft, Th., J. M. Chalovich, L. C. Yu, and B. Brenner. 1995a. Parallel inhibition of active force and relaxed fiber stiffness by caldesmon fragments at physiological ionic strength and temperature conditions. Additional evidence that weak cross-bridge binding to actin is an essential intermediate for force generation. *Biophys. J.* 68:2404–2418.

Kraft, T., M. Messerli, B. Rothen-Rutishauser, J.C. Perriard, T. Wallimann, and B. Brenner. 1995b. Equilibration and exchange of fluorescently labeled molecules in skinned skeletal muscle fibers visualized by confocal microscopy. *Biophys. J.* 69:1246–1258.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.

Leszyk, J., D. Mornet, E. Audemard, and J. H. Collins. 1989. Caldesmon structure and function: sequence analysis of a 35 kDa actin- and calmodulin-binding fragment from the C-terminus of the turkey gizzard protein. *Biochem. Biophys. Res. Commun.* 160:1371–1378.

Lynch, W. P., V. M. Riseman, and A. Bretscher. 1987. Smooth muscle caldesmon is an extended flexible monomeric protein in solution that can readily undergo reversible intra- and intermolecular sulfhydryl cross-linking. *J. Biol. Chem.* 262:7429–7437.

Mabuchi, K., J. J.-C. Lin, and C.-L. A. Wang. 1993. Electron microscopic images suggest both ends of caldesmon interact with actin filaments. *J. Muscle Res. Cell Motil.* 14:54–64.

Makuch, R., M. P. Walsh, and R. Dabrowska. 1989. Location of the calmodulin- and actin-binding domains at the C-terminus of caldesmon. *FEBS Lett.* 247:411–414.

Marston, S. 1988. Aorta caldesmon inhibits actin activation of thiophosphorylated heavy meromyosin Mg^{2+}-ATPase activity by slowing the rate of product release. *FEBS Lett.* 238:147–150.

Marston, S. B., and W. Lehman. 1985. Caldesmon is a Ca^{2+}-regulatory component of native smooth-muscle thin filaments. *Biochem. J.* 231: 517–522.

Marston, S. B., and C. S. Redwood. 1991. The molecular anatomy of caldesmon. *Biochem. J.* 279:1–16.

Marston, S. B., and C. S. Redwood. 1992. Inhibition of actin-tropomyosin activation of myosin MgATPase activity by the smooth muscle regulatory protein caldesmon. *J. Biol. Chem.* 267:16796–16800.

Marston, S. B., and C. W. J. Smith. 1984. Purification and properties of Ca^{2+}-regulated thin filaments and F-actin from sheep aorta smooth muscle. *J. Muscle Res. Cell Motil.* 5:559–575.

Martin, F., M.-C. Harricane, E. Audemard, F. Pons, and D. Mornet. 1991. Conformational change of turkey-gizzard caldesmon induced by specific chemical modification with carbodiimide. *Eur. J. Biochem.* 195: 335–342.

Moody, C., W. Lehman, and R. Craig. 1990. Caldesmon and the structure of smooth muscle thin filaments: electron microscopy of isolated thin filaments. *J. Muscle Res. Cell Motil.* 11:176–185.

Mornet, D., E. Audemard, and J. Derancourt. 1988. Identification of a 15 kDa actin binding region of gizzard caldesmon probed by chemical cross-linking. *Biochem. Biophys. Res. Commun.* 154:564–571.

Mornet, D., A. Bonet-Kerrache, G. M. Strasburg, V. B. Patchell, S. V. Perry, P. A. J. Huber, S. B. Marston, D. A. Slatter, J. S. Evans, and B. A. Levine. 1995. The binding of distinct segments of actin to multiple sites in the C-terminus of caldesmon: comparative aspects of actin interaction with troponin-I and caldesmon. *Biochemistry.* 34:1893–1901.

Pfitzer, G., C. Zeugner, M. Troschka, and J. M. Chalovich. 1993. Caldesmon and a 20-kDa actin-binding fragment of caldesmon inhibit tension development in skinned gizzard muscle fiber bundles. *Proc. Natl. Acad. Sci. USA.* 90:5904–5908.

Redwood, C. S., and S. B. Marston. 1993. Binding and regulatory properties of expressed functional domains of chicken gizzard smooth muscle caldesmon. *J. Biol. Chem.* 268:10969–10976.

Riseman, V. M., W. P. Lynch, B. Nefsky, and A. Bretscher. 1989. The calmodulin and F-actin binding sites of smooth muscle caldesmon lie in the carboxyl-terminal domain whereas the molecular weight heterogeneity lies in the middle of the molecule. *J. Biol. Chem.* 264:2869–2875.

Smith, C. W. J., K. Pritchard, and S. B. Marston. 1987. The mechanism of Ca^{2+} regulation of vascular smooth muscle thin filaments by caldesmon and calmodulin. *J. Biol. Chem.* 262:116–122.

Spudich, J. A., and S. Watt. 1971. The regulation of rabbit skeletal muscle contraction. Biochemical studies of the interaction of the tropomyosin-troponin complex with actin and the proteolytic fragments of myosin. *J. Biol. Chem.* 246:4866–4871.

Szpacenko, A., and R. Dabrowska. 1986. Functional domain of caldesmon. *FEBS Lett.* 202:182–186.

Takagi, T., M. Yazawa, T. Ueno, S. Suzuki, and K. Yagi. 1989. Amino acid sequence studies on cyanogen bromide peptides of chicken caldesmon which bind to calmodulin. *J. Biochem. (Tokyo).* 106:778–783.

Velaz, L., Y. Chen, and J. M. Chalovich. 1993. Characterization of a caldesmon fragment that competes with myosin-ATP binding to actin. *Biophys. J.* 65:892–898.

Velaz, L., M. E. Hemric, C. E. Benson, and J. M. Chalovich. 1989. The binding of caldesmon to actin and its effect on the ATPase activity of soluble myosin subfragments in the presence and absence of tropomyosin. *J. Biol. Chem.* 264:9602–9610.

Velaz, L., R. H. Ingraham, and J. M. Chalovich. 1990. Dissociation of the effect of caldesmon on the ATPase activity and on the binding of smooth heavy meromyosin to actin by partial digestion of caldesmon. *J. Biol. Chem.* 265:2929–2934.

Wang, C.-L. A., J. M. Chalovich, P. Graceffa, R. C. Lu, K. Mabuchi, and W. F. Stafford. 1991a. A long helix from the central regioin of smooth muscle caldesmon. *J. Biol. Chem.* 266:13958–13963.

Wang, C.-L. A., L-W.C. Wang, S. Xu, R. C. Lu, V. Saavedra-Alanis, and J. Bryan. 1991b. Localization of the calmodulin- and the actin-binding sites of caldesmon. *J. Biol. Chem.* 266:9166–9172.

Wang, Z., K. Y. Horiuchi, and S. Chacko. 1996. Characterization of the functional domains on the C-terminal region of caldesmon using full-length and mutant caldesmon molecules. *J. Biol. Chem.* 271:2234–2242.

Weeds, A. G., and R. S. Taylor. 1975. Separation of subfragment-1 isoenzymes from rabbit skeletal muscle myosin. *Nature.* 257:54–56.

Yazawa, M., K. Yagi, and K. Sobue. 1987. Isolation and characterization of a calmodulin binding fragment of chicken gizzard caldesmon. *J. Biochem. (Tokyo).* 102:1065–1073.

Yu, L. C., and B. Brenner. 1989. Structures of actomyosin cross-bridges in relaxed and rigor muscle fibers. *Biophys. J.* 55:441–453.

Zhan, Q., S. S. Wong, and C-L. A. Wang. 1991. A calmodulin-binding peptide of caldesmon. *J. Biol. Chem.* 266:21810–21814.