Strong Interaction between Caldesmon and Calponin*

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Caldesmon was labeled at either Cys-153 in the NH2-terminal domain or Cys-580 in the COOH-terminal domain with a 6-acryloyl-2-dimethylaminonaphthalene (acrylodan) fluorescence probe. The addition of smooth muscle calponin to Cys-580-labeled caldesmon resulted in an 18% drop in fluorescence intensity, which titrated with a stoichiometry of 0.9 and a binding constant of 9.5 × 10^6 M⁻¹. For Cys-153-labeled caldesmon, there was no change in fluorescence upon adding calponin. These findings indicate strong binding between calponin and the COOH-domain of caldesmon. The association was sensitive to ionic strength, suggesting that ionic interactions between calponin, a basic protein, and caldesmon, an acidic protein, contribute to the stabilization of the protein complex. That non-muscle acidic calponin interacts with caldesmon with a much reduced association constant of 3.5 × 10^3 M⁻¹ supports such a model. The binding between acidic calponin and caldesmon is strengthened to 1.8 × 10^7 M⁻¹ in the presence of Ca²⁺, which might bind to acidic residues of the calponin and partially neutralize its negative charge. The strong, specific binding between calponin and caldesmon suggests that this interaction occurs within smooth muscle cells and possibly plays a role in the regulation of contraction.

Smooth muscle contraction is regulated primarily by the phosphorylation of myosin light chains by a Ca²⁺/calmodulin-dependent myosin light chain kinase (1). However, under certain conditions there is a dissociation of contraction from myosin phosphorylation (for review, see Refs. 2–5) which has stimulated a search for additional regulatory mechanism(s). The search for a possible second regulatory pathway has focused on the actin-binding proteins caldesmon and calponin since, in vitro, they both inhibit actomyosin ATPase activity (for review, see Refs. in 6–8) which can be reversed by Ca²⁺-binding proteins (for review, see Ref. 9) or phosphorylation (for review, see Refs. 4, 10, and 11). There is growing physiological support for such a role for caldesmon (12–14) and calponin (15–18). The ability of caldesmon to cross-link actin and myosin by means of its COOH-terminal domain binding to actin and its NH2-terminal domain binding to myosin has also been postulated to serve several possible functions (see the Introduction of Ref. 19) including the promotion of myosin filament assembly in the vicinity of actin filaments, the enhancement of a productive interaction between myosin and actin, and the maintenance of tension, at the expenditure of very little energy, responsible for smooth muscle tone. Calponin has also been postulated to be responsible for this tension (20, 21). However, the in vivo mechanism whereby caldesmon and calponin take part in the regulation of smooth muscle contraction remains unclear.

The distribution of caldesmon and calponin in smooth muscle tissue is different. Smooth muscle has been regarded to contain two actin filament domains: a contractile domain that contains myosin and a cytoskeletal domain that does not (for review, see Refs. 22 and 23). Studies on the localization of caldesmon and calponin in smooth muscle tissue have revealed that whereas caldesmon is contained exclusively in the contractile domain (24–27), calponin is found in both regions (26, 27), although it is more predominant in the cytoskeletal region (27, 28). Furthermore, upon agonist stimulation of muscle, calponin redistributes to the periphery of the muscle cell (29), whereas caldesmon remains stationary (30).

Although there is a difference in distribution of caldesmon and calponin, evidence suggests that they could work together. First, they are both present in the contractile region of the muscle cell (see above). Second, at subsaturating concentrations, calponin and caldesmon can bind simultaneously to actin filaments (31). Finally, Vancomprenolle et al. (32) have reported that calponin and caldesmon interact, since calponin binds to a caldesmon affinity column, although the interaction was considered to be too weak to be relevant to calponin function (8). Therefore we have reinvestigated a possible interaction between calponin and caldesmon and, on the contrary, have found a very strong and specific interaction between them, raising, for the first time, the possibility that this interaction has a role in the regulation of smooth muscle contraction.

EXPERIMENTAL PROCEDURES

The preparation of caldesmon from fresh chicken gizzard or porcine stomach was carried out as described (33) with the addition of calmodulin-affinity chromatography (34). Recombinant chicken gizzard smooth muscle α-calponin was expressed, purified, and lyophilized as described by Gong et al. (35). Lyophilized calponin was dissolved in 6 M urea, 40 mM NaCl, 5 mM Mops, 0.2 mM EDTA, pH 7.5, 2 mM diethyothreitol and then dialyzed versus 0.1 M NaCl, 5 mM Mops, 0.2 mM EDTA, pH 7.5, plus the protease inhibitors, 0.25 mM phenylmethylsulfonyl fluoride, 0.3 mM benzamidine, 1 μg/ml leupeptin. In some cases it was necessary to centrifuge the final sample to clarify it from undissolved calponin. The concentrations of caldesmon and calponin were determined by the Lowry method using rabbit skeletal muscle tropomyosin as a standard (see Ref. 36). In some experiments, porcine stomach caldesmon was phosphorylated, using MAP kinase, to a level of 1.3 mol of phosphate/mol of protein, as described by Adam and Hathaway (37). Recombinant rat aorta acidic calponin was expressed and purified according to Applegate et al. (38) from a cDNA clone provided by Dr. Dianne Applegate. The purified acidic calponin was precipitated with ammonium sulfate and stored frozen. Before use the ammonium sulfate pellet was dialyzed against a 0.1 M NaCl buffer, pH 7.5, and then, in some cases, put

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† The abbreviations used are: Mops, 3-(N-morpholino)propanesulfonic acid; MAP, mitogen-activated protein; acrylodan, 6-acryloyl-2-dimethylaminonaphthalene.
through the urea treatment described above for muscle calponin. The concentration of acidic calponin was determined from its optical density at 280 nm using an extinction coefficient \( E^{1}\text{\textsc{m}} = 9.8 \), which was determined by Dr. Walter Stafford from its refractive index in the analytical ultracentrifuge\(^2\) according to Graceffa et al. (39).

Caldesmon was specifically labeled at either Cys-153 in the NH\(_2\)-domain or Cys-580 in the COOH-domain, with the sulphydryl-specific fluorescent probe 6-acyrly-2-

\( \text{dithiobis(2-nitrobenzoic acid)-modified actin whereupon Cys-153 was free to be modified (19, 33). The disulfide bond was then cleaved with dithiothreitol and the Cys-153-labeled caldesmon separated from the actin.}

The degree of labeling of acrylodan-caldesmon was determined from the extinction coefficient reported by Prendergast et al. (41). The degree of labeling of the Cys-153-labeled protein was 0.75 mol of acrylodan/mol of caldesmon, consistent with partial labeling of Cys-153. Evidence for the specific labeling of Cys-153 came from performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis of thrombin-digested Cys-153-labeled caldesmon as performed previously for Cys-153-labeled with a coumarin fluorescent probe (33). Thrombin cleaves (chicken) caldesmon primarily at residue 483 (42, 43), resulting in a large NH\(_2\)-terminal fragment containing Cys-153 and a small COOH-terminal fragment containing Cys-580. As we found previously for the coumarin probe (33), only the NH\(_2\)-terminal fragment was fluorescent on a gel of thrombin-digested labeled caldesmon (not shown). The labeling ratio of Cys-580-labeled (porcine) caldesmon was between 0.9 and 1.1 mol of acrylodan/mol of caldesmon, consistent with full and specific labeling of the single cysteine.

Fluorescence emission spectra of acrylodan-labeled caldesmon were obtained with a Spex Fluorolog 2/2/2 photon-counting fluorometer in the ratio mode at an excitation wavelength of 385 nm. Spectra were recorded at temperatures between 4 and 37 °C at a caldesmon concentration of about 0.4 \( \mu \text{M} \) in a solution containing 40–100 mM NaCl, 2 mM MgCl\(_2\), 5 mM Mops, pH 7.5. The fluorescence of acrylodan-labeled caldesmon primarily at residue 483 (42, 43), resulting in a large NH\(_2\)-terminal fragment containing Cys-153 and a small COOH-terminal fragment containing Cys-580. As we found previously for the coumarin probe (33), only the NH\(_2\)-terminal fragment was fluorescent on a gel of thrombin-digested labeled caldesmon (not shown). The labeling ratio of Cys-580-labeled (porcine) caldesmon was between 0.9 and 1.1 mol of acrylodan/mol of caldesmon, consistent with full and specific labeling of the single cysteine.

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**RESULTS AND DISCUSSION**

The fluorescence spectrum of acrylodan-labeled caldesmon shows a broad peak with a maximum fluorescence at about 522 nm (Fig. 1). The addition of chicken gizzard muscle \( \alpha \)-calponin to caldesmon labeled at Cys-580 in the COOH-domain, in 40 mM NaCl at 20 °C, results in an 18% drop in fluorescence compared with chicken gizzard muscle calponin (compare Figs. 2 and 3). Thus, although the

\( \text{Caldesmon-Calponin Interaction} \)

![Fluorescence spectrum of caldesmon labeled with acrylodan](image)

**FIG. 1. Fluorescence spectrum of caldesmon labeled with acrylodan at Cys-580 (panel A) or Cys-153 (panel B) as a function of added muscle \( \alpha \)-calponin at 20 °C in 40 mM NaCl, 2 mM MgCl\(_2\), 5 mM Mops, pH 7.5. In panel A the fluorescence intensity decreases as calponin is added to caldesmon;calponin molar ratios of 0.0, 0.25, 0.5, 0.75, 1.0, and 5.0. In panel B the spectra at a calponin/caldesmon ratio of 0.0 and 5.0 almost exactly coincide.**

In a later review article (8) they concluded that the interaction was apparently too weak to be relevant to calponin’s function. This conclusion is in contrast to our findings.

The calponin-caldesmon association was measured over a range of temperatures and ionic strengths (Table I). Increasing the salt concentration from 40 to 100 mM somewhat weakened the binding affinity at 20 °C but had no significant effect at 4 °C. The affinity was strengthened with increasing temperature. As a result, the association between calponin and caldesmon is strong at temperatures and ionic strength close to physiological conditions. The presence or absence of 0.5 mM Ca\(^{2+} \) had no effect on the calponin-caldesmon binding strength (data not shown). Since calponin is a basic protein with an isoelectric point of about 10 (8, 45), and caldesmon has a high acidic residue content with an isoelectric point around 5 (46), ionic interactions might contribute to the stabilization of the calponin-caldesmon complex and thus could account for the ability of salt to weaken the interaction.

Support for ionic interactions contributing to the calponin-caldesmon binding strength came from examining the association between caldesmon and an acidic isoform of calponin. This calponin has an additional highly acidic 38-residue COOH-terminal tail compared with the chicken gizzard muscle \( \alpha \)-calponin, which results in a protein with a much lower pI \( \sim 5 \) (8, 38). This calponin is thought to be a non-muscle isoform of the protein (38). The interaction between caldesmon and acidic calponin was characterized by a binding constant of \( K_a = 3.5 \times 10^{16} \) (Fig. 3), which is about 1.5 orders of magnitude less than that for the muscle calponin. (Treating or not treating acidic calponin with urea (see “Experimental Procedures”) had very little effect on the binding constant.) However, the binding of acidic calponin to caldesmon resulted in a 2-fold greater reduction in acrylodan fluorescence compared with the binding of muscle calponin (compare Figs. 2 and 3). Thus, although the

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\(^2\) W. Stafford, unpublished results.
increased negative charge of the acidic calponin weakens the interaction with caldesmon, it also results in a different interaction such that the acrylodan probe is more highly exposed to solvent (see below). The fact that an additional (acidic) COOH-terminal tail of calponin strongly affects its interaction with caldesmon suggests that it is the COOH-terminal region of muscle calponin which interacts with caldesmon.

It was considered that if Ca$^{2+}$ were to bind to the COOH-terminal tail of acidic calponin it might partially neutralize the negative charge and increase caldesmon binding. Therefore, the binding between acidic calponin and caldesmon was measured in the presence of 0.5 mM Ca$^{2+}$, and it was found that the association constant increased 5-fold to 1.8 × 10$^7$ M$^{-1}$ (Fig. 3), thereby supporting this idea. Furthermore, these results suggest the possibility that acidic calponin binds Ca$^{2+}$, probably in its COOH-terminal tail, although its sequence does not contain any recognized Ca$^{2+}$-binding motifs, and that this binding could affect its function. Further work is necessary to test this hypothesis.

The acrylodan probe is very sensitive to the probe’s environment, with the fluorescence shifting to the blue and increasing in intensity with an increasing hydrophobic environment and shielding from the solvent (41). The drop in caldesmon acrylodan (at Cys-580) fluorescence upon addition of calponin indicates that the binding of calponin causes a conformational change in caldesmon around Cys-580 with a concomitant increase in the exposure of the acrylodan probe to the solvent. There is no parallel red shift in the fluorescence peak, most likely because the peak position of acrylodan-caldesmon alone is already in the red of the wavelength range of the acrylodan probe (i.e. 435–540 nm; 41). In agreement with this conclusion, the complete unfolding of acrylodan-caldesmon in 4 M guanidine hydrochloride (47), which should highly expose the probe to solvent, results in only a slight red shift of acrylodan fluorescence to about 527 nm (data not shown). Calponin is probably not binding directly to the Cys-580 region of the caldesmon COOH-terminal domain since such an interaction would most likely lead to a shielding of the probe from the solvent and a resulting increase in fluorescence intensity and blue shift of the spectrum.

For the caldesmon-calponin interaction to have functional relevance it is necessary for such an interaction to take place in the presence of actin-tropomyosin. Therefore we titrated the fluorescence of Cys-580-labeled caldesmon bound to actin-tropomyosin (actin:caldesmon molar ratio = 15) with calponin, in 40 mM NaCl, 2 mM MgCl$_2$, pH 7.5. The addition of actin-tropomyosin to acrylodan-Cys-580-labeled caldesmon results in a large (75%) increase in fluorescence intensity with a large blue shift (from 522 to 485 nm) of the fluorescence maximum, indicating a shielding of the probe from the solvent by interaction with actin-tropomyosin. Upon further addition of calponin, up to a calponin/caldesmon molar ratio of 2, there was a very small (about 5%) decrease in fluorescence intensity without any change in peak position (data not shown). Furthermore, all of the caldesmon and calponin remained bound to the actin-tropomyosin, as determined by an actin sedimentation assay. This is consistent with calponin binding to caldesmon, with a much smaller decrease in fluorescence in the presence than in the absence of actin-tropomyosin because the actin-tropomyosin makes the probe less sensitive to calponin binding. However, another equally viable interpretation of these results is that calponin is indirectly affecting caldesmon fluorescence via actin. Thus, multiple interpretations of the results coupled with the small change in fluorescence make conclusions drawn from these experiments in the presence of actin-tropomyosin not definitive. Therefore, the possible interaction

Table I

| Temperature | 40 mM NaCl | 100 mM NaCl |
|-------------|-------------|-------------|
| °C          | $K_a \times 10^{-7}$ | $K_a \times 10^{-7}$ |
| 4           | 2.3         | 2.9         |
| 20          | 9.7         | 3.1         |
| 37          | ND$^a$      | 7.3         |

$^a$ ND, not determined.

The lines through the points are least squares fits to a simple binding equation described by Morris and Lehrer (51) and Grabarek et al. (52) to obtain a binding affinity constant $K_a$ of CaP/CaD. Solvent conditions are as in Fig. 1. Panel A, unphosphorylated caldesmon; panel B, caldesmon phosphorylated by MAP kinase. The lines through the points are least squares fits to a simple binding equation described by Morris and Lehrer (51) and Grabarek et al. (52) to obtain a binding affinity constant $K_a$ of CaP/CaD. Solvent conditions are as in Fig. 1. The data points were fit as in Fig. 2, which yielded a binding constant $K_a = 3.5 \times 10^7$ M$^{-1}$ for Ca$^{2+} = 0$ and $1.8 \times 10^7$ M$^{-1}$ for Ca$^{2+} = 0.5$ mM. It was considered that if Ca$^{2+}$ were to bind to the COOH-terminal of CaP/CaD, expressed as the molar ratio of CaP/CaD. Solvent conditions are as in Fig. 1. Panel A, unphosphorylated caldesmon; panel B, caldesmon phosphorylated by MAP kinase. The lines through the points are least squares fits to a simple binding equation described by Morris and Lehrer (51) and Grabarek et al. (52) to obtain a binding affinity constant $K_a$ and a binding stoichiometry $n$. In panel A, $K_a = 9.5 \times 10^7$ M$^{-1}$, $n = 0.9$. In panel B, $K_a = 8 \times 10^7$ M$^{-1}$, $n = 0.9$.

![FIG. 2. Fractional decrease in fluorescence intensity at 522 nm of caldesmon (CaD) labeled at Cys-580 with acrylodan, plotted as a function of added muscle acidic calponin (aCaP), expressed as the molar ratio of CaP/CaD. Solvent conditions are as in Fig. 1. Panel A, unphosphorylated caldesmon; panel B, caldesmon phosphorylated by MAP kinase. The lines through the points are least squares fits to a simple binding equation described by Morris and Lehrer (51) and Grabarek et al. (52) to obtain a binding affinity constant $K_a$ and a binding stoichiometry $n$. In panel A, $K_a = 9.5 \times 10^7$ M$^{-1}$, $n = 0.9$. In panel B, $K_a = 8 \times 10^7$ M$^{-1}$, $n = 0.9$.](image)

![FIG. 3. Fractional decrease in fluorescence intensity at 522 nm of caldesmon (CaD) labeled at Cys-580 with acrylodan, plotted as a function of added muscle acidic calponin (aCaP), expressed as the molar ratio of CaP/CaD. Open circles, Ca$^{2+} = 0$; closed circles, Ca$^{2+} = 0.5$ mM.](image)
of caldesmon and calponin in the presence of actin-tropomyosin awaits different experimental approaches.

That calponin and caldesmon interact so strongly suggests that such an interaction occurs in the smooth muscle cell and could play a regulatory or structural role in smooth muscle contraction. For such a role to be viable, it would be necessary for calponin to interact with caldesmon in the presence of actin. Such an interaction is possible, since the affinity between calponin and caldesmon is stronger than the affinity of either caldesmon (48, 49) or calponin (8, 50) for actin. Furthermore, it has been shown (see the Introduction) that a fraction of calponin saturates actin (31; see above). However, the possible role of a calponin-caldesmon complex in smooth muscle is presently unknown and awaits further characterization.

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