Smooth Muscle Calponin–Caltropin Interaction: Effect on Biological Activity and Stability of Calponin†

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ABSTRACT: Calponin inhibits actomyosin Mg^{2+} ATPase and is proposed to regulate smooth muscle contraction; however, the mechanism by which it exerts its effect and the regulation of its behavior is still under investigation. The proposed methods by which calponin regulation is effected include reversible phosphorylation of calponin which would allow contraction to occur and regulation by interaction with calcium–calmodulin. However, several investigators have been unable to find evidence of *in vivo* phosphorylation of calponin, and the affinity between calponin and calmodulin is not high enough to suggest that this interaction is biologically significant. In this paper, we present an alternative method of calponin regulation *via* calcium–caltropin and describe the calponin–caltropin complex for the first time. Caltropin, a calcium-binding protein isolated from smooth muscle, is a dimer under native conditions and interacts with calponin in a calcium-dependent fashion in the ratio of 2 mol of dimer:1 mol of calponin. The formation of this complex can be monitored by following the fluorescence of an acrylodan label on cysteine 273 of calponin, which undergoes a 35-nm blue shift in wavelength peak from 505 to 470 nm when calponin becomes complexed with caltropin. This fluorescence change when titrated with calcium indicates that the concentration of calcium required for complex formation is approximately 10^{-5} M, corresponding to the low-affinity calcium-binding sites of caltropin. This complex was further characterized by circular dichroism (CD). The CD spectrum of the complex has a negative ellipticity 1590° less than predicted for these two proteins in the presence of calcium, indicating secondary structure changes in one or both of the protein reactants, and these take the form of a decrease in α-helix and an increase in β-sheet. Circular dichroism was used to monitor both the guanidine hydrochloride (Gdn·HCl) and temperature denaturation of the complex. In both cases, the results indicated that calponin is more stable in the complex than when free in solution. The midpoint for the guanidine titration curve of the complex was 2.48 M Gdn·HCl vs 1.25 M Gdn·HCl for calponin alone. The midpoint of the melting curve for calponin alone is 55 °C, while the complex does not fully melt even at 80 °C. Caltropin is capable of regulating calponin's inhibition of the actomyosin ATPase, and it does this more efficiently than calmodulin. Thus, calponin and caltropin have the necessary properties to function as a regulatory complex in smooth muscle contraction.

Smooth muscle is known to contract in response to increased calcium levels in the cell. The primary mechanism of regulation of this activity occurs at the level of the thick filament *via* reversible phosphorylation of the myosin light chains (Adelstein & Eisenberg, 1980). When calcium levels increase in the cell, calcium binds to calmodulin, allowing calmodulin to then interact with myosin light chain kinase. This activates myosin light-chain kinase to phosphorylate myosin chains, which enables myosin to interact with actin, and contraction ensues (Hartshorne, 1987). However, this model for contractile regulation does not explain all of the observed behavior of smooth muscle. For example, it has been shown that when calcium levels decrease in the cell, there is a drop in phosphorylation of the myosin light chains, which does not necessarily result in a decrease in tension of the muscle [reviewed in Hai and Murphy (1989)]. In addition, smooth muscle thin filaments, used in conjunction with skeletal myosin (Marston & Smith, 1984), or thiophosphorylated smooth muscle myosin (Marston & Lehman, 1985) are still calcium sensitive, which indicates the thin filaments of smooth muscle must possess a calcium-sensitive component, the identity of which is yet to be established.

Preparations of chicken gizzard thin filaments have been shown to contain actin, tropomyosin, caldesmon, and calponin (Nishida *et al.*, 1990). Calponin and caldesmon demonstrate many similar properties in binding to actin, tropomyosin, and calcium-binding proteins (Takahashi *et al.*, 1986, 1988; Sobue *et al.*, 1981; Graceffa, 1987) and in inhibition of actomyosin ATPase activity (Ngai & Walsh, 1984; Winder & Walsh, 1990). It is well documented that caldesmon binds myosin (Hemric & Chalovich, 1990), while it has recently been proposed that calponin also binds myosin (Lin *et al.*, 1993; Szymanski & Tao, 1993), although the conditions may not be relevant in that the interaction does not occur at physiological salt concentrations. Both proteins can inhibit actin mobility on immobilized myosin, although their mechanism of action appears to differ in that calponin behaves in an all or none fashion while caldesmon produces a graded response (Shirinsky *et al.*, 1992). The relative importance and function of these two proteins have yet to be worked out.

In 1986, Takahashi *et al.* first identified calponin as a component of smooth muscle that bound to actin, tropomyosin, and calmodulin. Later, it was shown by Winder and Walsh (1990) that calponin was able to inhibit the actomyosin ATPase, suggesting that calponin was a prime candidate for a smooth muscle regulatory protein. Attention is now focused on elucidating how calponin becomes calcium sensitive, since it does not interact with calcium directly (Wills *et al.*, 1993)

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and a reconstituted thin-filament system consisting of only actin, tropomyosin, and calponin is not calcium sensitive (Winder & Walsh, 1990). Originally, calmodulin was identified as a possible calcium-sensitizing agent because it bound to calponin in a calcium-sensitive fashion. It turned out, however, that the mole ratio of calmodulin to calponin required to reverse calponin's inhibition is too great to suggest that this interaction could be of biological importance (Abe *et al.*, 1990; Makuch *et al.*, 1991). More recently, our laboratory has shown that calponin will interact with a variety of calcium-binding proteins which expose hydrophobic patches upon interaction with calcium. Among the proteins tested were two which displayed higher affinities for calponin than calmodulin did: S-100b and caltropin (Wills *et al.*, 1993). Since caltropin is isolated from smooth muscle tissue, it is a *bona fide* candidate as a regulator of calponin inhibition.

Caltropin was first described by Mani and Kay (1990). This smooth muscle protein binds 2 mol of calcium/mol of monomer and exposes a hydrophobic patch upon binding calcium. It has been shown, using analytical ultracentrifugation, that a molecular mass of 21 000 Da,[1] representing a dimer of caltropin, is observed under native conditions. Upon binding calcium, caltropin undergoes structural changes as reflected by circular dichroism, UV difference spectroscopy, and fluorescence. Therefore, this protein has the requirements to function as a calcium-sensitive protein in smooth muscle (Mani & Kay, 1990).

The characterization of the calponin–caltropin complex is of great interest if it is a regulatory complex of smooth muscle. In this study, hydrodynamic and spectroscopic techniques have been used to determine the properties of the complex, which included stoichiometry of the protein constituents in the complex and its stability to guanidine and temperature as well as its calcium sensitivity and secondary structural changes occurring upon interaction of the two relevant proteins.

MATERIALS AND METHODS

Protein Purifications. Calponin was purified from turkey gizzards by a modification of the method of Takahashi *et al.* (1986) in which a S-Sepharose fast-flow column was used in place of a SP-C50 cation-exchange column. The pool from the S-Sepharose column was dialyzed overnight vs 10 mM $NaPO_4$, 100 mM NaCl, 1 mM DTT, and 0.3 mM $CaCl_2$ and applied to a 3- × 30-cm Biorad hydroxylapatite column from which it eluted in the void volume. This fraction was lyophilized and then applied to a Synchropak reversed-phase C18 HPLC column in 0.1% TFA and eluted with a 0–70% acetonitrile/0.05% TFA gradient, from which calponin eluted at ~40% acetonitrile. This calponin was shown in spectroscopic and functional assays to behave the same as calponin isolated *via* the method of Takahashi *et al.* (1986). Caltropin was isolated from turkey gizzards according to the method of Mani and Kay (1990). Previously described methods were used to prepare bovine brain S-100b (Wills *et al.*, 1993), bovine brain calmodulin (Cachia *et al.*, 1986), rabbit skeletal actin (Pardee & Spudich, 1982), and rabbit skeletal S1 (Margossian & Lowey, 1982).

Protein Concentrations. The following extinction coefficients were used to determine protein concentrations: calponin, $A^{1\%}_{276nm} = 7.5$ (Wills *et al.*, 1993); calmodulin, $A^{1\%}_{277nm} = 1.95$ (Klee, 1977); actin, $A^{1\%}_{290nm} = 6.3$ (Lehrer & Kerwar, 1972); and S1, $A^{1\%}_{280nm} = 8.1$ (Margossian & Lowey, 1982). Absorption spectroscopy was performed on a Perkin-Elmer Lambda 5 spectrophotometer. S-100b and caltropin concentrations were determined from amino acid analysis from the compositions of Marshak *et al.* (1981) and Mani and Kay (1990), respectively. For amino acid analysis, protein samples were hydrolyzed for 1 h at 160 °C in 6 N HCl/0.1% phenol. The samples were analyzed on a Beckman 6300 ion-exchange HPLC amino acid analyzer. Amino acids were detected by postcolumn reaction with ninhydrin.

Analytical Ultracentrifugation. Studies were performed on a Beckman Spinco Model E analytical ultracentrifuge. Molecular masses were determined by low-speed sedimentation equilibrium employing Raleigh interference optics according to Chervenka (1969). Proteins were loaded at initial concentrations of 0.63–0.86 mg/mL, in 50 mM MOPS, pH 7.2, 100 mM NaCl, 1 mM DTT, and 1 mM EGTA or 2 mM $CaCl_2$. Molecular masses were calculated from the slope of the ln y vs r^2 plot. Proteins were combined in ratios of 2 mol of calponin to 1 mol of caltropin, and 2 mol of caltropin to 1 mol calponin, in the presence and absence of Ca^{2+}, in order to determine the nature of the complex.

Fluorescence Studies. Studies were performed on a Perkin-Elmer MPF 44B recording spectrofluorimeter as described previously (Wills *et al.*, 1993). The solvent used was 50 mM MOPS, pH 7.2, 100 mM NaCl, 1 mM DTT, and 1 mM EGTA, and the wavelength of excitation was 388 nm. Initial acrylodan calponin concentration in the cuvette was 0.5 μM. The fluorescence titrations of acrylodan calponin with calcium-binding protein were performed in the presence of 3 mM $CaCl_2$, and the emission was monitored at 460 nm. For the fluorescence scans, the caltropin concentration was 2.0 μM and $CaCl_2$ was added to a concentration of 3 mM. All spectra are corrected for solvent and for concentration changes. The calculation of dissociation constants were performed as previously described (Wills *et al.*, 1993), by curve fitting the fluorescence titrations assuming 2 mol of calcium-binding protein binds to 1 mol of calponin. The Ca^{2+} titration was performed with 0.5 μM calponin and 1.0 μM caltropin dimer and monitored at 460 nm.

Labeling of Calponin. Labeling with acrylodan (Molecular Probes, Eugene, OR) was performed as described previously (Wills *et al.*, 1993). The acrylodan concentration was determined using $A^{1M}_{387nm} = 16\,400$ (Prendergast *et al.*, 1983). The concentration of labeled calponin was established by amino acid analysis using the composition of the α-isoform sequenced by Takahashi and Nadal-Ginard (1991).

Determination of the Location of the Acrylodan Label. Determining the location of the label on calponin was achieved by doing a complete tryptic digest of acrylodan-labeled calponin in 50 mM MOPS, pH 7.2, 100 mM NaCl, 1 mM DTT, and 8 M urea; 1/25 trypsin (Sigma) to calponin (w/w) was added and the sample incubated at 37 °C for 24 h. The reaction was stopped by injecting onto a Zorbax 300SB C8, 4.6-mm × 25-cm RP HPLC column. A 1%/min gradient was run 0–50% acetonitrile in 0.1% TFA, and a peak that eluted at 40% acetontrile that had fluorescence characteristic of acrylodan in addition to protein absorption was collected. This peak was rerun at 0.5%/min acetonitrile to further purify

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; MOPS, 3-(*N*-morpholino)propanesulfonic acid; CD, circular dichroism; UV, ultraviolet; DTT, dithiothreitol; pCa, −log $[Ca^{2+}]_{free}$; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; Da, dalton; Gdn·HCl, guanidine hydrochloride; S1, subfragment 1 of myosin; HMM, heavy meromyosin; acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene; ATP, adenosine triphosphate; ΔG_D, observed free energy of unfolding; $\Delta G_D^{H_2O}$, free energy of unfolding in H_2O; T_m, midpoint of temperature melting.

it and then sequenced. Sequencing was performed on an Applied Biosystems 473A pulsed liquid/gas-phase protein sequencer. The sample was loaded on a precycled polybrene-coated glass fiber disc and subjected to standard Edman degradation chemistry.

Circular Dichroism Measurements. CD measurements were performed on a Jasco J-720 spectropolarimeter as previously described (Wills *et al.*, 1993). The computer averaged 10 scans, and the signal due to solvent was subtracted. The solvent used was 50 mM MOPS, pH 7.2, 100 mM NaCl, 1 mM DTT, and 1 mM EGTA with and without 4 mM $CaCl_2$. The concentration of the complex was 35.7 μM. Secondary structure analysis was performed using the algorithm of Provencher and Glöckner (1981). Theoretical curves were generated by adding the contributions of the constituent proteins in the ratios of their relative mass present during the experiment. The guanidine hydrochloride denaturation profile was monitored at 221 nm using a total protein concentration in the cell of 0.5 mg/mL. All of the samples were made up separately and allowed to equilibrate overnight before measurements were taken. Estimations of ΔG_D and $\Delta G_D^{H_2O}$, where $\Delta G_D^{H_2O}$ is the free energy of unfolding in H_2O, were calculated according to Pace (1986). The temperature denaturation was also followed at 221 nm using a total protein concentration in the cell of 0.5 mg/mL.

ATPase Assays. Assays were performed using 1 μM S1, 7 μM actin, 3.5 μM calponin, and either 3.5, 7.0, or 21.0 μM of the relevant calcium-binding protein. The buffer used was 25 mM Tris, pH 7.5, 60 mM KCl, 5 mM $MgCl_2$, and 0.5 mM DTT in a 500-μL reaction volume. All of the glassware was acid washed so the free-calcium concentration in the buffer was below the level at which calponin interacts with calcium-binding proteins for the experiments in the absence of calcium. To determine the activity in the presence of calcium, 3 mM $CaCl_2$ was added to the reaction tube. The reaction was started by adding 1 mM ATP and ended after 5 min by the addition of TCA. Inorganic phosphate was measured using the colorimetric assay with malachite green according to Itaya and Ui (1966).

Computer-Assisted Analysis. Analysis was performed as described previously (Wills *et al.*, 1993).

RESULTS AND DISCUSSION

Analytical Ultracentrifugation of the Calponin–Caltropin Complex. In order to determine the nature of the complex between caltropin and calponin, analytical ultracentrifugation was performed using varying ratios of the two proteins. Earlier studies had shown that calmodulin combines with calponin in a ratio of 2 mol of calmodulin:1 mol of calponin. This was demonstrated by the finding that in the presence of excess calponin to calmodulin, a molecular mass representing a 1:1 interaction was observed, while in the presence of 2 or 3 times the mole ratio of calmodulin to calponin, a molecular mass representative of a 2 calmodulin:1 calponin complex was demonstrated (Wills *et al.*, 1993). It was also shown in this earlier paper that the formation of the complex required the presence of calcium. In addition, experiments have been performed on troponin C and S-100b, which also form a complex of 2 calcium-binding proteins:1 calponin in a calcium-dependent fashion (data not shown). In the present study, the molecular mass of the caltropin–calponin complex also varies in a calcium-dependent fashion and depends as well upon the mole ratio of the interacting proteins. The nature of complex formation was established by analytical ultracentrifugation in the presence and absence of calcium. The

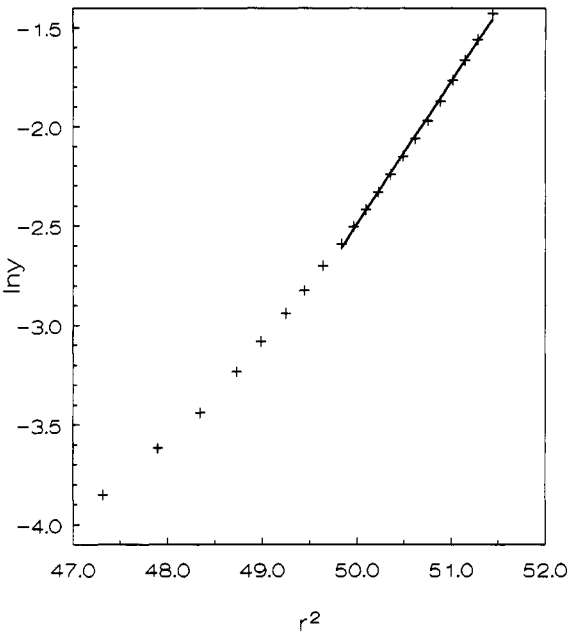

FIGURE 1: Representative plot of ln y vs r^2, where y is the concentration in fringe displacement units and r is the distance from the axis of rotation in centimeters. The slope of the line was determined from a least-squares fit of the data points through which the line passes and is used to calculate the molecular mass. The fit to the data had an R value of >0.999. A mole ratio of 2 caltropin dimers to 1 calponin was used at an initial concentration of 0.7 mg/mL, the speed was 13 000 rpm, and the cell was maintained at 20 °C.

molecular mass observed reflects the weighted contribution of all species in the cell, and since there is a heterogeneous population present including the two initial constituents plus any complexes formed, a range of molecular mass is obtained in any individual experiment. In the representative experiment shown in Figure 1, the proteins were combined in a ratio of 2 mol of caltropin dimer to 1 mol of calponin at an initial loading concentration of 0.7 mg/mL. The slope of the ln y vs r^2 plot was used to calculate the molecular mass, and deviation from the straight line near the meniscus indicates the presence of lower molecular mass species at this position in the cell due to uncomplexed protein. In the presence of calcium, a molecular mass of 70 668 Da was obtained, which is within experimental error of the combined mass of one calponin (32 333 Da) and two caltropins (21 000 Da/unit) for a total of 74 333 Da. If a third caltropin was able to bind to this complex, the molecular mass would increase to 95 333 Da, which did not occur in this experiment. In the absence of calcium, a molecular mass range of 20.4–27.1 kDa (data not shown), representative of the constitutive proteins, was observed, demonstrating that the formation of the complex was dependent upon the presence of calcium. In order to be certain the observed molecular mass, which indicated the complex formed was greater than 1 calponin:1 caltropin, did not arise from 2 calponin:1 caltropin, the proteins were combined in a ratio of excess calponin to caltropin. A range of molecular mass of 29.3–48.6 kDa (data not shown) was observed, representative of a 1:1 interaction together with uncomplexed protein, indicating the higher molecular mass observed for the complex in Figure 1 only occurs with excess caltropin and is truly indicative of a complex of two caltropins to one calponin. Previously, it has been shown in the analytical ultracentrifuge that calponin alone, in the presence or absence of calcium, has a molecular mass of 32.1 kDa (Wills *et al.*, 1993) and caltropin, in the presence or absence of calcium, has a molecular mass of 21.0 kDa (Mani & Kay, 1990).

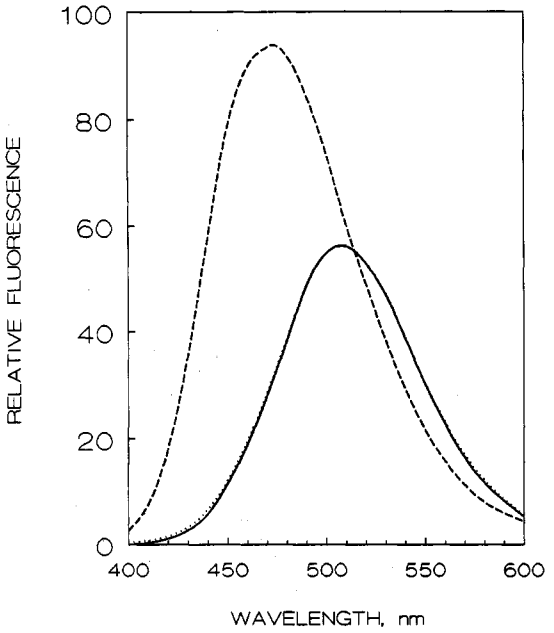

FIGURE 2: Fluorescence spectra of acrylodan-labeled calponin and acrylodan calponin in the presence of Ca^{2+} (—), acrylodan calponin in the presence of caltropin and EGTA (···), and acrylodan calponin in the presence of caltropin and Ca^{2+} (– – –). The wavelength of excitation is 388 nm.

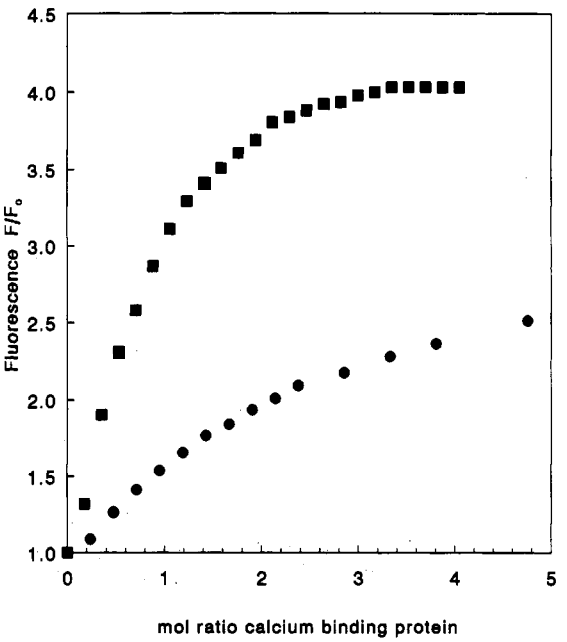

FIGURE 3: Titration of acrylodan calponin fluorescence at 460 nm, in the presence of Ca^{2+}, with increasing concentrations of caltropin (■) and calmodulin (●). Mole ratios for caltropin are calculated for the dimer. F is the observed fluorescence, while F_o is the initial fluorescence intensity.

Therefore, the higher molecular mass observed in this experiment is indeed due to complex formation and not simply the result of aggregation of the constituent proteins. Since calponin does not interact with calcium, as demonstrated previously (Wills *et al.*, 1993), complex formation is dependent upon a structural change that has been shown to occur in caltropin when this protein binds calcium (Mani & Kay, 1990). The complex is formed in the same fashion as the calponin–calmodulin complex in that it consists of 2 mol of calcium-binding protein to 1 mol of calponin, and its formation is calcium dependent. However, in this case, the functional unit of the calcium-binding protein, caltropin, is a dimer.

Fluorescence Spectroscopy. Since intrinsic static tryptophan fluorescence of calponin is not sensitive to interaction with calcium-binding proteins (Wills *et al.*, 1993), acrylodan was used to label calponin. The acrylodan fluorescence of calponin is sensitive to interactions of calponin with calcium-binding proteins and is therefore a useful probe to monitor complex formation. We have previously shown that reaction of calponin with the cysteine-specific probe acrylodan under native conditions resulted in a singly-labeled calponin that binds calcium-binding proteins in the same fashion as native calponin as shown by fluorescence titrations, maintains the same secondary structure as shown by circular dichroism, and behaves similarly in the ATPase biological assay as native calponin (Wills *et al.*, 1993). Acrylodan calponin, when excited at 388 nm, has an emission maximum at 505 nm (Figure 2). Neither the fluorescence intensity nor the peak wavelength are affected by the presence of caltropin/EGTA or Ca^{2+}. When caltropin is added in the presence of Ca^{2+}, however, there is a 35-nm blue shift in the peak of the spectrum from 505 to 470 nm. Since the greatest change in fluorescence intensity occurs at 460 nm, this wavelength was chosen for fluorescence titrations. Caltropin increases the fluorescence of acrylodan calponin $4^1/_2$ times at this wavelength. These effects are greater than those previously shown for calmodulin which causes a 25-nm blue shift and a $3^1/_2$ times increase in the fluorescence intensity of acrylodan calponin at 460 nm (Wills *et al.*, 1993). These results are indicative of the probe moving into a more nonpolar environment when calponin interacts with caltropin, perhaps becoming more buried in the protein as the complex forms (Prendergast *et al.*, 1983), a response which allows for a sensitive measure of complex formation. Titration of acrylodan calponin with caltropin in the presence of Ca^{2+} (Figure 3) shows that the response is saturated with 4 mole ratios of caltropin. This curve can be fitted using the model of 2 mol of calcium-binding protein binding to each mole of calponin, determined in the sedimentation equilibrium experiments, to calculate dissociation constants. A Kd_1 of ≤ 0.13 μM for the first mole of caltropin binding to calponin can be calculated, and this interaction accounts for 94% of the fluorescence change. Kd_2, calculated for the second mole of caltropin interacting with calponin, is 0.18–1.6 μM and produces 6% of the fluorescence change (Wills *et al.*, 1993). The ranges quoted represent the Kd ± the standard deviation. This affinity is significantly higher than the values found when the calmodulin titration is fitted to the model of ≤ 0.22 μM for Kd_1 and 2.5–3.4 μM for Kd_2 (Wills *et al.*, 1993) and is reflected in the fact that it takes 40 mole ratios of calmodulin over calponin to saturate the fluorescence effect due to the weaker binding affinities. Thus, caltropin is a more suitable candidate for regulating calponin's inhibitory behavior than is calmodulin.

Location of Acrylodan Label on Calponin. The location of this acrylodan label has now been identified by performing a complete tryptic digest of the acrylodan-labeled calponin and purifying the peptide labeled with acrylodan by monitoring for fluorescence off a reversed-phase HPLC column. The acrylodan-containing peptide was then sequenced, and the sequence found was -VYDPKY which is located uniquely at residues 267–272. Cysteine 273 in chicken gizzard calponin was identified as the labeled residue. Mezgueldi *et al.* (1992) have mapped the domains of interaction of calponin to actin, tropomyosin, and calmodulin through the use of affinity chromatography of proteolytic fragments. They found that calmodulin-, tropomyosin-, and actin-binding abilities were all maintained in a fragment constituting residues 1–184 and, further, fragment 1–145 was able to bind calmodulin and

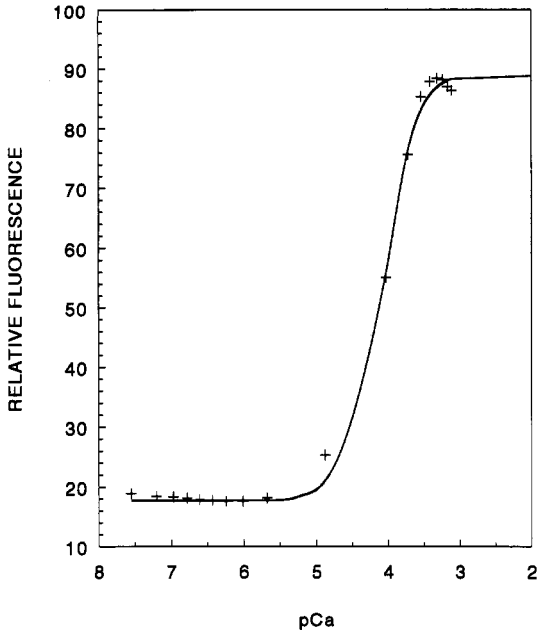

FIGURE 4: Calcium titration of the acrylodan calponin–caltropin complex (+). The data were fit to a sigmoidal transition equation (—).

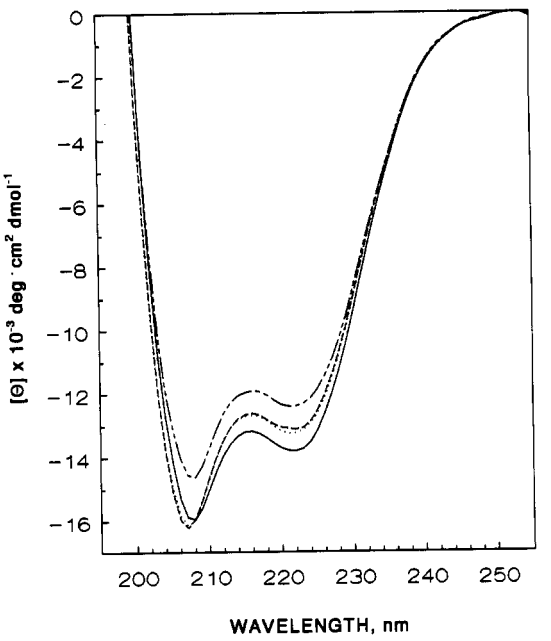

FIGURE 5: Far-ultraviolet circular dichroism spectra of the observed (···) and calculated (– – –) calponin–caltropin complex in the absence of calcium and the observed (– - -) and calculated (—) complex in the presence of calcium.

tropomyosin but not actin. Whether fragment 1–145 bound 1 or 2 mol of calmodulin was not determined. Nakamura *et al.* (1993) used the same method to determine that the carboxyl terminal 12-kDa comprising residues 183–292 did not bind actin, calmodulin, or tropomyosin. Cysteine 273 is not near the actin-, calmodulin-, or tropomyosin-binding sites as elucidated by these mapping experiments which indicates that the carboxyl terminal either is structurally affected by binding of these proteins of folds near the amino terminal region in the three-dimensional structure. Cysteine 273 is the most carboxy terminal of the cysteine residues in chicken gizzard calponin. Strasser *et al.* (1993) have sequenced mammalian isoforms of calponin from mouse and pig. They have shown that this cysteine is the last conserved residue between the various isoforms before a hypervariable region at the carboxy terminal begins in mammalian calponin isoforms.

Calcium Titration of the Calponin and Caltropin Complex. The acrylodan fluorescence of calponin increases in the presence of caltropin/calcium, whereas this does not occur in the presence of caltropin/EGTA. Therefore, complex formation can be monitored with increasing calcium levels in order to determine the concentration of calcium needed for interaction (Figure 4). The midpoint of this titration is at a pCa of 4.04. Caltropin has been shown to bind 2 mol of calcium/mol of monomer, and a pCa of 4.04 corresponds to the low-affinity calcium-binding sites of caltropin of 4.09 as determined by Mani and Kay (1990). The high-affinity sites of caltropin bind calcium at a pCa of 6.69. At this calcium concentration, there is no evidence of complex formation as judged by fluorescence change. This finding indicates that when the smooth muscle cell is at rest and the calcium levels are approximately 1×10^{-7} M (Bagshaw, 1993), there would be no interaction; the complex would form when calcium levels in the cell are elevated during contraction to 1×10^{-5} M and could therefore constitute a regulatory complex for smooth muscle contraction. The difference between observed calcium levels present during excitation (1×10^{-5} M) and those observed for complex interaction (9×10^{-5} M) could easily be accounted for by the difference in environment *in vitro* compared to the natural, more stable environment of the thin filament.

Circular Dichroism of the Complex. In order to determine if secondary structural changes occur upon interaction, far-ultraviolet circular dichroic spectra of the calponin–caltropin complex were determined in the presence and absence of calcium (Figure 5). The proteins were mixed as 2 mole ratios of caltropin dimer/mol of calponin since this is the stoichiometry of complex formation. In addition, theoretical spectra for the complex in the presence and absence of calcium were calculated in order to determine if the calculated and observed spectra are the same. A difference between the calculated and observed spectra indicates secondary structural changes occurred during complex formation. The calculated and observed spectra in the absence of calcium are superimposable, signifying that no secondary structure changes occur in the absence of calcium. Analysis of these spectra by the method of Provencher and Glöckner indicates there is 43% α-helix, 25% β-sheet, 8% β-turn, and 24% remainder when calponin and caltropin are mixed in the absence of calcium. However, in the presence of calcium, the observed spectrum has a negative ellipticity at 220 nm 1590° less than that deduced for the calculated spectrum, which is much greater than experimental error (±300°). Provencher–Glöckner analysis of these experimental curves results in the calculation of 36% α-helix, 31% β-sheet, 8% β-turn, and 24% remainder. These values indicate 6% less α-helix and 5% more β-sheet, with similar β-turn and remainder to that deduced from the calculated spectra, suggesting that there are significant structural changes that occur upon complex formation in the presence of calcium.

Guanidine Hydrochloride Denaturation. In order to study the stability of calponin and caltropin alone relative to the complex, a guanidine hydrochloride titration was monitored by circular dichroism ellipticity at 221 nm to observe the denaturation midpoint in the presence and absence of calcium (Figure 6). Calponin has a midpoint of denaturation of 1.25 M Gdn·HCl with a $\Delta G_D^{H_2O}$ of 3.0 kcal/mol, which is unaffected by the presence of calcium, in agreement with

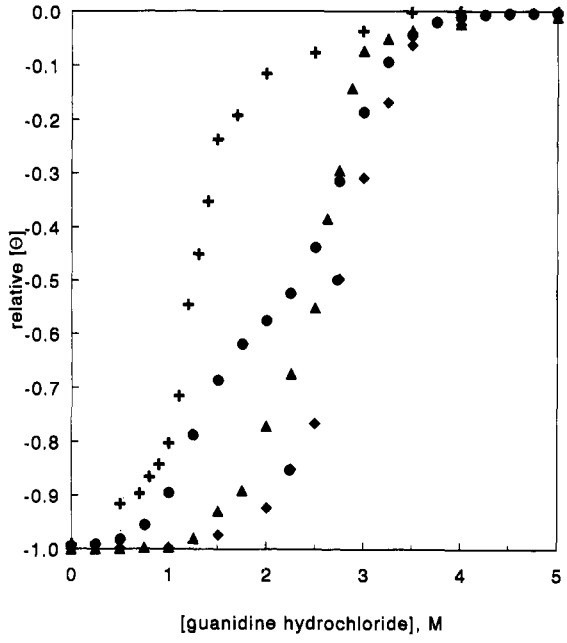

FIGURE 6: Titration of the calponin (+), caltropin (◆), and the calponin–caltropin complex (▲) in the presence of calcium with guanidine hydrochloride. The denaturation was monitored by circular dichroism changes at 221 nm. The theoretical denaturation profile for the complex calculated from the constituent proteins is also shown (●). Normalized data are plotted as relative [θ] vs concentration of guanidine hydrochloride.

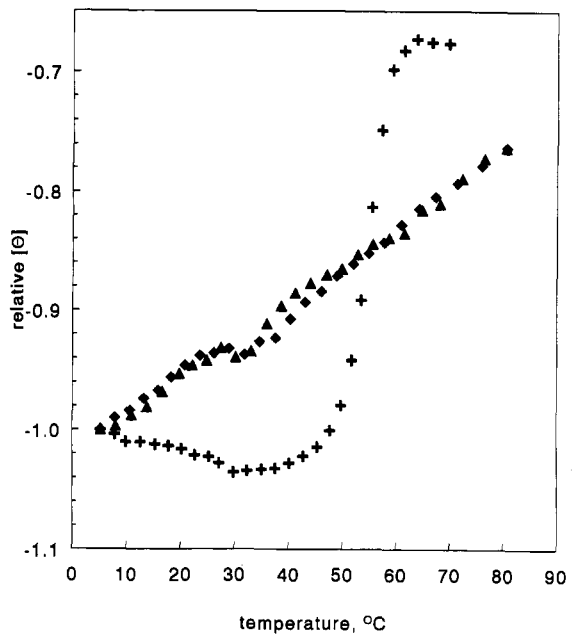

FIGURE 7: Temperature denaturation of calponin (+), caltropin (◆), and the calponin–caltropin complex (▲) in the presence of calcium. The denaturation was monitored by circular dichroism changes at 221 nm. Normalized data are plotted as relative [Θ] vs temperature.

earlier results which showed calponin does not interact with calcium (Wills *et al.*, 1993). Caltropin in the presence of calcium has a midpoint of 2.77 M Gdn·HCl with a $\Delta G_D^{H_2O}$ of 5.45 kcal/mol. The combination of calponin and caltropin in the absence of calcium possesses a midpoint of 1.91 M Gdn·HCl and a $\Delta G_D^{H_2O}$ of 4.09 kcal/mol and, in the presence of calcium, has a denaturation midpoint of 2.48 M Gdn·HCl with a $\Delta G_D^{H_2O}$ of 4.69 kcal/mol. These values indicate that calponin is stabilized in the presence of caltropin, since the complex does not begin to denature until beyond the midpoint of calponin denaturation. The theoretical curve for the guanidine denaturation of the complex shows significant deviation from the observed denaturation profile during the early stages of the titration when calponin would be expected to denature. Later on in the titration, the observed and calculated spectra are superimposable, indicating caltropin stability is not affected by complex formation; however, caltropin seems to confer stability upon calponin in the complex.

Temperature Denaturation of the Calponin–Caltropin Complex. The temperature denaturation profile of calponin as revealed by circular dichroism shows a sharp reversible melting transition with a T_m of 55 °C (Figure 7). Caltropin in the presence of calcium is very stable, is not completely denatured even at 80 °C, and does not exhibit a denaturation point at which it rapidly melts but, rather, experiences a gradual loss of structure with increasing temperature. This is similar behavior to that observed for other calcium-binding proteins, for example, troponin C (McCubbin *et al.*, 1980) and calmodulin (Tsalkova & Privalov, 1980; Brzeska *et al.*, 1983). When the complex of calponin and caltropin is heated, it shows very similar patterns to the denaturation of caltropin with no transition and is not completely denatured even at 80 °C. Thus, when calponin is in the complex, it does not demonstrate as great a stability at the beginning of the titration to about 40 °C, but the caltropin confers stability upon calponin during the later portion of the titration such that there is no sharp melting transition at 55 °C.

Effect of Caltropin on ATPase Assays. In order for the calponin–caltropin complex to be an important regulatory unit, caltropin must be able to modulate calponin's inhibitory activity on the actomyosin ATPase activity. In an acto-S1 ATPase assay, a 0.5 mol of calponin to 1 mol of actin ratio was able to inhibit ATPase 47.8%. Increasing ratios of caltropin, S-100b, and calmodulin were tested to determine to what extent these three proteins could affect calponin's activity. The ATPase assay was carried out with 0.5 mol of calponin:1 of mol actin, and then increasing amounts of the relevant three calcium-binding proteins were added in the presence and absence of calcium to determine if they had an effect. In the absence of calcium, calponin was able to inhibit ATPase activity with no effect of the calcium-binding proteins (data not shown); however, in the presence of calcium and calcium-binding proteins, there was recovery from inhibition. Figure 8 shows the recovery from inhibition at three ratios of calcium-binding protein to calponin. The graph indicates that at all three concentrations examined, S-100b and caltropin were more effective in reversing inhibition than calmodulin. At a 2 mole ratio of caltropin to calponin, which is the ratio of these components in the complex, caltropin is able to release 63% of the inhibition while S-100b is able to release 59% of the inhibition. Under the same conditions, calmodulin is able to release only 33% of the inhibition. Caltropin and S-100b are superior to calmodulin in releasing inhibition at all three ratios; however, the greatest difference is at the lowest ratios, which would be expected to be the physiologically important ones. At 1:1 ratios, caltropin releases 37% of the inhibition, S-100b 33%, and calmodulin 17%. At the higher 6:1 mole ratio, caltropin released 77% of the inhibition, S-100b 84%, and calmodulin 69%. Thus, the increase in release for the 6:1 ratio is not significantly higher than the release for the 2:1 ratio, considering 3 times the calcium-binding protein was used. Therefore, caltropin which is efficient at modulating calponin's inhibition, as well as being present in smooth muscle, is a prime candidate for forming a regulatory complex with calponin in a 2 mol of caltropin:1 mol of calponin ratio.

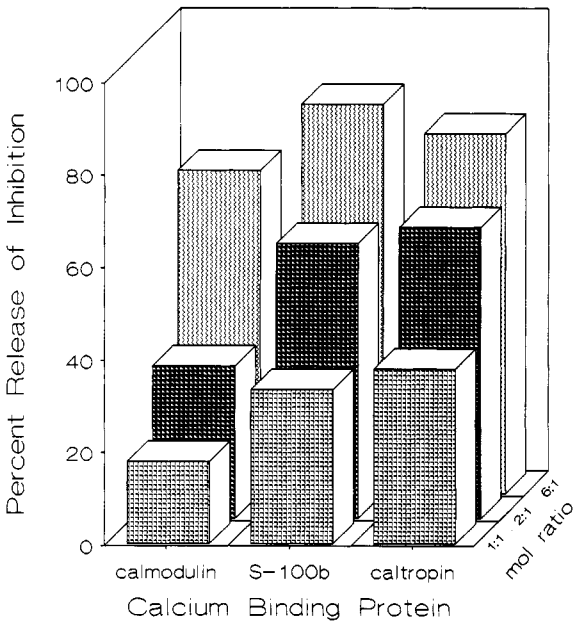

FIGURE 8: Reversal of calponin inhibition of the acto-S1 Mg^{2+}-ATPase by calmodulin, *S*-100b, and caltropin at a 1:1 (first row), 2:1 (second row), and 6:1 (third row) mole ratio of calcium-binding protein to calponin in the presence of calcium.

Currently, a great deal of work is being done to elucidate the mechanisms underlying thin-filament regulation of smooth muscle contraction, and it is of interest to reconcile the relative significance of the calponin–caltropin interaction in this process. While significant progress has been made in identifying potential participants in this process, methods of regulation which explain all the data have yet to be described. Due to the similarities between calponin and caldesmon, attention has focused upon the relationship between the two proteins. It has been found that at subsaturating concentrations relative to actin, the two proteins do not have any effect upon the function of the other, while at saturating conditions, they compete for binding to actin (Makuch *et al.*, 1991; Winder *et al.*, 1992). That the two proteins cannot coexist on the same filament means the two do not form a complex analogous to the troponin system and, also, indicates they are not working in concert. Further exploration of both proteins is required in order to understand their respective functions.

When calponin was first isolated by Takahashi *et al.* in 1986, it was described as an actin-, tropomyosin-, and calmodulin-binding protein. A parallel to the troponin system was made, and it was postulated that calponin would serve to inhibit contraction while calmodulin would function as a calcium-sensitizing agent (Abe *et al.*, 1990). Recent work revealing calponin can interact with myosin is intriguing; however, unphysiological salt conditions are required for interaction (Lin *et al.*, 1993; Szymanski & Tao, 1993). The role of calponin in binding actin has been borne out by experiments which have shown that calponin can bind to actin, inhibit actin's enhancement of myosin ATPase, and inhibit actin filament motility on immobilized myosin (Shirinsky *et al.*, 1992). Furthermore, it has been observed that calponin does not affect the phosphorylation level of myosin (Winder & Walsh, 1990) but, rather, seems to act by binding to actin and inhibiting myosin's interaction with actin (Abe *et al.*, 1990), thus representing a separate regulation system from myosin light-chain phosphorylation. The question of the manner by which calponin's function is regulated, however, has become the subject of much controversy.

Winder and Walsh (1990) found calponin could be phosphorylated *in vitro* by both protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase II. Phosphorylation by either of these two protein kinases abolished calponin's inhibition of actomyosin ATPase activity. Phosphorylation also blocked the actin–calponin interaction as shown by sedimentation assays (Winder & Walsh, 1990). These authors therefore proposed that calponin was regulated by reversible phosphorylation. Preliminary data on canine trachea provided evidence of calponin phosphorylation in response to stimuli in this tissue (Pohl *et al.*, 1991). However, the biological significance of this is uncertain because of a lack of confirmation of *in vivo* calponin phosphorylation by other investigators. Barany *et al.* (1991) found, using porcine carotid arterial muscles labeled with ^{32}P, that no phosphorylation of calponin takes place in contracting or resting arterial smooth muscle, indicating that the sites of calponin available for phosphorylation *in vitro* are blocked in the intact muscle. Gimona *et al.* (1992) also observed in living smooth muscle strips from chicken gizzard and guinea pig taenia coli, labeled with $^{32}PO_4$, that no phosphate incorporation could be detected in any of the calponin isoforms whether during contraction or relaxation.

An alternative regulatory proposal to phosphorylation is regulation by calcium–calmodulin which has been shown to reverse calponin's inhibitory activity (Abe *et al.*, 1990; Makuch *et al.*, 1991). Release of calponin's ATPase inhibition by calcium–calmodulin parallels release of actin binding by calponin as shown by cosedimentation assays, indicating that when calponin is bound to actin it inhibits ATPase and when it is released it is no longer active (Abe *et al.*, 1990; Makuch *et al.*, 1991). However, as much as a 10 molar excess of calmodulin over calponin is required to achieve this, indicating low association constants between these two proteins. Shirinsky *et al.* (1992) found in *in vitro* motility assays that inhibition of actin filament movement over immobilized smooth muscle myosin or skeletal muscle HMM by calponin could be reversed by calmodulin but large molar excesses of calmodulin are again required to exert this reversal, which calls into question whether this is physiologically possible. Calmodulin tends to interact with proteins it regulates with affinities as high as 10^{-10} M (Klee, 1988), making this system an unlikely regulatory mechanism, and a more effective one is required. In order for a regulatory complex to be effective, it requires interaction at high enough affinity for the formation of the complex to be biologically feasible, and it also requires sensitivity to the physiological state of the cell, so the complex can be switched on or off as the regulatory mechanism is required in the cell. Much work has been published on the calponin–calmodulin interaction. Our study suggests that caltropin, a smooth muscle calcium-binding protein, is capable of interacting with calponin with much higher affinity and, in so doing, regulates calponin's biological activity more effectively than calmodulin. Furthermore, since the complex only forms in the presence of calcium levels that occur in the cell during excitation, it meets the requirements of the regulatory mechanism. Thus, when calponin is bound to actin, it inhibits actin activation of the myosin Mg^{2+} ATPase. On the basis of this study, we propose that as calcium levels increase in the cell after excitation, calcium binds to caltropin which in turn interacts with calponin. The caltropin–calponin complex no longer inhibits actin–myosin interaction, and contraction ensues. Future work planned to further explore this putative regulatory mechanism includes defining the two

sites of interaction of caltropin with calponin and the relevance of each of these sites.

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