Calcium-Dependent Regulation of Caldesmon by an 11-kDa Smooth Muscle Calcium-Binding Protein, Caltropin†

Rajam S. Mani, William D. McCubbin, and Cyril M. Kay*

Department of Biochemistry, Medical Research Council Group in Protein Structure and Function, University of Alberta, Edmonton, Alberta, Canada T6R 2H7

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ABSTRACT: Caldesmon from chicken gizzard muscle has been examined for its ability to interact with caltropin using affinity chromatography and the fluorescent probe acrylodan. The action of caltropin on the inhibitory effect of caldesmon on actomyosin ATPase was also studied. Like calmodulin, caltropin could release the inhibitory effect of caldesmon in the presence of Ca^{2+}. Complete reversal was obtained when 1 mol of caltropin was added per mol of caldesmon. When caldesmon was applied to caltropin–Sepharose in the presence of Ca^{2+}, most of the caldesmon was bound to the column and could be eluted with EGTA, indicating that there is a direct interaction between caldesmon and caltropin. Acrylodan-labeled caldesmon, when excited at 375 nm, had an emission maximum at 504 nm. Addition of caltropin in the presence of Ca^{2+} resulted in a nearly 50% increase in fluorescence intensity, and this was accompanied by a blue shift in the emission maximum (i.e., $\lambda_{em,max}$ 492 nm), suggesting that the probe now occupies a more nonpolar environment. Titration of caltropin with labeled caldesmon indicated a strong affinity for this protein (K_d was in the order of 8×10^{-8}–2×10^{-7} M). However, when caltropin was added to labeled caldesmon in the presence of EGTA, there was no indication of any interaction. Caltropin was at least as potent as calmodulin, if not better, in reversing the inhibitory effect of caldesmon in the presence of calcium, making it a potential Ca^{2+} factor in regulating caldesmon in smooth muscle.

It is well known that the change in the intracellular Ca^{2+} concentration affects many physiological processes including muscle contraction. In smooth muscle, calcium exerts its control via mechanisms based on both the thick (myosin) filaments (Sobieszek & Bremel, 1975) and the thin (actin-based) filaments (Marston et al., 1980). Calmodulin is considered to be the Ca^{2+}-dependent regulatory component of the myosin-linked system (Dabrowska et al., 1977). Calmodulin, in the presence of calcium, activates the enzyme myosin light chain kinase which phosphorylates the 20 000-Da myosin light chain, and contractile activity ensues as actin filaments interact with cross-bridges arising from the myosin thick filaments. The thin filament linked pathway involves actin-mediated regulation by caldesmon, a protein that is bound to the thin filaments. Native smooth muscle thin filaments contain actin–tropomyosin and caldesmon in molar ratios of 14:2:1 (Marston et al., 1992). When purified caldesmon binds to actin–tropomyosin, it inhibits actomyosin ATPase activity. This inhibition is not Ca^{2+}-sensitive but can be rendered Ca^{2+}-dependent by a suitable Ca^{2+}-binding regulatory protein which can bind to caldesmon and thus reverse its inhibitory effect on the actomyosin ATPase activity. For example, in the presence of calcium, calmodulin can reverse the inhibitory effect of caldesmon on the actomyosin ATPase[1] activity. However, the affinity of calmodulin for caldesmon ($K = 1.6 \times 10^6$ M^{-1}) is rather weak, and for this reason, a very large molar excess of calmodulin over caldesmon (≈25-fold higher) is required to regulate caldesmon at 25 °C (Shirinsky et al., 1988; Pritchard & Marston, 1989). Hence, it is conceivable that the native thin filament is regulated not by calmodulin but by a different calcium-binding protein. Reconstitution of Ca^{2+}-sensitive thin filaments has never been demonstrated using a pure protein from smooth muscle. In this study, we have examined the regulation of reconstituted thin filaments by a novel 11-kDa smooth muscle calcium-binding protein to see if it could be responsible for thin filament linked regulation of actomyosin.

Calcium-binding proteins are known to bind to a hydrophobic matrix in a calcium-dependent manner and this property was utilized in isolating a novel 11-kDa smooth muscle calcium-binding protein from chicken gizzard (Mani & Kay, 1990). The 11-kDa SMCaBP is very similar to the calcium-binding protein isolated from Ehrlich ascites tumor cells (EAT cells) by Kuznicki and Filipek (1987) which they have identified as calcyclin on the basis of their sequence analysis. In fact, antibodies raised against (EAT cells CaBP) calcyclin recognized 11-kDa SMCaBP (J. Kuznicki, personal communication). We believe 11-kDa SMCaBP is a chicken calcyclin. The major difference between the two proteins is the absence of any sulfhydryl gorups in 11-kDa SMCaBP whereas calcyclin has a single cysteine residue in the N-terminal end at position 2. Recently, Todoroki et al. (1991) have also concluded that 11-kDa SMCaBP is a chicken calcyclin on the basis of their sequence analysis.

The function of calcyclin is unknown at present, but several functions have been suggested including cell-cycle regulation, cell differentiation, and cytoskeletal membrane interaction. The 11-kDa SMCaBP binds two calcium ions per subunit. The sequence of calcyclin reveals the presence of 2 EF-hand structural elements. Like calmodulin and TN-C, 11-kDa SMCaBP also has a high- and low-affinity calcium-binding site with K_d values of 10^{-7} M and 10^{-5} M, respectively (Mani & Kay, 1990).

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; MOPS, 4-morpholinepropanesulfonic acid; S_1, myosin subfragment-1; acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene, CD, circular dichroism; ATPase, adenosine triphosphatase; DTT, dithiothreitol, SDS, sodium dodecyl sulfate.

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In this paper, we report that 11-kDa SMCaBP can bind to caldesmon only in the presence of Ca^{2+}. In the presence of Ca^{2+}, 11-kDa SMCaBP released the caldesmon inhibition of actomyosin ATPase at 25 °C (in the presence of 60 mM KCl) when the two proteins were present in stoichiometric proportions, unlike calmodulin which required a 25-fold molar excess. In this aspect, 11 kDa SMCaBP mimics the role played by troponin–C in striated muscle. Troponin-C is known to overcome the inhibitory effect of troponin-I on the actomyosin ATPase activity (Mani et al., 1974). As 11-kDa SMCaBP can release the caldesmon-induced inhibition of the actomyosin ATPase in the presence of calcium, we refer to it as "caltropin", drawing analogy with its ability to bind calcium and its troponin-C like properties.

MATERIALS AND METHODS

Protein Purification. Caltropin was isolated from chicken gizzard using the procedure described in our earlier paper (Mani and Kay, 1990). Myosin, myosin subfragment–1 (S_1), and F-actin were purified from rabbit skeletal muscle by standard procedures (Margossian & Lowey, 1982; Pardee & Spudich, 1982). Skeletal tropomyosin was purified as described by Smillie (1982). Caldesmon from fresh or frozen chicken gizzards was prepared by a modification of the method of Bretscher (1984). Initial purification of caldesmon was carried out using an ion-exchange column as described by Clark et al. (1986). The caldesmon fraction obtained from the DEAE-Sephacel column was next applied to a 2.5 × 30 cm column of hydroxylapatite (Bio-Rad) in the presence of 10 mM sodium phosphate, pH 6.5, 0.05% sodium azide, 1 mM DTT, 0.3 mM $CaCl_2$. A linear gradient was formed from 500 mL of each of 10 mM and 250 mM sodium phosphate buffers. Caldesmon eluted as a sharp peak around 0.21 M phosphate and was homogeneous when tested by 12% SDS–polyacrylamide gel electrophoresis.

Affinity Chromatography. Caltropin was coupled to CNBr-activated Sepharose 4B (Pharmacia) following the procedures outlined by Pharmacia. About 4 mg of caltropin was linked to 1 mL of Sepharose. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and molecular weight determinations were carried out as described in one of our earlier publications (Mani & Kay, 1983). Protein concentrations were determined using an $E^{1\%}_{1cm,278nm}$ of 6.8 for caltropin (Mani & Kay, 1990) and $E^{1\%}_{1cm,280nm}$ of 3.8 for caldesmon (Bryan et al., 1989). Circular dichroism measurements were made on a J-720 spectropolarimeter (Jasco, Inc., Easton, MD) as described previously (Mani & Kay, 1992).

Fluorescence Studies. Fluorescence spectra were obtained with a Perkin-Elmer Model MPF-44 spectrofluorometer, and all measurements were made at 20 °C. The instrument was operated in a ratio mode. The A_{278nm} of the sample was less than 0.1. Caldesmon was labeled with acrylodan following the procedure of Prendergast et al. (1983). Acrylodan was first dissolved in dimethylformamide. Caldesmon in 0.1 M NaCl, 20 mM Tris (pH 7.5), and 1 mM DTT was incubated with excess acrylodan at 4 °C for 6 h. After labeling, the protein was subjected to exhaustive dialysis against the buffer used for labeling. The concentration of the label was determined from the absorbance at 380_{nm} using a molar extinction coefficient of 16 400 M^{-1} cm^{-1} (Prendergast, 1983). The ratio of the label to protein was determined to be 1.7, suggesting that both of the sulfhydryl groups in caldesmon were labeled under these labeling conditions.

Labeled caldesmon was titrated with caltropin in the presence of calcium by following the changes in the fluorescence intensity at 460 nm. The titration curve was analyzed for a 1:1 complex (molecular weights of 87 000 and 11 000 were used for caldesmon and caltropin) whose formation is described by the equation $P + M \overset{Kd}{\rightleftharpoons} PM$. The concentration of the complex is obtained by solving the quadratic equation

$$[PM] = \frac{(P_O + M_O + K_d) - [(P_O + M_O + K_d)^2 - 4\,P_O M_O]^{1/2}}{2}$$

where P_O is the total caldesmon concentration, M_O is the total caltropin, and K_d is the dissociation constant (Williams et al., 1985).

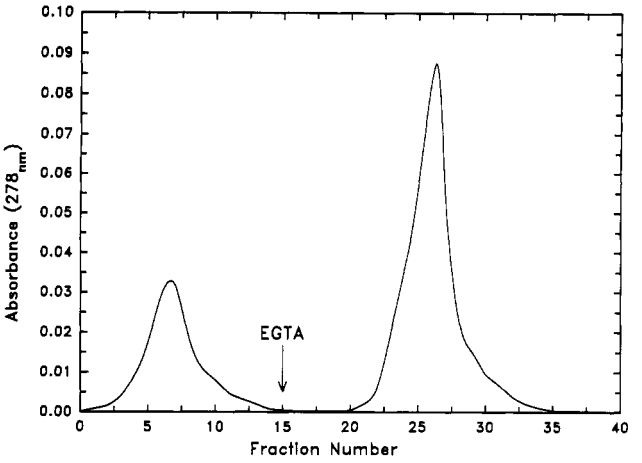

FIGURE 1: Caltropin–Sepharose affinity chromatography of caldesmon. Caldesmon (2 mg) was applied to a caltropin–Sepharose column equilibrated with the buffer containing 25 mM Tris-HCl (pH 7.5), 0.5 mM DTT, and 1 mM $CaCl_2$. The column was washed with the buffer, and at the point indicated by arrow, the bound protein was eluted with the same buffer containing 2 mM EGTA.

Reversal of Caldesmon Inhibition by Caltropin. Caltropin was tested for its ability to reverse caldesmon inhibition of actin–tropomyosin-activated myosin MgATPase activity. For the assay of skeletal muscle myosin, myosin subfragment (S_1) and tropomyosin were used. With skeletal muscle myosin assay tubes contained 0.25–0.5 μM myosin, 2.0–5.0 μM actin, 0.4–1.0 μM tropomyosin (tropomyosin/actin molar ratio of 0.25), and 0.4–1.0 μM caldesmon in order to induce sufficient inhibition. Caltropin was included at up to 0.006 mg/mL (3 μM). In assays involving skeletal muscle myosin subfragment-1, (1.0–2.0 μM) was incubated with actin (7.0–15.0 μM), (2.0–4.0 μM) tropomyosin, (0.2–0.5 μM) caldesmon, and up to (2 μM) caltropin. The reaction was initiated by adding MgATP to 2 mM and terminated by the addition of 5% trichloroacetic acid. The amount of P_i liberated was determined according to Itaya and Ui (1966) using the basic dye malachite green.

RESULTS

Interaction of Caltropin with Caldesmon. Evidence for the direct binding of caltropin to caldesmon was obtained using affinity chromatography (Figure 1). When caldesmon was applied to a caltropin–Sepharose 4B column in the presence of 1 mM Ca^{2+}, most of the protein was retained by the column. The bound caldesmon was eluted from the column by adding 2 mM EGTA in the buffer. SDS gel electrophoresis showed that caldesmon alone was present in the bound fraction (peak 2). These results clearly indicate that caltorpin–caldesmon interaction is calcium-dependent since caldesmon could be eluted from the caltropin affinity column only in the presence of EGTA.

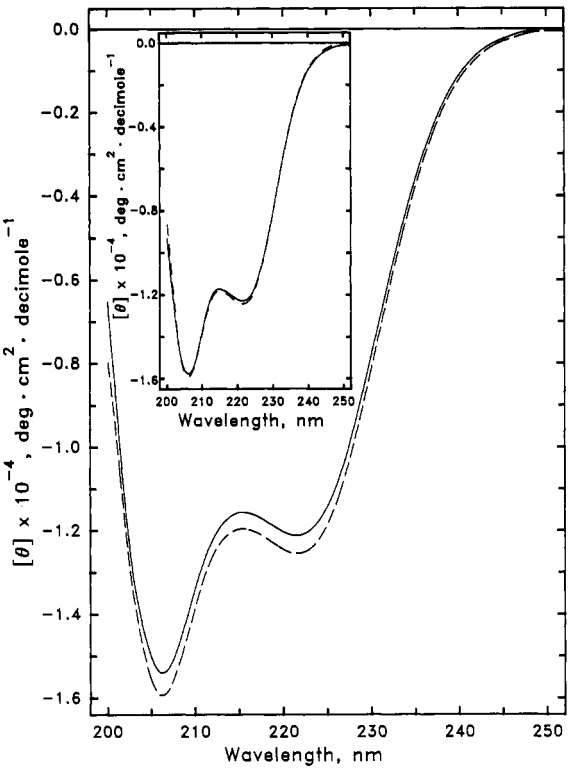

FIGURE 2: Farultraviolet circular dichroism spectrum for the caltropin–caldesmon complex in 10 mM MOPS (pH 7.1), 30 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM DTT (—) and the theoretical circular dichroism spectrum for the complex in the same solvent (– –). The far-ultraviolet circular dichroism spectrum for the caltropin–caldesmon complex in 10 mM MOPS (pH 7.1), 30 mM NaCl, 5 mM $MgCl_2$, 1 mM EGTA, and 1 mM DTT is shown in the inset (—). The theoretical circular dichroism spectrum for the complex in the same solvent (– –) is also shown in the inset.

Circular Dichroism Studies. Figure 2 shows the far-ultraviolet circular dichroism spectrum for the caltropin–caldesmon complex in the presence of Ca^{2+}. The two proteins were mixed in equimolar ratios using the subunit molecular weight of 11 000 for caltropin and a molecular weight of 87 000 for caldesmon. This figure also includes a theoretical curve for caltropin–caldesmon in the presence of Ca^{2+}, calculated from the ellipticity value of caltropin and caldesmon alone. The experimentally observed ellipticity values deviate from the theoretical values particularly in the 222- and 207-nm wavelength region, suggesting that the interaction has produced a conformational change. For instance, at 222 nm the difference between the observed and the theoretical ellipticity value was nearly 700 deg cm^2 $dmol^{-1}$ while the experimental error in these measurements is only ±300 deg cm^2 $dmol^{-1}$, thus clearly indicating that the interaction has produced a conformational change. However, when the two proteins were mixed in the absence of Ca^{2+}, i.e., in the presence of 1 mM EGTA (please see the inset in Figure 2), the experimentally observed CD spectrum is virtually identical to the theoretical curve, implying that the two proteins do not interact in the absence of Ca^{2+}, and this finding is in line with our affinity column results.

Fluorescence Spectroscopy. Tryptophan residues in caldesmon can be selectively excited by exposing the protein solution to monochromatic light at 295 nm. Since caltropin has no tryptophan, one can study the interaction between these two proteins by monitoring the intrinsic tryptophan fluorescence of caldesmon. However, addition of caltropin had no significant effect on either the tryptophan fluorescence intensity or on the emission maximum which was at 345 nm for

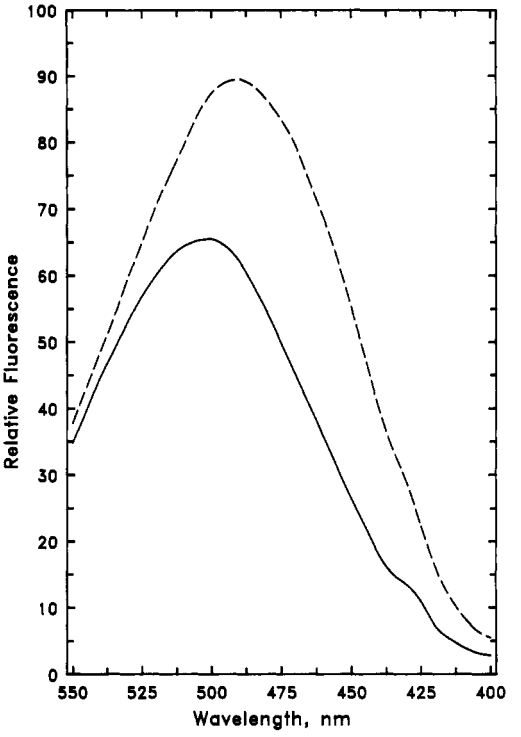

FIGURE 3: Fluorescence emission spectra of acrylodan–caldesmon (—) and acrylodan–caldesmon–caltropin complex (– –) in 20 mM Tris (pH 7.5), 100 mM NaCl, 1 mM $CaCl_2$, and 1 mM DTT at 20 °C. The excitation wavelength was 375 nm.

caldesmon. For this reason, we decided to use acrylodan, a sulfhydryl fluorescent probe, to label caldesmon. Under the experimental conditions used, the ratio of the label to caldesmon obtained was 1.7, suggesting that both the sulfhydryl groups present in caldesmon were labeled with acrylodan. When the labeled caldesmon was excited at 375 nm, the emission maximum occurred at 504 nm in the presence of Ca^{2+}. Addition of caltropin to labeled caldesmon in the presence of calcium resulted in a nearly 50% increase in fluorescence intensity, and the emission maximum was now centered at 492 nm (Figure 3). Since the emission maximum is blue shifted, one may infer that the probe in the presence of caltropin is located in a more nonpolar environment. Even though the emission maximum was at 492 nm the observed increase in relative fluorescence intensity was maximal (nearly 2-fold) at 460 nm (Figure 3). For this reason, during the titration of labeled caldesmon with caltropin the fluorescence intensity at 460 nm was monitored.

Analysis of the titration curve by curve fitting showed that (Figure 4) the best fit was obtained when the molar ratio of caltropin to caldesmon in the complex at saturation was 1/1 assuming molecular masses for caltropin and caldesmon of 11 000 Da and 87 000 Da, respectively. Also in bioassay involving acto-S_1 MgATPase measurements, the same stoichiometry was observed (see below). A K_d value of $(9 \pm 2) \times 10^{-8}$ M was obtained from the titration. Addition of caltropin to labeled caldesmon in the absence of calcium produced no significant change either in the fluorescence intensity or in the emission maximum suggesting that the two proteins do not interact in the absence of Ca^{2+}.

Reversal of Caldesmon Inhibition by Caltropin. Caldesmon produced nearly 40% inhibition on the actin–tropomyosin activated ATPase rate of skeletal muscle myosin subfragment-1 (S_1). Caltropin in the presence of 0.2 mM Ca^{2+} could release caldesmon's inhibition at 25 °C in buffers containing 60 mM KCl. Complete recovery in the ATPase rate was

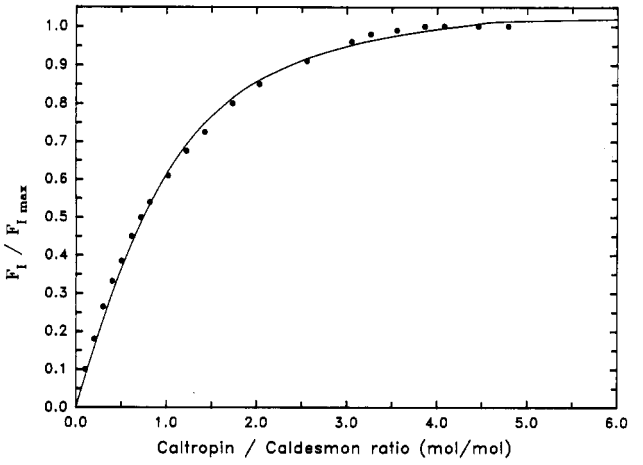

FIGURE 4: Influence of caltropin on acrylodan–caldesmon fluorescence. The initial caldesmon concentration was 4×10^{-7} M. Measurements were carried out in 20 mM Tris (pH 7.5) 100 mM Nacl, 1 mM $CaCl_2$, and 1 mM DTT at 20 °C. Relative fluorescence intensifies (F_I/F_{Imax}) at 460 nm are plotted as a function of molar ratios of caltropin added to caldesmon. The smooth curve through the data points was calculated for the binding of 1 subunit of caltropin/mol of caldesmon (i.e., for 0.5 mol of caltropin/mol of caldesmon). The excitation wavelength was 375 nm.

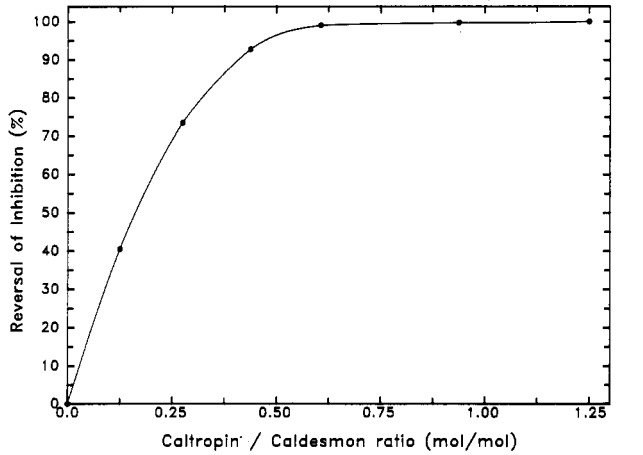

FIGURE 5: Caltropin-induced reversal effect on acto-S_1 ATPase inhibition by caldesmon. ATPase measurements were carried out at room temperature in the presence of 8 μM skeletal muscle actin, 2 μM skeletal muscle tropomyosin, 1 μM skeletal muscle myosin subfragment 1 (S_1), and 0.6 μM caldesmon. The solvent system used consisted of 60 mM KCl, 1.0 mM DTT, 25 mM Tris (pH 7.5), 2 mM ATP, 5 mM $MgCl_2$, and 0.2 mM $CaCl_2$. One hundred percent is equivalent to an ATPase rate of 180 nmol of P_i/min (mg of subfragment-1)$^{-1}$ at room temperature.

observed when 1 mol of caltropin was added per mol of caldesmon (Figure 5). In fact, more than 90% recovery was noticed by the time 0.5 mol of caltropin was added; i.e., one polypeptide chain of caltropin per mol of caldesmon was sufficient to release most of caldesmon's inhibition at 25 °C since caltropin exists as a dimer of 21 000 molecular weight in native solvents (Mani & Kay, 1990). Thus, caltropin is very potent compared to calmodulin since nearly a 25-fold molar excess of calmodulin was required to release caldesmon's inhibition at 25 °C (Pritchard & Marston, 1989).

The actin–tropomyosin-activated ATPase rate of skeletal muscle myosin was also inhibited by caldesmon, and the extent of inhibition obtained ranged from 40% to 50%. Addition of caltropin in the presence of calcium at 25 °C could only bring about partial recovery in the ATPase rate. Maximum recovery obtained was nearly 50% of the initial caldesmon-induced inhibition (Figure 6). This observed effect of caltropin on

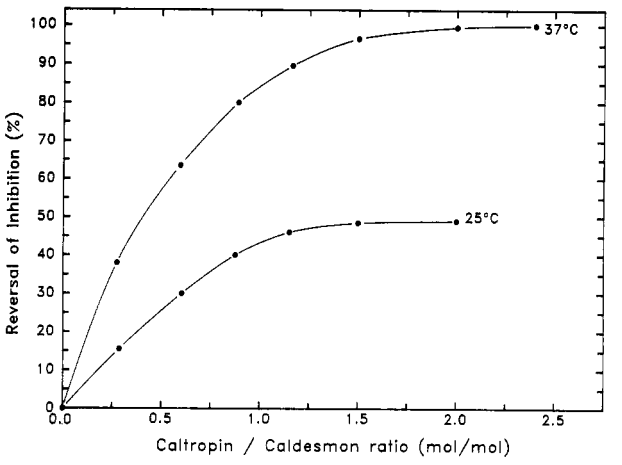

FIGURE 6: Reversal of caldesmon's inhibition of actin–tropomyosin activated myosin MgATPase by caltropin. The assay was carried out using 0.5 μM of skeletal myosin, 4.0 μM skeletal actin, 1.0 μM skeletal tropomyosin, and 0.5 μM caldesmon. The solvent system used at 25 °C was 60 mM KCl, 25 mM Tris (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, and 0.2 mM $CaCl_2$, and at 37 °C the solvent consisted of 120 mM KCl, 25 mM Tris (pH 7.5), 5 mM $MgCl_2$, 1 mM DTT, 2 mM ATP, and 0.2 mM $CaCl_2$. The myosin MgATPase rate remained linear during the incubation period (5 min at 37 °C; 10 min at 25 °C). One hundred percent is equivalent to an ATPase rate of 310 nmol of P_i/min (mg of myosin)$^{-1}$ at room temperature and 920 nmol of P_i/min (mg of myosin)$^{-1}$ at 37 °C.

caldesmon's inhibition of the actomyosin ATPase could be a reflection on the affinity with which caldesmon binds to myosin and myosin subfragment-1 (S_1). The affinity of caldesmon for myosin is greater than S_1 (Hemric & Chalovich, 1990), and this could explain as to why only a partial recovery from caldesmon's inhibition was observed upon caltropin addition when myosin is used instead of S_1 in the assay. The ability of caltropin to release caldesmon-dependent ATPase inhibition at 37 °C in buffer containing 120 mM KCl is also shown in Figure 6. Addition of caltropin in the presence of Ca^{2+} could now restore normal ATPase level; i.e., caldesmon-induced inhibition was completely eliminated, and for this we needed 1 mol of caltropin per mol of caldesmon.

DISCUSSION

Caldesmon was initially isolated using a calmodulin affinity column (Sobue et al., 1981). Calmodulin in the presence of calcium binds to caldesmon and can reverse caldesmon's inhibition of the actin-activated myosin ATPase. Initially a simple "flip-flop" mechanism was proposed (Sobue et al., 1982) according to which, in the presence of calcium, calmodulin competed with actin for caldesmon binding and hence reversed inhibition by displacing caldesmon from actin. The binding affinity between caldesmon and actin at 25 °C in 60 mM KCl is 10^7 M^{-1} (Pritchard & Marston, 1991), and this is an order of magnitude higher than for calmodulin (K_{aff} 10^6 M^{-1}), with the result that for flip-flop binding, a very large molar excess of calmodulin would be required to reverse caldesmon's inhibition. This is indeed the case since nearly a 25-fold molar excess of calmodulin was required to regulate caldesmon at 25 °C in the presence of 60 mM KCl (Pritchard & Marston 1989; Shirinsky et al., 1988). Since the native thin filaments do not contain the large excess of calmodulin which would be required for Ca^{2+} sensitivity at 25 °C/60 mM KCl, it is conceivable that the native thin filament is regulated not by calmodulin but by a diffeent smooth muscle calcium-binding protein. This has led many investigators to look for new calcium-binding proteins in smooth muscle. The fact that

some calcium-binding proteins expose a hydrophobic site in the presence of Ca^{2+} has resulted in the use of Ca^{2+}-dependent hydrophobic interaction chromatography for isolating new calcium-binding proteins (Moore & Dedman, 1982; Mani & Kay, 1992). Such an approach was used in isolating two novel calcium binding proteins with molecular weights of 12 000 and 11 000. Of these two calcium-binding proteins, the 12-kDa protein gave no evidence for interaction with caldesmon, on the basis of our spectroscopic studies and bioassay, which involved studying the effect of the 12-kDa protein on caldesmon's inhibition of the actin-activated myosin ATPase (Mani & Kay, 1992). Caltropin gave direct evidence for interaction with caldesmon since most of the caldesmon was retained by a caltropin–Sepharose affinity column in the presence of calcium. Bound caldesmon could be eluted from this column in the presence of EGTA, suggesting that the two proteins interact only in the presence of Ca^{2+}.

Fluorescence spectroscopy is a powerful technique to study protein–protein interaction. In the present study, both of the sulfhydryl groups in caldesmon were labeled with acrylodan. Addition of caltropin to labeled caldesmon in the presence of Ca^{2+} resulted in perturbing the environment around the fluorescent probe. Titration of caltropin with caldesmon indicated that it binds to caldesmon with high affinity and the K_d value obtained was $(9 \pm 2) \times 10^{-8}$ M. This is at least half an order of magnitude higher than the affinity with which calmodulin binds to caldesmon, since the K affinity for calmodulin is approximately 10^6 M^{-1} (Pritchard & Marston, 1981; Shirinsky et al., 1988).

In the present study, the effect of caltropin on caldesmon's inhibition of the actomyosin ATPase was studied by measuring the inorganic phosphate that is liberated as outlined in the Materials and Methods section. Caltropin in the presence of Ca^{2+} was very effective in releasing caldesmon's inhibition on the actin-activated S_1 Mg^{2+}-ATPase activity. Most of the inhibition was removed by the time 0.5 mol of caltropin was added per mol of caldesmon; i.e., one polypeptide chain of caltropin per mol of caldesmon was sufficient to restore normal ATPase level. The effect of caldesmon on the actin-activated myosin ATPase was also studied. In this instance, only a partial recovery in the ATPase rate was noticed upon caltropin addition; i.e., caldesmon's inhibition was not completely eliminated. Even for this partial recovery, we needed 1 mol of caltropin/mol of caldesmon. Hence, when myosin is used instead of S_1 in the actomyosin ATPase measurements, we seem to require more than double the amount of caltropin. However, when the assay was carried out at 37 °C in 120 mM KCl, caltropin was effective in restoring the normal ATPase level; i.e., complete recovery from caldesmon's inhibition was achieved. Calmodulin is also known to overcome the inhibition produced by caldesmon at 37 °C/120 mM KCl. Direct binding experiments at 37 °C/120 mM KCl have shown that calmodulin can bind to a caldesmon–actin complex and hence it can release caldesmon's inhibition whereas at 25 °C/160 mM KCl calmodulin is not known to bind to a caldesmon–actin complex, with the result that calmodulin has to compete with actin for caldesmon (Marston & Redwood, 1991). Since the affinity of caldesmon for actin ($K \approx 10^7$ M^{-1}) is stronger than for calmodulin ($K \approx 10^6$ M^{-1}), a large molar excess of calmodulin is required for releasing caldesmon's inhibition. It is possible that a similar phenomenon exists with caltropin–caldesmon interaction since complete recovery is achieved at 37 °C while at 25 °C/60 mM KCl, only a partial recovery is noticed. The main difference is that with caltropin nearly 50% recovery in the ATPase is obtained at 25 °C/60 mM KCl when 1 mol of caltropin is added per mol of caldesmon, whereas with calmodulin at 25 °C/60 mM KCl nearly a 25-fold molar excess of calmodulin was required even for partial recovery (Pritchard & Marston, 1989). This observation in our view can be interpreted by concluding that caltropin is very potent and is in fact better than calmodulin in regulating caldesmon's inhibition of the actomyosin ATPase in a Ca^{2+}-dependent manner.

Pritchard and Marston (1991) have recently isolated a low molecular weight calcium-binding protein from sheep aorta which when tested had no significant effect on caldesmon's inhibition. As this protein was not characterized by including its amino acid composition, it is not possible to compare it with our protein preparation. Since their protein did not release caldesmon's inhibition, it is possible that they were studying the 12-kDa calcium-binding protein which, in our hands, also had a very minimal effect on caldesmon's inhibition when used in stoichiometric proportions.

In conclusion, caltropin, which is modulated by Ca^{2+}, binds to caldesmon in a Ca^{2+}-dependent manner. Evidence for direct binding was obtained using a caltropin–Sepharose affinity column. Caltropin which binds to caldesmon in the presence of Ca^{2+} with high affinity is present in significant amounts (~10 mg/350 g of wet tissue) in chicken gizzard, and this represents roughly 20–25% of the calmodulin content. Caltropin which is very potent in regulating caldesmon's inhibition in a calcium-dependent manner could conceivably be the Ca^{2+} factor that controls the thin filament linked Ca^{2+} regulation in smooth muscle.

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REFERENCES

Bretscher, A. (1984) *J. Biol. Chem. 259*, 12873–12880.
Bryan, J., Imai, M., Lee, R., Moore, P., Cook, R. G., & Lin, W. G. (1989) *J. Biol. Chem. 264*, 13873–13879.
Clark, T., Nagi, P. K., Sutherland, C., Grožchel-Stewart, V., & Walsh, M. P. (1986) *J. Biol. Chem. 261*, 8028–8035.
Dabrowska, R., Aromatorio, D., Sherry, J. M. F. & Hartshorne, D. J. (1977) *Biochem. Biophys. Res. Commun. 78*, 1263–1272.
Hemric, M. E. & Chalovich, J. M. (1990) *J. Biol. Chem. 265*, 19672–19678.
Itaya, K. A., & Ui, M. (1966) *Clin. Chim. Acta 14*, 361–366.
Kuznicki, J., & Filipek, A. (1987) *Biochem. J. 247*, 663–667.
Mani, R. S., & Kay, C. M. (1983) *Biochemistry 22*, 3902–3907.
Mani, R. S., & Kay, C. M. (1987) *Methods Enzymol. 139*, 168–187.
Mani, R. S., & Kay, C. M. (1990) *Biochemistry 29*, 1398–1404.
Mani, R. S., & Kay, C. M. (1992) *Arch. Biochem. Biophys. 296*, 442–449.
Mani, R. S., McCubbin, W. D. & Kay, C. M. (1974) *Biochemistry 13*, 5003–5007.
Margossian, S. S., & Lowey, S. (1982) *Methods Enzymol. 85*, 55–71.
Marston, S. B., Trevett, R. M., & Walters, M. (1980) *Biochem. J. 185*, 355–365.
Marston, S., Pinter, K., & Bennett, P. (1992) *J. Muscle Res. Cell Motil. 13*, 206–218.
Moore, P. B., & Dedman, J. R. (1982) *J. Biol. Chem. 257*, 9663–9667.

Pardee, J. D., & Spudich, J. A. (1982) *Methods Enzymol. 85*, 164–181.
Prendergast, F. G., Meyer, M., Carlson, G. L., Lida, S., & Potter, J. D. (1983) *J. Biol. Chem. 258*, 7541–7544.
Pritchard, K., & Marston, S. B. (1989) *Biochem. J. 257*, 839–843.
Pritchard, K., & Marston, S. B. (1991) *Biochem. J. 277*, 819–824.
Shirinsky, V. P., Bushueva, T. C. & Frolova, S. I. (1988) *Biochem. J. 255*, 203–208.
Smillie, L. B. (1982) *Methods Enzymol. 85*, 234–241.
Sobieszek, A., & Bremel, R. D. (1975) *Eur. J. Biochem. 55*, 49–60.
Sobue, K., Muramoto, Y., Fujita, M., & Kakiuchi, S. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5652–5655.
Sobue, K., Morimoto, K., Inui, M., Kanda, K., & Kakiuchi, S. (1982) *Biomed. Res. 3*, 188–196.
Todoroki, H., Kobayashi, R., Watanabe, M., Minami, H., & Hidaka, H. (1991) *J. Biol. Chem. 266*, 18668–18673.
Williams, T. C., Shellin, J. G. & Sykes, B. D. (1985) *NMR in Life Sciences* (Bradbury, E. M., & Nicolini, C., Eds.) pp 93–103, Plenum Press, New York, London.