Smooth Muscle Calponin

INHIBITION OF ACTOMYOSIN MgATPase AND REGULATION BY PHOSPHORYLATION*

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Calponin isolated from chicken gizzard smooth muscle inhibits the actin-activated MgATPase activity of smooth muscle myosin in a reconstituted system composed of contractile and regulatory proteins. ATPase inhibition is not due to inhibition of myosin phosphorylation since, at calponin concentrations sufficient to cause maximal ATPase inhibition, myosin phosphorylation was unaffected. Furthermore, calponin inhibited the actin-activated MgATPase of fully phosphorylated or thiophosphorylated myosin. Although calponin is a Ca++-binding protein, inhibition did not require Ca++. Furthermore, although calponin also binds to tropomyosin, ATPase inhibition was not dependent on the presence of tropomyosin. Calponin was phosphorylated in vitro by protein kinase C and Ca++/calmodulin-dependent protein kinase II, but not by cAMP- or cGMP-dependent protein kinases, or myosin light chain kinase. Phosphorylation of calponin by either kinase resulted in loss of its ability to inhibit the actomyosin ATPase. The phosphorylated protein retained calmodulin and tropomyosin binding capabilities, but actin binding was greatly reduced. The calponin-actin interaction, therefore, appears to be responsible for inhibition of the actomyosin ATPase. These observations suggest that calponin may be involved in regulating actin-myosin interaction and, therefore, the contractile state of smooth muscle. Calponin function in turn is regulated by Ca++-dependent phosphorylation.

Primary regulation of contraction in smooth muscle involves phosphorylation of the 20,000-dalton light chains of myosin by Ca++/calmodulin-dependent myosin light chain kinase (1, 2). It is becoming increasingly clear, however, that other regulatory systems, having both direct and indirect calcium dependence, may have a role to play in the regulation of smooth muscle contraction (3). These mechanisms of regulation include caldesmon/calmodulin (4, 5), the calcium- and phospholipid-dependent protein kinase (protein kinase C) (6), and perhaps the direct binding of Ca++ to myosin (7, 8). Recently, another smooth muscle protein has been described which interacts with F-actin and tropomyosin in a Ca++-independent manner and with calmodulin in a Ca++-dependent manner. It is present in smooth muscle at the same molar concentration as tropomyosin (9). Electron microscopy supports the idea that calponin is a bona fide thin filament protein: electron microscopy of smooth muscle tropomyosin paracrystals indicated that calponin binds to a site 16-17 nm from the C terminus of tropomyosin with 40 nm periodicity, i.e. identical to the binding pattern of skeletal muscle tropinin T (11). The thin filament-bound form of calponin is degraded 500 times more slowly by calpain than is the free form of calponin, suggesting a very close association between calponin and the thin filament similar to the association of tropinin T with the skeletal muscle thin filament (12). Calponin is clearly a distinct protein from caldesmon and myosin light chain kinase (10), but it is antigenically related to the C-terminal half of rabbit skeletal and bovine cardiac tropinin T (13). Recently, however, Lehman (14) has suggested that calponin may be a cytoskeletal or nuclear matrix protein rather than a thin filament component (see "Discussion").

During characterization of native thin filaments prepared from chicken gizzard smooth muscle, we observed a 32-kDa protein in addition to actin, tropomyosin, and caldesmon (15). This protein was identified as calponin (13), a conclusion which we have confirmed using specific polyclonal antibodies to isolated gizzard calponin. The properties, including the binding to actin and tropomyosin, suggested that calponin may function in the regulation of smooth muscle contraction. To investigate this possibility, we carried out preliminary studies of the effects of purified calponin on the actin-activated myosin MgATPase in vitro using purified smooth muscle proteins: actin, myosin, tropomyosin, calmodulin and myosin light chain kinase (16). In the presence of 6 μM actin, 2 μM tropomyosin, and 1 μM myosin, calponin (2 μM) inhibited the actin-activated myosin MgATPase by 78%.

We report here further characterization of the inhibitory activity of calponin on the actin-activated myosin MgATPase of smooth muscle and demonstrate that phosphorylation of calponin reverses this inhibitory effect. Data presented indicate that the inhibitory action of calponin is due to its ability to bind actin which is lost upon phosphorylation.

EXPERIMENTAL PROCEDURES

Materials—[γ-32P]ATP (20-40 Ci/mmol) was purchased from Amersham (Oakville, Ontario, Canada). Sephadex G-75 was purchased from Pharmacia (Mississauga, Ontario, Canada) and CM-Sephadex, protein A-Sepharose, ribonuclease A, and chymotrypsinogen A from Sigma. Dithiothreitol and ATPγS were purchased from

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1 The abbreviations used are: ATPγS, adenosine 5’-O-(3-thiotriphosphate); EGTA, [ethylenedioxytetramethylene]tetraacetic acid; SDS PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Boehringer Mannheim (Dorval, Quebec, Canada) and electrophoresis reagents from Bio-Rad. General laboratory reagents used were of analytical grade or better and were purchased from Fisher Scientific (Scientific, Calgary, Alberta, Canada).

**Protein Purifications**—Calmodulin was purified from frozen bovine brains by a modification of the procedure of Gopalakrishna and Anderson (17) as described in detail by Walsh et al. (18). The following proteins were purified by previously described methods: chicken gizzard actin (19), tropomyosin (20), myosin (21) and myosin light chain kinase (22), and the bovine cardiac catalytic subunit of type II cAMP-dependent protein kinase (23). Chicken gizzard caldesmon containing endogenous Ca2+/calmodulin-dependent kinase activity, and rat brain protein kinase C were purified as previously described (24, 25) and generously provided by Drs. Gisèle Scott-Scott and Janice Parente, respectively, of this laboratory. Cyclic GMP-dependent protein kinase (bovine lung) was generously provided by Dr. Tom Lincoln, University of South Alabama and Ca2+/calmodulin-dependent protein kinase II (bovine brain) by Dr. R. K. Sharma, University of Calgary. Calponin was purified as described by Taka-hashi et al. (9) with the modifications that CM-Sephadex was used for the ion-exchange chromatography step and Sephadex G-75 was used in place of Ultrogel ACA 4A for the gel filtration step.

**Actin-activated Myosin MgATPase Assay**—ATPase activities were measured as described previously (26) under the following conditions: 25 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 60 mM KCl, 0.1 mM CaCl2, or 0.1 mM EGTA, 1 mM [γ-32P]ATP (~10 cpm/pmol), 1 μM myosin, 6 μM actin, 2 μM tropomyosin, 1 μM calmodulin, 74 mM myosin light chain kinase in reaction volumes of 1.0 ml at 30 °C. Other additions are indicated in the figure legends. Reactions were started by addition of ATP. For the determination of ATPase rates, aliquots (0.1 μl) of reaction mixtures were withdrawn at 1-min intervals following the addition of ATP up to 9 min. Rates were calculated by linear regression analysis of the time-course data. In one case, ATPase rates were determined at various actin and tropomyosin concentrations, maintaining a 3:1 molar ratio of actin/tropomyosin, under otherwise standard conditions.

**Myosin Phosphorylation Assay**—Levels of myosin phosphorylation were measured on the same samples used for myosin ATPase measurements as described previously (27). Wherever necessary, the remainder of each reaction mixture was added to an equal volume of SDS gel sample buffer and boiled. Samples (50 μl) were subjected to SDS-PAGE and autoradiography.

**Superprecipitation Assay**—Reaction conditions were exactly as described for the actomyosin ATPase assay with the exception that nonradiolabeled ATP was used. A acti was recorded in an LKB Ultraspec.

**Phosphorylation of Calponin**—Phosphorylation by Ca2+/calmodulin-dependent protein kinase II was carried out under the following optimal conditions (24): 20 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 0.1 mM CaCl2, 1 mM [γ-32P]ATP, 5.9 μM calponin, 4.6 μM calmodulin, 0.2 mM dithiothreitol, and phosphokinase reaction mixtures were allowed to incubate at 30 °C for 10 min. Following incubation at 30 °C for 10 min the supernatants were subjected to SDS-PAGE. Immunoprecipitation assays were performed using the whole reconstituted in vitro smooth muscle system were carried out in the presence of 2 μM phosphorylated or unphosphorylated calponin and nonradiolabeled ATP as described for the MgATPase assay above. Following incubation at 30 °C for 7 min the samples were centrifuged at 109,000 × g in a Beckman TL 100 centrifuge for 1 h at 2 °C and pellets and supernatants were subjected to SDS-PAGE.

**Immunoprecipitation of Calponin**—Polyclonal antibodies to chicken gizzard calponin (which had been cut out of SDS-polyacrylamide gels) were raised in rabbits using standard procedures. The polyclonal antibody preparation of anti-calponin was used to precipitate calponin (31). Calponin (0.25 mg) and anti-calponin (1.25 mg) were incubated for 4 h at 22 °C in a reaction volume of 0.455 ml. Protein A-Sepharose (0.5 ml) was added and incubated in a bench-top Eppendorf centrifuge for 2 min. The effect of the supernatant on actomyosin ATPase activity was measured under standard conditions. A calponin concentration of 3.9 μM would be present in the ATPase reaction mixture if the protein was not immunoprecipitated. Two control immunoprecipitation reactions were also carried out. In one case calponin was omitted (buffer control) and in the other anti-calponin was omitted (antibody control).

**Other Procedures**—The following enzymatic activities were assayed using published methods: cAMP-dependent protein kinase (23), cGMP-dependent protein kinase (32), myosin light chain kinase (27), protein kinase C (38), and Ca2+/calmodulin dependent protein kinase II (24). Protein concentrations were determined by the Coomassie Brilliant Blue dye-binding assay (33) using dye reagent purchased from Fierce Chemical Co. or by spectrophotometric measurements, using the following absorption coefficients: calmodulin (ε 280 nm = 1.9 (19)), myosin (ε 280 nm = 4.5 (35)), tropomyosin (ε 280 nm = 2.9 (36)), caldesmon (ε 280 nm = 3.3 (37), and calponin (ε 280 nm = 11.3. The absorption coefficient of calponin was determined by recording the protein’s UV absorption spectrum after dialysis overnight against two changes (5 liters each) of 20 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol. The protein concentration was determined by scanning laser densitometry following SDS-PAGE. The calponin preparation was kept to 97.6% pure by scanning laser densitometry following SDS-PAGE. The calponin concentration was determined, by amino acid analysis (38), to be 0.49 mg/ml. Electrophoresis was performed in 7.5–20% polyacrylamide gradient slab gels (1.5 mm thick) with a 5% acrylamide stacking gel in the presence of 1% SDS at 36 μA using the discontinuous buffer system of Laemmli (39). Gels were stained in 45% (v/v) ethanolic 0.25% (v/v) acetic acid containing 0.14% (w/v) Coomassie Brilliant Blue R-250 and diffusion destained in 10% (v/v) acetic acid. Destained gels were sealed in plastic bags and autoradiographed, if necessary, using Kodak X-OMAT AR film in DuPont-Cronex cassettes fitted with DuPont Quanta III intensifying screens. Films were allowed to develop until a clear image was visible. The exposure times were determined by scanning laser densitometry equipped with a Hewlett-Packard model 3390A integrator.

**RESULTS**

**Inhibition of Actomyosin ATPase by Calponin**—We have shown previously that calponin has an inhibitory effect on smooth muscle actomyosin MgATPase activity (16). This effect was examined in greater detail using a reconstituted system comprising the purified contractile and regulatory proteins of chicken gizzard smooth muscle: myosin, actin, tropomyosin, calmodulin, and myosin light chain kinase. Fig. 1 shows that calponin produced a concentration-dependent inhibition of actomyosin ATPase with maximal (79%) inhibition being reached at concentrations of calponin ≥ 2 μM. Inhibition was shown to be due to calponin since inhibitory activity could be removed by immunoprecipitation with specific polyclonal antibodies to calponin (Table I). The inhibitory effect of calponin is not a nonspecific effect due to its basic nature (pI = 8.4–9.1 (13)). Two other basic proteins, ribonuclease A (pI = 9.6) and chymotrypsinogen (pI = 9.5), exhibited no inhibitory effects on the actomyosin MgATPase, even at concentrations as high as 10 μM: ATPase rates of
Phosphorylation level was determined to be 1.82 ± 0.05 mol of Pi/mg myosin. Phosphorylation was unaffected (Fig. 1), even at calponin concentrations as high as 17.5 μM, in which case the phosphorylation level was determined to be 1.82 ± 0.05 mol of Pi/mg myosin (n = 9). SDS-PAGE followed by autoradiography verified that specific phosphorylation of the 20,000 dalton myosin light chain occurred in all cases (data not shown).

Table I

| Immuno precipitation of calponin and actomyosin ATPase inhibitory activity |
|---|
| Assay conditions | Actomyosin ATPase |
|---|---|
| Actomyosin - Ca²⁺ | 6.6 |
| Actomyosin + Ca²⁺ | 141.7 |
| Actomyosin + Ca²⁺ + supernatant | 125.0 |
| Actomyosin + Ca²⁺ + buffer control | 133.5 |
| Actomyosin + Ca²⁺ + antibody control | 37.6 |

*Actomyosin refers to the standard actomyosin ATPase reaction system.

Calponin was maximally phosphorylated by incubation for 8 min under the ATPase assay conditions described under "Experimental Procedures" with the exception that actin was omitted (tropomyosin was included). The following additions were then made simultaneously: 6 μM actin (O, △) and 6 μM actin + 5 μM calponin (△). Samples of reaction mixtures were withdrawn at the indicated times following actin addition for quantification of ATP hydrolysis. In one case (△) calponin (5 μM) was added 5 min after the addition of actin.

These results indicate that inhibition of the actomyosin ATPase is not due to inhibition of myosin phosphorylation as a consequence, for example, of removal of calmodulin by binding to calponin. The mechanism of inhibition was further investigated by examining the effect of calponin on the actin-activated MgATPase activity of prephosphorylated myosin (Fig. 2). Myosin was first phosphorylated in the presence of myosin light chain kinase and Ca²⁺-calmodulin to a level of 1.7 mol of Pi/mol myosin. Actin was then added in order to activate the myosin MgATPase and the incubation continued in the presence or absence of calponin. In a third experiment, calponin was added 5 min after the addition of actin rather than with actin. As expected, the MgATPase of phosphorylated myosin was strongly activated by actin, to 181.7 nmol of Pi/mg myosin-min (Fig. 2, O); in a separate experiment the ATPase rate of unphosphorylated myosin in the presence of actin was determined to be only 1.1 nmol of Pi/mg myosin-min. Calponin added with actin caused a 78% decrease in the rate of ATP hydrolysis, to 39.7 nmol of Pi/mg myosin-min (Fig. 2, △), i.e. comparable to the inhibition seen in Fig. 1 where ATP hydrolysis and myosin phosphorylation occurred concomitantly. Calponin is capable not only of inhibiting actin activation of the MgATPase of prephosphorylated myosin, but also inhibits the actin-activated MgATPase when added to the fully activated system: addition of calponin 5 min after actin resulted in 96% inhibition of the maximal rate of ATP hydrolysis, i.e. from 173 to 7 nmol of Pi/mg myosin-min (Fig. 2, △).
Myosin was prephosphorylated as described in the legend to Fig. 3 prior to the addition of 6 μM actin with or without 5 μM calponin in the presence of 0.1 mM CaCl₂ or 1 mM ETYA. ATPases and myosin phosphorylation levels were quantified as described under "Experimental Procedures." We have also found that calponin immobilized on nitrocellulose binds Ca²⁺ when incubated with 45CaCl₂ as was observed (data not shown).

Having established the inhibitory effect of calponin on the actomyosin ATPase in the presence of Ca²⁺, we investigated the possibility that calponin inhibition of the ATPase may be regulated by phosphorylation. Calponin was found to be phosphorylated by protein kinase C (to 1.11 mol of P_i/mol), by Ca²⁺/calmodulin-dependent protein kinase II (to 1.0 mol of P_i/mol) and by a Ca²⁺/calmodulin-dependent protein kinase copurifying with smooth muscle caldesmon (to 1.9 mol of P_i/mol), but not by cAMP- or cGMP-dependent protein kinases or myosin light chain kinase (≤0.01 mol of P_i/mol). In each case, specific phosphorylation of calponin was verified by SDS-PAGE and autoradiography (data not shown). We previously published evidence suggesting that the Ca²⁺/calmodulin-dependent protein kinase activity copurifying with caldesmon actually resides within the caldesmon molecule itself (24). However, this conclusion is not consistent with the recently detected amino acid sequence of caldesmon (43). The properties of this kinase most closely resemble those of Ca²⁺/calmodulin-dependent protein kinase II (44). We, therefore, examined the site specificity of phosphorylation of calponin by this kinase activity and by purified bovine brain Ca²⁺/calmodulin-dependent protein kinase II using two-dimensional phosphopeptide mapping of limit trypptic peptides (Fig. 4). Identical phosphopeptide maps were obtained (compare Fig. 4, A and B), so we conclude that the kinase activity copurifying with caldesmon is a smooth muscle form of Ca²⁺/calmodulin-dependent protein kinase II. Phosphopeptide mapping of calponin phosphorylated by protein kinase C revealed a simpler pattern of phosphopeptides (Fig. 4C), only three major phosphopeptides being detected. If trypptic digests of calponin phosphorylated by protein kinase C and Ca²⁺/calmodulin-dependent protein kinase II were combined prior to two-dimensional phosphopeptide mapping, the three phosphopeptides (labeled 1-3) comigrated (data not shown) suggesting that the two major sites of phosphorylation by Ca²⁺/calmodulin-dependent protein kinase II (sites 1 and 2) are also phosphorylated by protein kinase C. Site 3 which is strongly phosphorylated by protein kinase C is poorly phosphorylated by Ca²⁺/calmodulin-dependent protein kinase II. Additional sites of phosphorylation by Ca²⁺/calmodulin-dependent protein kinase II are apparent (Fig. 4, A and B), but are minor relative to sites 1 and 2.

Thin layer electrophoresis of acid hydrolysates of phosphorylated calponin followed by autoradiography indicated that Ca²⁺/calmodulin-dependent protein kinase II and protein kinase C phosphorylated both serine and threonine residues. Neither kinase incorporated radiolabeled phosphate onto tyrosine residues.

The effect of phosphorylation of calponin on its ability to inhibit the actomyosin MgATPase was then investigated. Calponin was phosphorylated to 1.11 mol of P_i/mol calponin by Ca²⁺/calmodulin-dependent protein kinase II and to 1.20 mol of P_i/mol calponin by protein kinase C. The effects of phosphorylation during reactions in the absence of Ca²⁺, we repeated the experiments of Table II using thiophosphorylated myosin which is resistant to the action of myosin phosphatase (42).
FIG. 4. Peptide mapping of phosphorylated forms of calponin. Approximately 1 µg of each limit tryptic digest of phosphorylated calponin was subjected to two-dimensional phosphopeptide mapping as described by Colburn et al. (29). Thin layer electrophoresis (TLE) was performed from anode  to cathode  followed by ascending thin layer chromatography (TLC). Autoradiographs presented are: A, calponin phosphorylated by Ca\(^{2+}\)/calmodulin-dependent protein kinase II (1.0 mol of P\(_i\)/mol calponin); B, calponin phosphorylated by caldesmon with copurifying kinase activity (1.9 mol of P\(_i\)/mol calponin); C, calponin phosphorylated by protein kinase C (1.0 mol of P\(_i\)/mol calponin). 0, represents the origin and 1, 2, and 3 represent the major phosphopeptides referred to in the text.

FIG. 5. Inhibition of the actomyosin MgATPase by calponin is blocked by phosphorylation. ATPase rates were measured under standard conditions in the presence of the indicated concentrations of unphosphorylated calponin (○) or calponin phosphorylated by either Ca\(^{2+}\)/calmodulin-dependent protein kinase II (●) or protein kinase C (▲).

various concentrations of phosphorylated and unphosphorylated calponins on the actomyosin ATPase were examined (Fig. 5). At concentrations as high as 5 µM, calponin phosphorylated by Ca\(^{2+}\)/calmodulin-dependent protein kinase II or protein kinase C had little or no effect on the actin-activated myosin MgATPase when compared with unphosphorylated calponin. Partial inhibition (from 117 to 89 nmol of P\(_i\)/mg myosin-min) was observed in other experiments in the presence of 8 µM phosphorylated calponin. The same concentration of unphosphorylated calponin reduced the ATPase rate to 6 nmol of P\(_i\)/mg myosin-min. Therefore, calponin loses its ability to inhibit the actomyosin ATPase upon phosphorylation.

Although regulation of actomyosin MgATPase by calponin is not mediated directly by calcium, but via calcium-dependent kinases, the possibility exists that calponin could be acting in a troponin-like manner by immobilizing tropomyosin on the actin filament, i.e. not permitting tropomyosin to move relative to the actin filament in such a way as to permit normal actin-myosin interaction. In the presence of actin (7 µM) and presence or absence of 1 µM tropomyosin, calponin produced a concentration-dependent inhibition of the actin-activated myosin MgATPase (Fig. 6A) reaching maximum (70–80%) inhibition in both cases at or above 2 µM calponin. This effect can be seen more clearly in Fig. 6B where the data have been normalized in order to compensate for the lower ATPase rates seen in the absence of tropomyosin. Calponin inhibition of the actin-activated MgATPase of myosin is not, therefore, dependent on the presence of tropomyosin.

It is known from the work of Takahashi et al. (9, 13) that calponin binds to calmodulin, tropomyosin, and actin. Examination of the effects of phosphorylation on these binding properties of calponin may help to shed light on the mechanism whereby calponin inhibits the actin-activated myosin MgATPase. We found that phosphorylation of calponin did not affect its interaction with either calmodulin-Sepharose or tropomyosin-Sepharose (data not shown) but, as shown in Fig. 7A, did affect its binding to actin. Unphosphorylated calponin bound to actin and to actin-tropomyosin as determined in a sedimentation assay (Fig. 7A, lanes 1 and 2, 5 and 6). Upon phosphorylation calponin bound to actin and to actin-tropomyosin (Fig. 7A, lanes 3 and 4, 7 and 8). Similarly in sedimentation experiments performed in the presence of actin, tropomyosin, myosin, calmodulin and myosin light chain kinase, i.e. the MgATPase assay system, unphosphorylated calponin bound to actin-

FIG. 6. Inhibition of actin-activated myosin MgATPase by calponin in the presence and absence of tropomyosin. Actomyosin ATPase rates were measured as described under "Experimental Procedures" at the indicated concentrations of calponin in the presence of actin (7 µM) and in the presence (○, ■) or absence (●) of 1 µM tropomyosin and in the presence (○, ●) or absence (■) of Ca\(^{2+}\). A, data presented as ATPase rate in nanomoles of P\(_i\)/mg myosin-min. B, data normalized by expressing ATPase rate in the presence or absence of tropomyosin as a percentage of the maximum.
FIG. 7. Binding of phosphorylated and unphosphorylated calponin to actin, actin-tropomyosin and the reconstituted actomyosin system. A, actin and phosphorylated or unphosphorylated calponin were sedimented at high speed in the presence and absence of tropomyosin as described under "Experimental Procedures." Supernatants (S) and pellets (P) were analyzed by SDS-PAGE. Lanes 1 and 2, actin + unphosphorylated calponin; lanes 3 and 4, actin + phosphorylated calponin; lanes 5 and 6, actin + tropomyosin + unphosphorylated calponin; lanes 7 and 8, actin + tropomyosin + phosphorylated calponin. In this experiment, calponin was phosphorylated to 1.0 mol of Pi/mol calponin by Ca\(^2+\)/calmodulin. Identical results were obtained if calponin was phosphorylated to 1.0 mol of Pi/mol calponin by protein kinase C. In separate experiments, calponin alone (phosphorylated or unphosphorylated) did not sediment under these conditions. B, phosphorylated or unphosphorylated calponin (2 \(\mu\)M) was sedimented at high speed in the presence of actin (6 \(\mu\)M), myosin (1 \(\mu\)M), tropomyosin (2 \(\mu\)M), myosin light chain kinase (74 nM) and calmodulin (1 \(\mu\)M) as described under "Experimental Procedures." Supernatants (S) and pellets (P) were analyzed by SDS-PAGE. Lane 1, molecular weight markers (a, phosphorylase b, 97,400; b, bovine serum albumin, 66,200; c, ovalbumin, 45,000; d, carbonic anhydrase, 29,000; e, soybean trypsin inhibitor, 20,100; f, lysozyme, 14,400); lanes 2 and 3, myosin, actin, tropomyosin, myosin light chain kinase, and calmodulin alone; lanes 4 and 5, as lanes 2 and 3 but plus unphosphorylated calponin; lanes 6 and 7, as lanes 2 and 3 but plus phosphorylated calponin. In this experiment calponin was phosphorylated to 2.3 mol of Pi/mol calponin by Ca\(^2+\)/calmodulin-dependent protein kinase II. M, myosin; A, actin; Tm, tropomyosin; CaP, calponin; LC\(_{20}\), 20-kDa light chain of myosin; LC\(_{17}\), 17-kDa light chain of myosin.

DISCUSSION

In spite of being a major protein component of smooth muscle, calponin was only recently reported (9). Its properties, as determined by Takahashi and coworkers (9-13), suggest that it is a bona fide thin filament protein, although this has recently been challenged by Lehman (14) who suggested that a 35-kDa protein identified as calponin may be part of the insoluble cytoskeleton or possibly a component of the nuclear matrix. This suggestion was based on two observations: first, thin filaments immunoprecipitated with anti-caldesmon did not contain calponin and, secondly, gizzard "ghost" cells from which actin and myosin were extracted were enriched in the 35-kDa protein. However, the results of the immunoprecipitation experiments suggest that calponin may have been completely proteolyzed, a reasonable possibility given the prolonged incubation at 25 °C and its known susceptibility to proteolysis. Furthermore, if comparable amounts of the histones in washed muscle and ghost cells were loaded on the gels in Fig. 6, a and b, of Ref. 14, it would be apparent that the amount of calponin retained in the ghost cells is small relative to that extracted with actin and myosin. The weight of evidence, therefore, favors calponin as a thin filament-associated protein which, as shown by Takahashi et al. (10), is specific to smooth muscle and is, therefore, of interest as a potential regulator of smooth muscle contraction. This possibility is supported by the in vitro binding properties of calponin: it interacts with F-actin and tropomyosin in the presence or absence of Cal\(^2+\), and with calmodulin only in the presence of Cal\(^2+\) (9, 13). We initially encountered this protein in preparations of native thin filaments from chicken gizzard (15); it is clear that the 32-kDa protein observed in such preparations is identical to calponin (13). Consequently, we have investigated the effects of purified calponin on the actin-activated myosin MgATPase in an in vitro system reconstructed from purified smooth muscle contractile and regulatory proteins: actin, myosin, tropomyosin, calmodulin, and myosin light chain kinase.

Calponin inhibits the actomyosin ATPase maximally (~80%) when present in the assay system at a concentration equimolar to tropomyosin. Takahashi et al. (9) have shown that the molar concentrations of calponin and tropomyosin in chicken gizzard smooth muscle are equal. The inhibitory effect of calponin on the actomyosin ATPase is apparently due to its interaction with actin and is clearly not due to inhibition of myosin phosphorylation. Furthermore, although calponin can bind Cal\(^2+\) directly (40), its ability to inhibit the actomyosin ATPase is not Cal\(^2+\)-dependent. This observation prompted us to search for an alternative mechanism of regulating calponin function. Phosphorylation was an obvious one to investigate. We found that calponin could be phosphorylated by protein kinase C and Cal\(^2+\)/calmodulin-dependent protein kinase II, but not by cAMP- or cGMP-dependent protein kinases or by myosin light chain kinase. Most interestingly, phosphorylation by either protein kinase C or Cal\(^2+\)/calmodulin-dependent protein kinase II abolished inhibition of actomyosin ATPase activity by calponin. Phosphorylation also blocked the actin-calponin interaction. However, phosphorylation did not affect the interaction of calponin with tropomyosin (Fig. 7B, lanes 4 and 5) and was recovered predominantly in the pellet along with myosin. Phosphorylated calponin clearly does not bind to actin-tropomyosin and was recovered in the supernatant along with some unphosphorylated actin (Fig. 7B, lanes 6 and 7). Quantitative data (Table III) were obtained by laser densitometry of the gels shown in Fig. 7.

TABLE III  
Dissociation of calponin from actin induced by phosphorylation

| Conditions                  | Supernatant | Pellet |
|-----------------------------|-------------|-------|
| A. A + CaP                  | 2.5         | 97.5  |
| A + P-CaP                   | 70.8        | 29.2  |
| A + Tm + CaP                | 10.0        | 90.0  |
| A + Tm + P-CaP              | 81.1        | 18.9  |
| B. ATPase system + CaP      | 24.7        | 75.3  |
| ATPase system + P-CaP       | 100.0       | 0.0   |

* Results were obtained by laser densitometry of the gels shown in Fig. 7, A and B.

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in immobilized tropomyosin or calmodulin. In separate experiments (data not shown) we have found that calponin, whether phosphorylated or not, does not bind to an affinity column of smooth muscle myosin (phosphorylated or unphosphorylated) coupled to Sepharose 4B.

The electron microscopic observations made by Takahashi et al. (11) indicate that calponin is distributed regularly along tropomyosin paracrystals with \( \pm 40 \) nm periodicity and is located 16-17 nm from the C terminus of each tropomyosin molecule. This is remarkably similar to the distribution of tropinin T along striated muscle thin filaments (45). Furthermore, calponin and tropinin T exhibit immunological cross-reactivity (13). We have also observed that our polyclonal antibodies to chicken gizzard calponin cross-react with immobilized tropomyosin or calmodulin. In separate experiments, we have found that calponin binds to tropomyosin and inhibits the actomyosin ATPase, it may activate the MgATPase of phosphorylated myosin. Several investigators have suggested that caldesmon may be involved in latch-bridge formation (4,49-51), i.e., the formation of slowly cycling or noncycling cross-bridges which are responsible for force maintenance at low levels of ATP consumption and in the presence of intermediate \( Ca^{2+} \) concentrations (52-56). A similar argument could be made for calponin but neither is, as yet, entirely satisfactory.

A great deal of evidence now exists supporting the central role of myosin phosphorylation in the regulation of smooth muscle contraction (3). It is, however, clear that at least one secondary or modulatory mechanism exists which can alter the contractile state of a smooth muscle cell or its response to extracellular signals. Some such mechanisms also utilize \( Ca^{2+} \) as the second messenger. Possibilities which involve \( Ca^{2+} \) include caldesmon (4,5), protein kinase C (6), and direct binding of \( Ca^{2+} \) to myosin (7,8). Calponin may be added to this list.

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