Calponin has been implicated in the regulation of smooth muscle contraction through its interaction with F-actin and inhibition of the actin-activated MgATPase activity of phosphorylated myosin. Both properties are lost following phosphorylation (primarily at serine 175) by protein kinase C or calmodulin-dependent protein kinase II. To evaluate further the functional importance of serine 175, wild-type calponin and three site-specific mutants (S175A, S175D, and S175T) were expressed in Escherichia coli and compared with calponin purified from chicken gizzard smooth muscle in terms of actin binding, actomyosin MgATPase inhibition, and phosphorylation by protein kinase C and calmodulin-dependent protein kinase II. The affinities of skeletal muscle F-actin for wild-type and S175T calponins were similar to that for the tissue-purified protein (Kd = 0.8, 1.3, and 1.0 μM, respectively), whereas the affinities for S175A and S175D calponins were much lower (Kd = 26.8 and 44.2 μM, respectively). Tissue-purified, wild-type, and S175T calponins displayed comparable inhibition of the smooth muscle actin-activated myosin MgATPase, whereas S175A and S175D calponins were much less effective. Phosphorylation confirmed serine 175 as the principal site of phosphorylation by both kinases. These results indicate that the hydroxyl side chain at position 175 of calponin plays a critical role in the binding of calponin to actin and inhibition of the cross-bridge cycling rate.

Calponin is a 32-kDa, thin filament-associated protein that has been implicated in the regulation of smooth muscle contraction (1–4). Through its interaction with actin, calponin inhibits the actin-activated MgATPase activity of phosphorylated smooth muscle myosin (1) and inhibits the relative movement of actin and myosin in vitro motility assays (5, 6). Exogenous calponin attenuated Ca2+-induced contractions of permeabilized rabbit mesenteric arterial smooth muscle strips in a concentration-dependent manner (7). Inhibition of the actomyosin ATPase occurs predominantly by a reduction of Vmax rather than an effect on the affinity of phosphorylated myosin heads for actin (8–10). The inhibitory effect of calponin can be alleviated by phosphorylation catalyzed by protein kinase C (PKC) or Ca2+/calmodulin-dependent protein kinase II (CaM kinase II) (11) and restored following dephosphorylation by type 2A (12) or 2B protein phosphatases (13). Phosphorylation-induced loss of inhibition results from a marked reduction in the affinity of phosphorylated calponin for actin (1). Several examples of phosphorylation of calponin in intact muscle in response to various contractile stimuli have been reported (11, 14–16), and in response to phenylephrine (an α1-adrenergic agonist known to trigger activation of PKC) calponin translocates from cytosolic filamentous structures to a region underlying the sarcolemma (17). Other investigators, however, have reported that calponin is not phosphorylated in intact muscle (18–20). The principal site of phosphorylation by either kinase is serine 175 (11), although Nakamura et al. (21) suggested that threonine 184 was the principal residue phosphorylated by PKC. Mergueli et al. (22) have defined the actin-binding domain of calponin as residues 145–182, which includes serine 175.

To investigate further the importance of serine 175 in the interaction of calponin with actin and inhibition of the actin-activated myosin MgATPase, we have expressed in bacteria wild-type calponin and three site-specific mutants in which serine 175 was replaced by alanine, aspartic acid, or threonine. The following properties of the expressed proteins were compared with calponin purified from chicken gizzard smooth muscle: (i) protein conformation, (ii) actin binding, (iii) inhibition of actin-activated myosin MgATPase activity, and (iv) phosphorylation by PKC and CaM kinase II. The results indicate that serine 175 plays a critical role in the interaction of calponin with actin and ATPase inhibition; in particular, a hydroxyl group in this position is essential for these functions. The results also confirm serine 175 as the principal site of phosphorylation by PKC and CaM kinase II.

**EXPERIMENTAL PROCEDURES**

Materials—[γ-32P]ATP (≥5000 Ci/mmol) was purchased from Amer sham Corp. (Oakville, Ontario, Canada). I-ω-Phosphatidyl-d3-serine and 1,2-diolen were purchased from Serva Feinbiochemica Hanshoff (Heidelberg, Germany). PKC (a mixture of α, β, and γ isozymes) was purified from rat brain as described previously (23). Myosin light chain kinase (24), actin (25), myosin (26), tropomyosin (27), and calmodulin (1) were purified from chicken gizzard smooth muscle. CaM kinase II was co-purified from chicken gizzard with caldesmon because this preparation of the kinase was found to be much more stable than the isolated kinase (28). Actin was also purified from rabbit skeletal muscle as described by Zol and Potter (29). Calmodulin was purified

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1 The abbreviations used are: PKC, Ca2+- and phospholipid-dependent protein kinase C; CaM kinase II, Ca2+/calmodulin-dependent protein kinase II; MOPS, 3-(N-morpholino)propanesulfonic acid; PDB-T, phosphate-buffered saline containing Tween-20; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.
from bovine brain (30). Monoclonal antibody CP-93 to turkey gizzard calponin was purchased from Sigma. Oligonucleotide primers were synthesized at the University Core DNA Services, University of Calgary. Electrophoresis reagents were purchased from Bio-Rad. All other chemicals were reagent grade or better and were purchased from Can Lab (Edmonton, Alberta, Canada).

PCR—Thermal Cycler using the following primers: 5’-ATGTCGAACGCGA-3’ primer. PCR was performed in a Perkin-Elmer System 2400 DNA Thermal Cycler using the following primers: 5’-ATGTCGAACGCGA-3’ (primer 5) and 5’-GAATTCCTTATTGTGAGTT- GCTGTTGTTGAGGCC-3’ (primer 3). These Tris-PCRs are derived from the nucleotide sequence of calponin reported by Takahashi and Nadal-Ginard (31) with an EcoRI site incorporated at the 5’ end of the 3’ primer. The 50-μl reaction mixture contained 50 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM MgCl₂, 0.6 mM of each dNTP, 1 μM of each primer, AmpliTag polymerase (Perkin-Elmer), and cDNA templates (the reverse transcription products). Denaturation of the sample was affected at 94°C for 1 min followed by 35 cycles of 1 min of denaturation at 94°C, 1 min annealing at 55°C, and 2 min of extension at 72°C. A single PCR product of the expected size for α calponin (879 base pairs) was produced, as shown by agarose gel electrophoresis. This cDNA hybridized with a unique 1.3 kb mRNA upon Northern blot analysis with chicken gizzard mRNA as expected (31). The PCR product was subcloned into the pBSII(SK) for sequencing using the Perkin-Elmer Applied Biosystems, Inc. (Mississauga, Ontario, Canada) automated fluorescent sequencing methodology and Genetics Computer Group, Inc. (Madison, WI) sequence analysis software. The nucleotide sequence was identical to that reported by Takahashi and Nadal-Ginard (31) except for four nucleotide substitutions: nucleotides 78, 95, 96, and 197 were C, G, T, and A, respectively, rather than T, G, C, and W. We have confirmed our sequence in several independent experiments, and Gong et al. (32) have independently confirmed nucleotides C and A at positions 78 and 197, respectively. Only one of the four nucleotide changes alters the encoded amino acid (nucleotide 197): threonine 66 becomes lysine, in agreement with the peptide sequences reported by Meczewski et al. (32) and Vancomerndle et al. (33) and our own peptide sequencing data (3).

**PCR Mutagenesis**—S175A, S175D, and S175T mutants were produced by PCR mutagenesis using the overlap extension method (34). The complete nucleotide sequences of all mutants were confirmed to ensure that only the desired mutations were obtained.

**Bacterial Expression and Protein Purification**—Wild-type and mutant calponins were expressed in Escherichia coli and purified from bacterial lysates essentially as described by Gong et al. (32) by sequential chromatography on columns of CM-Sephadex, hydroxylapatite, and Superose 12. Calponin-containing fractions were identified by SDS-PAGE in 12.5% acrylamide mini-slab gels (35). The purified proteins, shown to be electrophoretically homogeneous, were concentrated and dialyzed against 0.1 M glycine hydrochloride, pH 7.5, 60 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, and 1 mM EDTA (pH 7.5), 60 mM KCl, 10 mM MgCl₂, 0.2 mM CaCl₂, 1 mM dithiothreitol, and 1 mM ATP (3,500–5,300 cpm per μl) in a reaction volume of 0.6 ml. Reactions were started by the addition of ATP, and samples (50 μl) were withdrawn at 1-min intervals up to 9 min for quantification of [γ-32P]ATP as described previously (37). ATPase rates in the absence of calponin were 1.69 μM (μmol Pi·min⁻¹·mg protein⁻¹) (mean ± S.D.; n = 18) in the presence of Ca²⁺ and 8.4 ± 5.5 nmol Pi·min⁻¹·mg protein⁻¹ (n = 10) in the presence of Ca²⁺ (1 mM EGTA replaced 0.2 mM CaCl₂ in the ATPase reaction mixture).

**Proteolysis**—Calponin (0.2 mg/ml) was incubated at 0°C with trypsin (0.5 μg/ml), chymotrypsin (0.5 μg/ml), protease K (0.5 μg/ml), or Staphylococcus aureus V8 protease (2 μg/ml) in 20 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM EGTA, and 1 mM dithiothreitol. Samples (25 μl) were withdrawn at t = 5, 10, 15, 30, 45, and 60 min, and added to an equal volume of SDS gel sample buffer, and boiled prior to SDS-PAGE on full-sized 7.5–20% polyacrylamide gradient slab gels (35). Phosphorylation by PKC—Calponin (0.1 mg/ml) was incubated at 30°C with PKC (0.2 μg/ml) in 20 mM MOPS (pH 7.0), 5 mM MgCl₂, 0.3 mM CaCl₂, 0.3 mM phosphatidylserine, 62 μg/ml 1,2-diolein, 0.03% (w/v) Triton X-100, and 0.1 mM [γ-32P]ATP (~110 cpm/μmol). Samples (20 μl) were withdrawn at selected times for quantification of protein-bound [γ-32P]ATP as described previously (38).

**Phosphorylation by CaM kinase II**—Calponin (0.1 mg/ml) was incubated at 30°C with CaM kinase II (123 μM/ml) and calmodulin (2.5 μM) in 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 0.3 mM CaCl₂, and 0.5 mM [γ-32P]ATP (76 cpm/μmol). Phosphorylation was quantified as described above. Duplicate samples were subjected to SDS-PAGE in 12.5% acrylamide mini-slab gels (35).

Monoclonal Antibody Epitope Competition Assays—Four monoclonal antibodies against different epitopes on chicken gizzard calponin² were used in an enzyme-linked immunosorbant assay-mediated competition experiment to evaluate the structural conservation of the mutant calponins under native conditions. Recombinant wild-type calponin was coated on 96-well microtiter plates (Falcon 3915) at 5 μg/ml in 50 mM carbonate buffer (pH 9.6), 100 μl/well, and incubated overnight as described previously (39) at 4°C. After blocking with 1% bovine serum albumin in phosphate-buffered saline (137 mM NaCl, 2.7 mM KH₂PO₄, 8.0 mM Na₂HPO₄, pH 7.4) containing 0.05% Tween-20 (PBS-T) and washing three times with PBS-T, a constant concentration (predetermined in the indirect enzyme-linked immunosorbant assay to give a binding in the upper one-third of the linear range against the coated wild-type calponin) of anti-calponin monoclonal antibodies CP1, 3, 4, or 8 mixed with serial dilutions of wild-type, S175A, S175D, and S175T calponins (0.001-10 μg/ml) were added and incubated at 4°C for 6 h. Following three washes with PBS-T, horseradish peroxidase-labeled rabbit anti-mouse polyclonal immunoglobulin (Sigma) second antibody was added and incubated at 37°C for 1 h. Following three further washes with PBS-T, H₂O₂/2'-azinobis(3-ethylbenzthiazolinesulfonic acid) substrate was added and color developed at room temperature. The A₄₀₅ nm was recorded using a Bio-Rad model 3500 UV automated microplate reader at a series of time points, and the values in the linear range were plotted against the concentration of the blocking protein.

Protein Concentrations—Concentrations of calponin and its mutants were determined by amino acid analysis using endogenous phenylalanyl-nine and exogenous norleucine for quantification. PKC and myosin light chain kinase concentrations were determined by the Coomassie blue dye binding assay with dye reagent and γ-globulin standard purchased from Pierce. The differences were quantified using the following values for the absorbance of a 1% solution with a path length of 1 cm: myosin, 4.5 at 280 nm (40); tropomyosin, 2.9 at 278 nm (40); calmodulin, 1.95 at 277 nm (42); skeletal muscle actin, 6.3 at 290 nm (43); and smooth muscle actin, 6.38 at 290 nm (44).

**RESULTS**

Two experimental approaches were used to determine whether or not the conformations of the mutant recombinant calponins were similar to that of wild-type calponin or calponin

²) J. P. Walsh, M. P. Resek, M. E., and McMartin, G. A. (1996) Biochem. Cell Biol. 74, in press.
binding of four monoclonal antibodies to recombinant wild-type calponin and the two mutants, S175A and S175D, which exhibited functional differences compared with wild-type calponin (see below). The titration curves showed similar binding patterns for all four monoclonal antibodies by the wild-type and S175A calponins, indicating no detectable structural differences. The S175D mutant showed slightly decreased affinity for monoclonal antibodies CP1, CP3, and CP4 as compared with that of the wild-type or S175A calponins, indicating a minor structural change induced by introduction of a negatively charged residue at position 175. In additional experiments, it was observed that these four and an additional three monoclonal antibodies gave similar binding patterns to solid phase-immobilized wild-type, S175A, and S175T calponins. However, the S175D mutant exhibited lower maximal binding to CP1, 3, 4, 5, and 7 (data not shown).

The binding of bacterially expressed calponins to smooth muscle actin or actin/tropomyosin was evaluated by a co-sedimentation assay. Each calponin was mixed with chicken gizzard actin with or without tropomyosin as described under “Experimental Procedures” and centrifuged at high speed to separate F-actin and bound proteins from G-actin and unbound proteins. Calponin purified from chicken gizzard (4 μM) bound to smooth muscle actin-tropomyosin as expected (Table I); 74% of the calponin was recovered in the pellet fraction under these conditions. Wild-type calponin and the S175T mutant showed similar binding to actin/tropomyosin; 71 and 69% of the respective calponins was recovered in the pellet. On the other hand, S175A and S175D showed much reduced binding to actin/tropomyosin: 14% of S175A and 30% of S175D was recovered in the pellet. Similar results were obtained in the absence of tropomyosin: the proportions of wild-type calponin, S175A, S175D, and S175T mutant calponins recovered in the pellet associated with smooth muscle F-actin were 86, 5.6, 33, and 69%, respectively (means of two experiments). Similar results were also obtained with skeletal muscle actin (Table I); the proportions of tissue-purified calponin, wild-type calponin, S175A, S175D, and S175T mutant calponins recovered in the pellet were 71, 72, 28, 20, and 66%, respectively.

We noticed that the proportion of sedimented smooth muscle actin was reduced in the presence of the three calponin mutants compared with tissue-purified or wild-type calponin, whereas the sedimentation of skeletal muscle actin was unaffected by the different calponins (Table I), suggesting that the three mutant calponins may have a minor effect on smooth muscle actin polymerization or cause a small degree of actin filament severing producing short filaments that do not sediment upon high speed centrifugation. Consequently, skeletal muscle actin/smooth muscle tropomyosin was chosen as the system to quantify the affinities of actin for the various calponin species to facilitate accurate quantification of the amount of actin-bound calponin as the calponin concentration is varied over the range 1.75–4.5 μM as described under “Experimental Procedures.” The following Kd values for actin/tropomyosin binding were determined from the Scatchard plots shown in Fig. 4; 1.0 μM for calponin purified from chicken gizzard, 0.8 μM for wild-type calponin, and 1.3 μM for S175T. These values are not significantly different, indicating that the bacterially expressed wild-type protein and the S175T mutant bind to actin with the same affinity as the native protein. The S175A and S175D mutants, on the other hand, bound to actin with much lower affinity; Kd = 26.8 μM for S175A and 44.2 μM for S175D.

The effects of calponin and its mutants on the actin-activated MgATPase activity of smooth muscle myosin are compared in Fig. 5. As expected, calponin purified from chicken gizzard markedly inhibited the ATPase. A similar degree of inhibition...
was observed with the bacterially expressed wild-type calponin (20.8% of control ATPase activity was retained at 4 μM and 12.4% at 5 μM calponin) and the S175T mutant (Fig. 5). The S175A and S175D mutants, however, were relatively ineffective in inhibiting the actomyosin ATPase, consistent with their much reduced affinities for actin. SDS-PAGE and autoradiography of samples at the end of the ATPase reactions verified that phosphorylation of the 20-kDa light chain of smooth muscle myosin was unaffected by any of the calponin species (data not shown).

Fig. 6 compares the time courses of phosphorylation of the various calponin species by PKC. Tissue-purified calponin and bacterially expressed calponin exhibit similar phosphorylation time courses with maximal stoichiometry of ~1.1 mol P/mol calponin under these conditions. No phosphorylation was observed in the absence of Ca²⁺, phospholipid, and diacylglycerol,
which are required by PKC (a mixture of α, β, and γ isoenzymes) for activity. Much less phosphate incorporation into S175A (~0.3 mol P/mol) or S175D (~0.4 mol P/mol) was observed, consistent with our earlier conclusion that serine 175 represents the principal site of phosphorylation by PKC. The S175T mutant is clearly a substrate of PKC, although the kinetics of its phosphorylation are much slower than for the native or wild-type protein.

In the case of phosphorylation by CaM kinase II (Fig. 7), again the native and wild-type proteins are very similar, and phosphorylation required the presence of Ca\(^{2+}\) and calmodulin. The S175A and S175D mutants were very poor substrates of this enzyme, even poorer than for PKC. The S175T mutant, however, proved to be as good a substrate for CaM kinase II as the native or wild-type protein.

**DISCUSSION**

Serine 175 has previously been implicated in the binding of calponin to actin and inhibition of the actin-activated myosin MgATPase (11). Phosphorylation by PKC or CaM kinase II occurs predominantly at this site with substoichiometric phosphorylation occurring at other sites: Thr\(^{180}\), Thr\(^{184}\), Ser\(^{215}\), Ser\(^{254}\), and Thr\(^{259}\) by PKC and Ser\(^{71}\), Ser\(^{254}\), and Ser\(^{215}\) by

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**TABLE I**

| Calponin          | Protein recovered in pellet* % | Calponin | Smooth muscle actin | Calponin | Skeletal muscle actin |
|-------------------|--------------------------------|----------|---------------------|----------|-----------------------|
| Chicken gizzard   | 73.7 ± 10.9                    | 77.4 ± 3.2| 70.9 ± 5.5         | 88.5 ± 1.4 |
| Wild-type         | 70.9 ± 5.3                     | 79.1 ± 2.6| 72.3 ± 6.2         | 82.2 ± 1.9 |
| S175A             | 13.9 ± 8.9                     | 61.2 ± 13.6| 28.3 ± 3.9         | 87.1 ± 2.1 |
| S175D             | 30.3 ± 21.1                    | 61.8 ± 15.3| 20.3 ± 5.3         | 87.4 ± 1.6 |
| S175T             | 69.2 ± 17.1                    | 66.2 ± 12.8| 66.3 ± 7.4         | 86.5 ± 1.6 |

*Values represent the mean ± S.D. (n = 4–24).
Structure-Function Relations of Calponin

Fig. 6. Phosphorylation of tissue-purified, wild-type, and mutant calponins by PKC. Chicken gizzard (C), wild-type (●), S175A (○), S175D (▲), and S175T (□) calponins were incubated with PKC as described under "Experimental Procedures," and phosphate incorporation was quantified at the indicated times. The results are representative of eight independent experiments.

CaM kinase II. Ser175 is located within the actin-binding domain (residues 145–182) defined by Mezgueldi et al. (22). It should be noted, however, that Nakamura et al. (21) concluded that Thr184 is the major site of phosphorylation by PKC. To address the functional importance of Ser175 directly, we have expressed and characterized three site-specific mutants of calponin and compared their properties with those of bacterially expressed wild-type calponin and calponin purified from chicken gizzard smooth muscle. Ser175 was mutated (i) to alanine to assess the importance of the hydroxyl side chain and to determine whether or not this residue indeed represents the principal phosphorylation site, (ii) to aspartic acid to assess the conformational changes in the protein (i.e., in an attempt to mimic, at least in part, the effects of phosphorylation), and (iii) to threonine to assess the importance of a hydroxylated side chain at this position and, ultimately, of the calponin-actin complex. The results indicate that bacterially expressed wild-type calponin is structurally and functionally indistinguishable from the protein purified from smooth muscle, thus confirming and extending the work of Gong et al. (32).

The properties of the S175A mutant are particularly intriguing. This mutant calponin binds very weakly to smooth or skeletal muscle actins similarly to the protein purified from chicken gizzard. The \( K_d \) of skeletal muscle actin for wild-type calponin (0.8 \( \mu M \)) was found to be similar to that for native calponin (1.0 \( \mu M \)). Consistent with these observations, wild-type calponin inhibited the actin-activated MgATPase activity of smooth muscle myosin to a similar extent as did the native protein. Furthermore, phosphorylation of wild-type and tissue-purified calponins by PKC and CaM kinase II occurred with similar time courses and maximal stoichiometries. These results indicate that bacterially expressed wild-type calponin is structurally and functionally indistinguishable from the protein purified from smooth muscle, thus confirming and extending the work of Gong et al. (32).

The stoichiometry of S175A phosphorylation presumably represents phosphate incorporation into minor, nonfunctional

Fig. 7. Phosphorylation of tissue-purified, wild-type, and mutant calponins by CaM kinase II. A, chicken gizzard (○), wild-type (●), S175A (○), S175D (▲), and S175T (□) calponins were incubated with CaM kinase II as described under "Experimental Procedures," and phosphate incorporation was quantified at the indicated times. Phosphorylation of the tissue-purified protein was also measured in the absence of Ca\(^2+\), phospholipid, and diacylglycerol (▲). The results are representative of six independent experiments. GG, chicken gizzard.
sites identified earlier (11).

The properties of the S175D mutant are very similar to those of S175A and substantiate the conclusions that Ser 175 plays a crucial role in binding to actin and that it is the principal site of phosphorylation by PKC and CaM kinase II. In particular, the slightly decreased affinity of S175D calponin for monoclonal antibodies CP1, 3, and 4 suggests that introduction of negative charge at position 175 disrupts the conformation that together with loss of the hydroxyl group contributes to the marked reduction in affinity for actin. Similar effects probably account for the dramatic consequences of phosphorylation of calponin at Ser 175.

In conclusion, our results indicate that serine 175 of calponin plays a crucial role in the interaction of calponin with actin and inhibition of the actin-activated MgATPase activity of smooth muscle myosin and that this residue is the principal site of phosphorylation by PKC and CaM kinase II. In particular, the integrity of the hydroxyl group in the side chain at position 175 is critically important for high affinity binding to actin.

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