Identification of the Regulatory Site in Smooth Muscle Calponin That Is Phosphorylated by Protein Kinase C*

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F-actin and tropomyosin inhibited the phosphorylation of calponin by protein kinase C, and the phosphorylation reduced the binding of calponin to F-actin and tropomyosin. Labeled phosphate from \( \gamma^{32P} \)ATP was retained both on the chymotryptic NH\(_2\)-terminal 22-kDa fragment, which contains the actin-, tropomyosin-, and calmodulin-binding regions, and on the COOH-terminal 12-kDa fragment. Fractionation of tryptic \( \alpha^{32P} \)-labeled peptides by high performance liquid chromatography allowed isolation of three phosphopeptides (designated T\(_1\), T\(_2\), and T\(_3\)), each of which was located in three repeating amino acid motifs of calponin. Both the relative initial rates and extent of phosphorylation decreased in the order T\(_2\) > T\(_3\) > T\(_1\). Both serine and threonine residues were phosphorylated in T\(_1\) (GASQAGMTAPGTK), and only a threonine residue was phosphorylated in T\(_2\) (FASQQGMTPGTYTR) and in T\(_3\) (GASQGGMTYGLPR). As the 22-kDa fragment contained only T\(_2\), the phosphorylation site in T\(_2\) appeared to regulate the binding of calponin to F-actin and tropomyosin. The amino acid sequence of T\(_2\) indicates that protein kinase C phosphorylates Thr\(_{184}\). Thus Thr\(_{184}\) is the preferred site of phosphorylation and is functionally the most important of the sites phosphorylated by protein kinase C in smooth muscle calponin.

The reversible phosphorylation of the 20-kDa regulatory light chain of smooth muscle myosin by a calcium- and calmodulin-dependent myosin light chain kinase has been established as the mechanism for activation of smooth muscle contraction (1–3). However, regulation by the phosphorylation of myosin light chain does not explain all aspects of the contractile functions of smooth muscle, in particular, the tonic contractile responses. Therefore, additional regulatory mechanisms have been postulated (4–6). Such mechanisms involve, for example, the calcium- and phospholipid-dependent protein kinase (protein kinase C (PKC))\(^1\) (6)), the direct binding of calcium to myosin (7, 8), and the actions of calcium-sensitive factors that are associated with the thin filament. There are likely candidates for thin filament-linked regulatory proteins in smooth muscle, namely, caldesmon (9–16) and the recently discovered tropinin-T-like protein, calponin (17, 18).

Calponin, first purified from chicken gizzard (19) and later from bovine aorta (20), has been shown to interact with actin, tropomyosin, and Ca\(^{2+}\)-calmodulin (19). More recently, cDNA clones for chicken gizzard calponin have been sequenced, and the deduced amino acid sequences represent the two isoforms of chicken gizzard calponin (21). Smooth muscle calponin is known to be an excellent substrate for PKC in vitro (17, 22), and this protein inhibits the actin-activated myosin Mg-ATPase of smooth muscle (17). Moreover, the phosphorylation of calponin by PKC results in loss of its activity via dissociation of calponin from actin (17). The binding of calponin to F-actin, tropomyosin, and calmodulin has been well documented (19), but the domains of interaction with these proteins have not been studied in detail. Chymotryptic proteolysis generates a 22-kDa fragment of turkey gizzard calponin, which has been shown to interact with tropomyosin (23). A cDNA sequence of chicken gizzard calponin (21) indicates that this 22-kDa fragment contains the region that corresponds to residues 7–182 of chicken gizzard calponin. Also, it is reported that NH\(_2\)-terminal calponin region of residues 52–168 seems to contain the major determinants for F-actin and Ca\(^{2+}\)-calmodulin binding (24).

In contracting or resting arterial smooth muscle, calponin is not phosphorylated (25). However, in intact canine tracheal smooth muscle, the potentially important thin filament proteins calponin and caldesmon are phosphorylated and dephosphorylated at a rate sufficient to indicate a role in contraction and relaxation (26).

In the present study, we determined that F-actin and tropomyosin as well as calmodulin decrease the rate of phosphorylation of calponin by PKC, and that the phosphorylation of calponin reduces its ability to bind not only to F-actin but also to tropomyosin. We prepared fragments of calponin by digestion with \( \alpha \)-chymotrypsin and analyzed their binding to F-actin, tropomyosin, and calmodulin. Furthermore, the phosphorylation of calponin at multiple sites by PKC was analyzed, and the functionally most important and preferred site of phosphorylation was identified.

**EXPERIMENTAL PROCEDURES**

**Materials**—\( \gamma^{32P} \)ATP was purchased from Amersham Corp., Cyanogen bromide-activated Sepharose 4B, CM-Sephadex, and TPCK-treated trypsin were purchased from Sigma. \( \alpha \)-Chymotrypsin was purchased from Worthington. Phosphatidylinositol (brain) was purchased from Serva Research Laboratories. All other chemicals were of the highest grade commercially available.

**Purification of Proteins**—Calponin was purified from chicken gizzard as described by Takahashi et al. (19) with a single modification,
namely, CM-Sephadex was used for the ion-exchange chromatography. PKC was purified from rat brain by the method previously described (27). Tropomyosin was purified from chicken gizzard by a modification of the procedure described by Brecher (28). The following proteins were purified by previously described methods: rabbit skeletal actin (29), bovine brain calmodulin (30), chicken gizzard myosin without light chain (31), and tropomyosin. The samples were spotted on a 20 mm Tri-HCl (pH 7.5), 5.8 mM calmodulin, 1 μg/ml PKC, 100 mM [γ-32P]ATP (25,000-30,000 cpm/nmol) or 100 μM ATP, 5 mM MgCl₂, 0.8 mM CaCl₂, and 50 μg/ml phosphatidylinositol. The reaction was initiated by the addition of [γ-32P]ATP. Incorporation of radio-labeled phosphate was monitored by adding aliquots of the reaction mixture to 20% trichloroacetic acid at appropriate times. The precipitates, after centrifugation, were washed twice with 5% trichloroacetic acid, and then radioactivity was determined by scintillation counting. For the preparation of phosphorylated calponin, we took advantage of the heat stability of calponin (19). The reactions were stopped by the addition of 5 mM EGTA which was followed by boiling at 95 °C for 5 min. Then the mixtures were centrifuged for 20 min and used for subsequent binding or fluorescence assays.

Phosphorylation of Calponin—Phosphorylation of purified chicken gizzard calponin was carried out at 30 °C in a reaction mixture that contained 20 mM Tri-HCl (pH 7.5), 5.8 mM calmodulin, 1 μg/ml PKC, 100 mM [γ-32P]ATP (25,000-30,000 cpm/nmol) or 100 μM ATP, 5 mM MgCl₂, 0.8 mM CaCl₂, and 50 μg/ml phosphatidylinositol. The reaction was initiated by the addition of [γ-32P]ATP. Incorporation of radio-labeled phosphate was monitored by adding aliquots of the reaction mixture to 20% trichloroacetic acid at appropriate times. The precipitates, after centrifugation, were washed twice with 5% trichloroacetic acid, and then radioactivity was determined by scintillation counting. For the preparation of phosphorylated calponin, we took advantage of the heat stability of calponin (19). The reactions were stopped by the addition of 5 mM EGTA which was followed by boiling at 95 °C for 5 min. Then the mixtures were centrifuged for 20 min and used for subsequent binding or fluorescence assays.

Chymotryptic Digestion of Calponin—Calponin (1.0 mg/ml) was incubated at 25 °C for 0.5 to 30 min in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EGTA, and 0.5 mM DTT and used for subsequent binding or fluorescence assays.

Chromatographic Digestion of Calponin—Calponin (1.0 mg/ml) was incubated at 25 °C for 0.5 to 30 min in 20 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EGTA, 1 mM DTT, and 1.0 μg/ml α-chymotrypsin. Digestion was stopped by addition of diisopropyl fluorophosphate to a final concentration of 2 mM. The major 22- and 12-kDa fragments of calponin were purified by affinity chromatography on tropomyosin-Sepharose 4B.

Purification of Tryptic Phosphopeptides by HPLC—32P-Labeled calponin was purified by HPLC. In brief, 1 ml of calponin (0.3 mg/ml) was phosphorylated by incubation with PKC at 30 °C for various times. The reactions were stopped by addition of 5 mM EGTA, boiling, and subsequent clarification, and 0.1% trifluoroacetic acid was added to 10% v/v. After each supernatant was dialyzed exhaustively against buffer that contained 20 mM Tri-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EGTA, and 0.5 mM DTT and used for subsequent binding or fluorescence assays.

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Phosphorylation of Calponin by PKC—In a previous study we reported that smooth muscle calponin is a good substrate for PKC and that the phosphorylation of calponin by PKC is inhibited by calmodulin in a noncompetitive fashion with respect to calponin (22). We investigated whether or not actin and tropomyosin can also inhibit the phosphorylation of calponin, and we found that indeed they can. Kinetic analysis of actin-induced inhibition of the phosphorylation of calponin by PKC revealed that actin inhibited the phosphorylation of calponin in a competitive fashion with respect to calponin (Fig. 1A) and that the Kᵢ value was 2.2 μM. In contrast to actin, tropomyosin inhibited the phosphorylation in an uncompetitive fashion (Fig. 1B). When using histone type III-S from calf thymus (Sigma) as the substrate for PKC, no inhibition of PKC was observed in the presence of actin, tropomyosin, or calmodulin (data not shown). These results indicate that the sites of binding of actin, tropomyosin, and calmodulin to the calponin molecule may be different from one another. Furthermore, we
examined the phosphorylation of calponin by PKC in the presence of both actin and tropomyosin. In the presence of excess concentrations of both actin and tropomyosin, the phosphorylation was markedly reduced, but not completely.

Measurement of Chemical Phosphate in Purified Calponin—We determined whether the purified calponin is already partially phosphorylated or not, by the chemical phosphate determinations according to the method described by Buss and Stull (36), before determining the effect of phosphorylation of calponin on its binding abilities to F-actin, tropomyosin, and calmodulin. We found that endogenous phosphate in purified calponin was not detected.

Effect of Phosphorylation on the Ability of Calponin to Bind F-Actin, Tropomyosin, and Calmodulin—The binding of phosphorylated and unphosphorylated calponin to F-actin in the presence and absence of tropomyosin were compared by the ultracentrifugation method, as described under "Experimental Procedures." As shown in Table 1, the amount of phosphorylated calponin remaining unbound was 59.2 ± 2.5%, as compared with 12.2 ± 3.4% in control reactions with unphosphorylated calponin. The apparent dissociation constant \(K_d\) value of calponin for F-actin in the absence of tropomyosin, which was obtained from a double-reciprocal plot (Fig. 2A), increased from 0.19 ± 0.06 \(\mu\)M for the unphosphorylated protein to 1.6 ± 0.54 \(\mu\)M for the phosphorylated protein. These differences are statistically significant \((p < 0.05)\). The corresponding values of calponin for F-actin in the presence of tropomyosin also increased from 0.18 ± 0.04 \(\mu\)M to 1.2 ± 0.16 \(\mu\)M \((p < 0.01)\) (Fig. 2B). Tropomyosin did not affect the \(K_d\) value of unphosphorylated and phosphorylated calponin for F-actin statistically. These results suggest that phosphorylation of calponin by PKC can reduce the binding affinity for F-actin both in the presence and absence of tropomyosin. These results were in good agreement with the findings, reported previously (17), that phosphorylated calponin has a much reduced affinity for F-actin.

To determine whether the phosphorylation of calponin affects its binding to tropomyosin and calmodulin, we examined the effect of the phosphorylation of calponin on its ability to bind dansyl-tropomyosin and dansyl-calmodulin. The effects of tropomyosin-binding proteins on the fluorescence of dansyl-calmodulin have been reported previously (35). In the presence and absence of Ca\(^{2+}\), the fluorescence spectrum of dansyl-tropomyosin exhibited a maximum at 505 nm when it was excited at 365 nm. Addition of calponin caused an increase in fluorescence intensity of dansyl-tropomyosin but did not cause any shift in the emission maximum of the spectrum (data not shown). The effects of calcium and calmodulin-binding proteins on the fluorescence of dansyl-calmodulin have been also reported previously (34). With excitation at 340 nm, the fluorescence spectrum of dansyl-calmodulin in the presence of 0.1 mM EGTA exhibited a maximum at 520 nm; in the presence of 0.3 mM Ca\(^{2+}\), the maximum shifted to 490 nm and the fluorescence intensity at 490 nm increased. In the presence of Ca\(^{2+}\), the addition of calponin caused a blue shift in the fluorescence spectrum and the fluorescence intensity at 490 nm of dansyl-calmodulin increased (data not shown). In the absence of Ca\(^{2+}\), the addition of calponin did not alter the spectrum of dansyl-calmodulin (data not shown). These results correspond closely to earlier observation that calponin can bind to a tropomyosin affinity column in a Ca\(^{2+}\)-independent manner and to a calmodulin affinity column in Ca\(^{2+}\)-dependent manner (19).

The effects on the fluorescence intensity of the addition of increasing amounts of calponin to dansyl-tropomyosin and dansyl-calmodulin are illustrated in Fig. 3, A and B. In the case of binding to dansyl-tropomyosin (Fig. 3A), the apparent dissociation constant \(K_d\) value for calponin, which was obtained from a double-reciprocal plot, increased from 1.8 ± 0.19 \(\mu\)M for the unphosphorylated protein to 4.1 ± 0.94 \(\mu\)M for the phosphorylated protein significantly \((p < 0.05)\), and the maximum change in the fluorescence intensity \((\Delta F_{max}/F_0)\) decreased from 0.79 ± 0.074 for the unphosphorylated protein to 0.25 ± 0.034 for the phosphorylated protein significantly \((p < 0.01)\). In the case of binding to dansyl-calmodulin (Fig. 3B), \(\Delta F_{max}/F_0\) decreased from 0.64 ± 0.026 for the unphosphorylated protein to 0.50 ± 0.061 for the phosphorylated protein significantly \((p < 0.05)\), but similar apparent \(K_d\) values were obtained for both unphosphorylated (0.64 ± 0.026 \(\mu\)M) and phosphorylated calponin (0.55 ± 0.032 \(\mu\)M). These results suggest that the phosphorylation of calponin by PKC reduces the binding affinity of calponin not only for F-actin but also for tropomyosin. In the case of calmodulin, phosphorylation of calponin does not reduce the binding affinity for calmodulin. However, phosphorylation of calponin probably reduced the conformational change of calmodulin-calponin complex, because the increase in fluorescence intensity is directly related to complex formation (39).
The binding of phosphorylated and unphosphorylated 22- and 12-kDa fragments to F-actin was compared by the ultracentrifugation method described in the "Experimental Procedures." As shown in Table I, although the 22-kDa fragment has weaker affinity for F-actin than intact calponin, this fragment co-sediments with F-actin in a Ca²⁺-independent manner. In contrast to the 22-kDa fragment, the 12-kDa fragment does not co-sediment with F-actin. Moreover, as shown in Table I, 68.3 ± 5.9% of the phosphorylated 22-kDa fragment remained unbound, as compared with 31.3 ± 2.7% in control reactions with the unphosphorylated 22-kDa fragment. These differences are statistically significant (p < 0.01). These results suggest that the actin-binding region(s) is located on the 22-kDa fragment, and that the phosphorylation of the 22-kDa fragment, as well as that of intact calponin, reduces its ability to bind to F-actin.

To identify the fragments that bind tropomyosin and calmodulin, the digestion mixture containing unphosphorylated calponin was subjected to affinity chromatography on immobilized tropomyosin and immobilized calmodulin. The 22-kDa fragment was retained on the immobilized tropomyosin in a Ca²⁺-independent manner, and on the immobilized calmodulin in a Ca²⁺-dependent manner, but the 12-kDa fragment flowed through the two affinity columns in both the presence and the absence of Ca²⁺ (Fig. 5, A and B). The changes in fluorescence intensity of dansyl-tropomyosin and dansyl-calmodulin, as a function of the concentration of calponin, of the 22- and 12-kDa fragments, are shown in Fig. 5, C and D. The 12-kDa fragment had essentially no effect on the fluorescence of either dansyl-tropomyosin or dansyl-calmodulin. By contrast, calponin and the 22-kDa fragment caused substantial increases in the intensity of fluorescence. These findings are in agreement with the results of binding assays using affinity chromatography. These results indicate that the tropomyosin- and calmodulin-binding regions are located in the 22-kDa fragment.

Purification and Analysis of Tryptic Phosphopeptides—To identify the sites in calponin that are phosphorylated by PKC, calponin was phosphorylated (1.2 mol of phosphate/mol of calponin) and subjected to the complete tryptic hydrolysis (see "Experimental Procedures"). The tryptic peptides were separated by C-8 reverse-phase HPLC. Fractionation of tryptic phosphopeptide peaks of calponin that had been phosphorylated by PKC are shown in Fig. 6, A and B. It is clear from Fig. 6B that three major radioactive peaks were found. Each radioactive peak fraction was rechromatographed using more gradual CH₃CN gradient to achieve further purification by reverse-phase HPLC. As a result, each of the three peaks contains only one phosphopeptide. The three purified phosphopeptides were designated T₁, T₂, and T₃ on the basis of the order of their elution from the first reverse-phase column. The recovery of ³²P from the column was 80–90% of the applied radioactivity. To determine the relative initial rates
of phosphorylation and the extent of phosphorylation of these phosphopeptides, we prepared calponin that was phosphorylated by PKC to different extents and then we repeated the analysis after tryptic hydrolysis. From Fig. 7, it is clear that both the relative initial rates and the extent of phosphorylation of these three phosphopeptides decreased in the order T2 > T3 > T1. Among these three phosphopeptides, the preferred site of phosphorylation is located in T2, which accounts for about 50% of the total phosphorylation by PKC after 60 min and about 67% of the total phosphorylation within the first 5 min.

Amino Acid Sequencing and Determination of Phosphoamino Acids—The actual amino acid sequences of the phosphopeptides generated by tryptic digestion of chicken gizzard calponin that had been phosphorylated by PKC are shown in Table II. To identify the location of the phosphorylated amino acids, phosphoamino acid analysis of these phosphopeptides was carried out. Both serine and threonine residues were found to be the phosphorylated amino acids in T1, and only threonine was phosphorylated in T2 and T3 (Fig. 8). This finding is in good agreement with our previous report that PKC preferentially phosphorylated threonine in calponin from chicken gizzard (22). Moreover, similar results were obtained in the presence of both 50 μM CaCl2 and 1 μM phorbol 12,13-dibutyrate or 30 μg/ml 1-oleoyl-2-acetyl-rac-glycerol in the reaction mixture for the phosphorylation of

FIG. 4. Time course of chymotryptic digestion of phosphorylated calponin. Calponin (1 mg/ml), phosphorylated by PKC (1.2 mol phosphate/mol of calponin), was treated with α-chymotrypsin (1 μg/ml) at 25 °C. At various times, 2 mM diisopropyl fluorophosphate was added to aliquots of the digestion mixture. Lanes 1-6, aliquots of the digestion mixture taken at 0, 1, 5, 10, 30, and 60 min after the start of the reaction, respectively. A, 12.5% SDS-PAGE stained with Coomassie Blue; B, corresponding autoradiograph. The positions of molecular mass markers (kDa) are as indicated. CaP, calponin; 31K, 31-kDa fragment; 22K, 22-kDa fragment; 12K, 12-kDa fragment.

FIG. 5. Binding of α-chymotryptic fragments to immobilized tropomyosin and calmodulin and effects of these fragments on the fluorescence of dansyl-calmodulin and dansyl-tropomyosin. Calponin was digested with α-chymotrypsin as described under “Experimental Procedures.” Digestion was stopped by the addition of 2 mM diisopropyl fluorophosphate, and the digests were dialyzed against buffer A (see “Experimental Procedures”) for the binding assay with a tropomyosin affinity column and against buffer A that contained 100 mM NaCl for the binding assay with a calmodulin affinity column. The dialyzed digests were loaded on columns of tropomyosin-Sepharose 4B and calmodulin-Sepharose 4B. After washing of unbound proteins from the columns, bound proteins were eluted as described under “Experimental Procedures.” A, 12.5% gel after SDS-PAGE of fractions from tropomyosin affinity column; lane 1, total fragments; lane 2, flow-through; lanes 3 and 4, bound fraction eluted at 20 mM NaCl (lane 3) and at 100 mM NaCl (lane 4) from the affinity column. B, 12.5% gel after SDS-PAGE of the fractions from calmodulin affinity column; lane 1, total fragments; lane 2, flow-through; lane 3, bound fraction. The numbers on the side of the lanes represent molecular mass in kDa. C and D, calponin, purified 22- and 12-kDa fragments were added to a 0.5 μM solution of dansyl-calmodulin or a 0.2 μM solution of dansyl-tropomyosin in buffer as described in the legend to Fig. 2. Fluorescence titrations of dansyl-tropomyosin (C) and dansyl-calmodulin (D) with calponin (○), 22-kDa fragment (●), and 12-kDa fragment (△).
The site of phosphorylation, T₁, was determined to be calponin (Gly₁²¹₅-Lys₂²²₅), with Ser²¹⁵ and Thr²²⁰ and/or Thr²²⁴ being the sites of phosphorylation. Thus multiple sites of phosphorylation were identified and those contained within the 22-kDa fragment, which had the actin-, tropomyosin-, and calmodulin-binding regions, appeared to be functionally important. However, two threonine residues, Thr¹⁸⁰ and Thr¹⁸⁴, were present within the phosphopeptide T₂. Therefore, it was important to establish the site phosphorylated by PKC in tryptic T₂ peptide. The most probable sites of phosphorylation were identified by failure to identify a Thr when peptide sequences were compared with the known sequence (40). To eliminate the possibility that either of these residues (Thr¹⁸⁰ and Thr¹⁸⁴) was phosphorylated, we used the synthetic peptide corresponding to T₂. As shown in Table III, Thr¹⁸⁰ in the tryptic phosphopeptide T₂ and both Thr¹⁸⁰ and Thr¹⁸⁴ in the corresponding synthetic peptide were detected. But the yield of Thr¹⁸⁴ in the tryptic phosphopeptide T₂ was found to be reduced markedly. These data suggest that Thr¹⁸⁴ is the phosphorylated amino acid in the T₂, which accounts for about 50% of the total phosphorylation (about 0.6 mol of phosphate/mol of calponin) (Fig. 7).

**DISCUSSION**

PKC is now recognized as a major regulatory enzyme and it has been implicated in the control of a wide variety of physiological processes (41). There are numerous examples of endogenous substrates, including cytoskeletal proteins (41), that can be phosphorylated by PKC. Since this kinase may be implicated in smooth muscle functions, such as contraction and relaxation (6, 42-44), substrates of PKC in smooth muscle are of particular interest. We reported previously that calponin from chicken gizzard smooth muscle is an excellent substrate for PKC (22).

The experiments reported herein demonstrate that three tryptic phosphopeptides can be generated from chicken gizzard calponin, (T₁, (Gly₁²¹₅-Lys₂²²₅); T₂, (Phe₁⁷₃-Arg₁⁸⁵); and T₃, (Gly₂⁵²-Arg₂⁶⁵)), when calponin has been phosphorylated by PKC. This result is in good agreement with the results of two-dimensional phosphopeptide mapping by Winder and Walsh (17), in which three major tryptic phosphopeptides were detected. From our results of amino acid sequence and phosphoamino acid analysis of these phosphopeptides, we have determined that PKC phosphorylates Thr¹⁸⁴ in T₂, Ser²¹⁵ and Thr²²⁰ and/or Thr²²⁴ in T₁, and Thr²⁵⁹ in T₃. From these findings, together with the observations that (i) the F-actin-, tropomyosin-, and calmodulin-binding domains are all located in the 22-kDa fragment; (ii) the binding of F-actin and tropomyosin to calponin inhibits the phosphorylation of calponin by PKC; (iii) the phosphorylation of calponin by PKC reduces its ability to bind F-actin and tropomyosin; and (iv) the phosphorylation of the 22-kDa fragment also reduces its ability to bind F-actin, we argue that the site of phosphorylation located in the 22-kDa fragment is functionally the most important of all the sites of phosphorylation in calponin.

**Table II**

*Sequence analysis of phosphopeptides generated by tryptic proteolysis of calponin that had been phosphorylated by PKC*

Calponin was exhaustively phosphorylated by PKC and was digested with trypsin, and phosphopeptides were separated by reverse-phase HPLC, as described under "Experimental Procedures." Purified phosphopeptides were subjected to amino acid sequencing.

| Phosphopeptide | Amino acid sequence                      |
|----------------|------------------------------------------|
| T₁ (213-225)  | Gly-Ala-Ser-Gln-Ala-Gly-Met-Thr-Ala-Pro-Gly-Thr-Lys |
| T₂ (175-185)  | Phe-Ala-Ser-Gln-Glu-Met-Thr-Ala-Tyr-Gly-Thr-Arg   |
| T₃ (252-265)  | Gly-Ala-Ser-Gln-Glu-Met-Thr-Ala-Pro-Arg         |

*The number is based on the published sequence (21).
PKC. Further, it was confirmed that Thr\textsuperscript{184} was the phospho-
rylated amino acid in the T\textsubscript{2}. This conclusion is in good
agreement with the findings of House \textit{et al.} (45) based upon their
studies that PKC can recognize primary specificity
determinants on either the COOH- or NH\textsubscript{2}-terminal side of the
phosphorylatable residue. As described under "Results,"
we cannot position the COOH terminus of the 22-kDa frag-
ment. But on the basis of the available information about the
amino-terminal sequence of the 22-kDa fragment, its esti-
mated molecular mass, the sequence of calponin (21), and
protease specificity, it is suggested that only Thr\textsuperscript{184} is included
in the 22-kDa fragment, and the other phosphorylated amino
acids are included in the 12-kDa fragment. Moreover, it is of
interest to note that the three phosphopeptides that were
preferentially phosphorylated by PKC were located within
the repeating motifs of amino acid sequence within the cal-
ponin molecule.

When calponin was phosphorylated by PKC, we observed
that the phosphorylation was inhibited by the presence of
either F-actin or tropomyosin. We evaluated the kinetics of
the actin- and tropomyosin-induced inhibition of the phos-
phorylation of calponin under the condition that most of the
\textsuperscript{32}P was incorporated into the T\textsubscript{2} (Fig. 7). The results revealed
that actin inhibits the phosphorylation in a competitive fash-
ion while tropomyosin inhibits it in an uncompetitive fashion
with respect to calponin. We reported previously (22) that
calmodulin inhibits phosphorylation in a noncompetitive
fashion with respect to calponin. From these results, we
speculate that the site of phosphorylation by PKC located
within the T\textsubscript{2} of calponin are affected by the binding of F-
actin, tropomyosin, and calmodulin to calponin and that the
T\textsubscript{2} may be adjacent to the actin-binding domain, but not to
the calmodulin- and tropomyosin-binding domains, because
of the types of their inhibitory effects on the phosphorylation
of calponin by PKC.

We also found that the phosphorylation of calponin by
PKC alters the capacity of this protein for binding tropomy-
osin, as well as actin. Although phosphorylated calponin
retained its tropomyosin-binding capacity, phosphorylation
caused a decrease in the affinity of calponin for tropomyosin,
as indicated by results obtained using fluorescence techniques.
In contrast to our result, it was shown previously (17) that
the phosphorylation of calponin by PKC does not affect
the interaction of this protein with immobilized tropomyosin.
The discrepancy between our result and the other may be due to
difference in the binding assay, namely, the use of fluores-
cence technique in our study and affinity chromatography
in the other. It has been reported that calponin inhibits the
actin-activated myosin Mg-ATPase of smooth muscle and
that the phosphorylation of this protein by PKC results in
loss of this ability via the dissociation of calponin from actin
(17). Our findings suggest that the inhibitory effect of cal-
ponin on the actin-activated myosin Mg-ATPase in smooth
muscle is due to its ability to bind not only actin but also
tropomyosin, which is reduced when calponin is phosphory-
lated by PKC.

Calponin interacts with F-actin and tropomyosin in a Ca\textsuperscript{2+}-
deependent manner and with calmodulin in a Ca\textsuperscript{2+}-depend-
ent manner (19). In the present study, using binding assays,
we found that F-actin-, tropomyosin-, and calmodulin-binding
domains were all located in the chymotryptic 22-kDa frag-
ment. These findings are in good agreement with other recent
findings (21, 23, 24). Mezgueldi \textit{et al.} (24) reported that NH\textsubscript{2}-
terminial region of residues 52–168 in chymotryptic NH\textsubscript{2}-
terminial 22-kDa fragment contains the major determinants for
F-actin and Ca\textsuperscript{2+}-calmodulin binding. Takahashi and Nadal-
Girard (21) published the sequence of a cDNA clone for
calponin from chicken gizzard and suggested the locations of
the putative actin-, tropomyosin-, and calmodulin-binding
domains of this protein. They suggested that amino acid
residues 129–149 of calponin may be responsible for the
interaction with actin filaments and calmodulin, and that the
tropomyosin-binding structure of calponin may be composed
of several regions, which extend to the amino-terminal half
of the molecule. In another report, Vancompernolle \textit{et al.} (23)
demonstrated that the chymotryptic NH\textsubscript{2}-terminal 22-kDa
fragment of turkey gizzard calponin binds to tropomyosin-

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**Table III**

Sequence analysis of tryptic phosphopeptide T\textsubscript{2} in comparison to the corresponding synthetic peptide

| Cycle No. | Amino acid | Phosphopeptide T\textsubscript{2} | Synthetic peptide |
|-----------|------------|----------------------------------|------------------|
| 1         | F          | 102.6 pmoles                      | 103.9 pmoles     |
| 2         | A          | 86.1 pmoles                       | 106.3 pmoles     |
| 3         | S          | 17.8 pmoles                       | 12.8 pmoles      |
| 4         | Q          | 56.4 pmoles                       | 64.6 pmoles      |
| 5         | Q          | 61.1 pmoles                       | 111.9 pmoles     |
| 6         | G          | 71.7 pmoles                       | 86.8 pmoles      |
| 7         | M          | 44.5 pmoles                       | 40.4 pmoles      |
| 8         | T          | 17.3 pmoles                       | 8.5 pmoles       |
| 9         | A          | 41.0 pmoles                       | 35.4 pmoles      |
| 10        | Y          | 32.8 pmoles                       | 33.0 pmoles      |
| 11        | G          | 29.3 pmoles                       | 35.3 pmoles      |
| 12        | T          | 0.7 pmoles                        | 5.4 pmoles       |
| 13        | R          | 9.8 pmoles                        | 2.9 pmoles       |

\*The repetitive yield of phosphopeptide T\textsubscript{2} and synthetic peptide was 80.1% and 79.7%, respectively.
Sepharose. In addition, Takahashi et al. reported that amino acids residues 185–193 of calponin, which are homologous to the calmodulin-binding domain of troponin modulin, are possible candidates for the site of binding of calmodulin in a Ca²⁺- independent manner. However, our results indicated that the chymotryptic 12-kDa fragment, which contained amino acid residues 185–193 of calponin, did not interact with calponin either in the presence or in the absence of Ca²⁺, as evidenced both by the inability to affect the fluorescence spectra of dansyl-calmodulin and by the inability to bind to immobilized calmodulin on an affinity column.

Finally, it is reported that in vivo calponin is not phosphorylated in contracting arterial smooth muscle (25). By contrast, in tracheal smooth muscle, calponin phosphorylation increased rapidly in response to the contraction of smooth muscle by carbacbol, and remained elevated at steady state (26). In our present study, it is suggested that calponin is phosphorylated by PKC in vitro and that the phosphorylation is inhibited partially in the presence of both actin and tropomyosin. These data suggest that calponin can be phosphorylated by PKC under some physiological conditions. Further investigations are needed to establish the physiological relevance of calponin phosphorylation.

The ability of PKC to phosphorylate calponin and to affect the functional properties of smooth muscle calponin suggests that this enzyme may also regulate certain calponin-mediated functions of smooth muscle. It appears that the site of phosphorylation in the 22-kDa fragment, which contains the F-actin-, tropomyosin-, and calmodulin-binding regions, is functionally the most important of all the sites phosphorylated by PKC.

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