Dephosphorylation of Calponin by Type 2B Protein Phosphatase†

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ABSTRACT: Calponin is a smooth muscle-specific, thin filament-associated protein which has been implicated in the regulation of contraction via its interaction with actin and inhibition of the cross-bridge cycling rate. Calponin is phosphorylated by protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II), primarily at S175, with loss of actin binding and inhibition of the actin-activated myosin MgATPase. We previously isolated calponin phosphatase from chicken gizzard smooth muscle and identified it as a type 2A protein phosphatase [Winder et al. (1992) *Biochem. J. 286,* 197−203]. The methods used to detect phosphatase activity in that study would additionally have detected type 1 and 2C phosphatases, but not type 2B phosphatase (Ca^{2+}/CaM-dependent phosphatase or calcineurin). We have, therefore, examined the expression of type 2B phosphatase in smooth muscle and its ability to dephosphorylate calponin. Western blotting with polyclonal antibodies to the brain enzyme revealed the expression of type 2B phosphatase in chicken gizzard, and immunofluorescence microscopy confirmed the presence of the phosphatase in isolated smooth muscle cells (rabbit and toad stomach). The purified brain phosphatase dephosphorylated calponin (phosphorylated by PKC or CaM kinase II) in a Ca^{2+}/CaM-dependent manner. Dephosphorylation by calcineurin restored actin-binding and actin-activated myosin MgATPase inhibition which had been reduced by PKC-catalyzed phosphorylation. We conclude that calponin dephosphorylation may be catalyzed not only by type 2A phosphatase but also by type 2B phosphatase, raising the possibility that both phosphorylation and dephosphorylation of calponin could be regulated by Ca^{2+}/CaM.

Calponin is a smooth-muscle specific (Gimona et al., 1990; Takahashi & Nadal-Ginard, 1991) actin-, Ca^{2+}/calmodulin (CaM[1])-, and tropomyosin-binding protein (Takahashi et al., 1986, 1988; Vancompernolle et al., 1990; Winder & Walsh, 1990; Childs et al., 1992; Wills et al., 1993; Winder et al., 1993b) which has been implicated in the regulation of smooth muscle contraction since *in vitro* it inhibits the actin-activated MgATPase activity of smooth muscle myosin (the cross-bridge cycling rate) (Abe et al., 1990; Winder & Walsh, 1990; Makuch et al., 1991; Marston, 1991; Winder et al., 1992b) and actin filament movement over immobilized myosin in the *in vitro* motility assay (Shirinsky et al., 1992; Haeberle, 1994). Calponin binds to F-actin or F-actin/tropomyosin with high affinity ($K_d \approx 50$ nM; Winder et al., 1991) and has been shown by confocal immunofluorescence microscopy to colocalize with actin and tropomyosin in single toad stomach smooth muscle cells (Walsh et al., 1993). Calponin-mediated inhibition of the cross-bridge cycling rate results from its interaction with actin and is due to reduction of the V_{max} of the actomyosin ATPase rather than an effect on the affinity of actin for myosin (Nishida et al., 1990; Horiuchi & Chacko, 1991; Miki et al., 1992). The physiological significance of the interactions of calponin with tropomyosin (Childs et al., 1992) and Ca^{2+}/CaM (Wills et al., 1993; Winder et al., 1993b) is unknown. Calponin's potential as a regulator of smooth muscle contraction is supported by its presence in smooth muscle tissues at a molar ratio of 1 calponin:7 actin monomers (Takahashi et al., 1986), i.e., the same as for the troponin complex in striated muscles. Furthermore, purified calponin had an inhibitory effect on contraction of saponin- or β-escin-permeabilized rabbit mesenteric arterial smooth muscle strips (Itoh et al., 1994).

Calponin is phosphorylated *in vitro* by PKC and CaM kinase II (Naka et al., 1990; Winder & Walsh, 1990; Nakamura et al., 1993; Walsh et al., 1993; Winder et al., 1993a) with loss of actin binding and alleviation of inhibition of the actomyosin ATPase (Winder & Walsh, 1990; Winder et al., 1993a). The principal site of phosphorylation is S175 (Winder et al., 1993a). Although calponin phosphorylation has been demonstrated in intact smooth muscle strips in response to stimuli such as carbachol, endothelin-1, and okadaic acid (Rokolya & Moreland, 1993, 1994; Winder et al., 1993a; Carmichael et al., 1994; Gerthoffer & Pohl, 1994), others have reported that calponin is not phosphorylated in intact muscle (Bárány et al., 1991; Gimona et al., 1992; Bárány & Bárány, 1993; Adam et al., 1994). Parker et al. (1994) have recently provided independent supportive evidence for PKC-catalyzed phosphorylation of calponin in intact single cells of the ferret portal vein: in resting cells, calponin was distributed throughout the cytosol, associated with filamentous structures; when stimulated with phenylephrine, which activates PKC via diacylglycerol generation, calponin distribution changed from primarily cytosolic to

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[1] Abbreviations: BSA, bovine serum albumin; CaM, calmodulin; CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; CaN, calcineurin; CaP, calponin; DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PKC, Ca^{2+}- and phospholipid-dependent protein kinase C; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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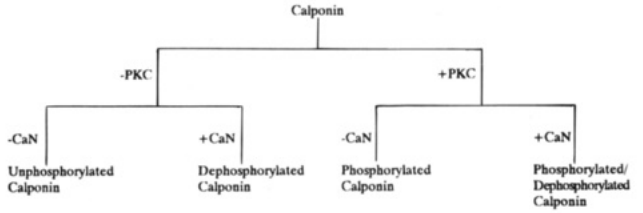

FIGURE 1: Scheme summarizing the phosphorylation and dephosphorylation of calponin with appropriate controls. PKC, protein kinase C; CaN, calcineurin.

primarily surface cortex associated.

If calponin phosphorylation indeed plays a physiological role, there must also be a phosphatase(s) in smooth muscle which dephosphorylate(s) calponin, thereby restoring its binding to actin and inhibition of the actomyosin ATPase. We detected and isolated calponin phosphatase from chicken gizzard smooth muscle and showed it to be a type 2A protein phosphatase (Winder et al., 1992a). Dephosphorylation of calponin by this phosphatase restored actin binding and actomyosin ATPase inhibition. The assay conditions used to detect calponin phosphatase activity would have detected type 1 and 2C phosphatases as well as the type 2A phosphatase. However, the type 2B (Ca^{2+}/CaM-dependent) phosphatase would have escaped detection due to its requirement for Ca^{2+} and calmodulin: assays were carried out in the absence of calmodulin or added Ca^{2+} (Winder et al., 1992a). Type 2B phosphatase, also known as calcineurin, was originally isolated from brain (Wang & Desai, 1977; Klee & Krinks, 1978; Klee et al., 1979; Stewart et al., 1982) and was later identified in heart (Wolf & Hofmann, 1980), skeletal muscle (Stewart et al., 1982, 1983), liver, adipose tissue (Ingebritsen et al., 1983), placenta (Pallen et al., 1985), pancreas (Burnham, 1985), platelets (Tallant & Wallace, 1985), and T lymphocytes (Fruman et al., 1992). Low levels of calcineurin-like proteins were also detected by radioimmunoassay in tongue, adrenal gland, lung, spleen, testis, and thyroid (Wallace et al., 1980). To our knowledge, type 2B phosphatase expression has not been examined in smooth muscle although an analog of calcineurin B (the Ca^{2+}-binding subunit) was detected by Western blot analysis of a crude smooth muscle homogenate (reported in abstract form: Krinks et al., 1985). In this study, we report that (1) type 2B phosphatase is expressed in smooth muscle, (2) this phosphatase dephosphorylates calponin in a Ca^{2+}/CaM-dependent manner, and (3) calponin dephosphorylated by type 2B phosphatase binds to actin and inhibits the actin-activated myosin MgATPase without affecting myosin phosphorylation.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (>5000 Ci/mmol) was purchased from Amersham Corp. CaM was coupled to CNBr-activated Sepharose 4B (Sigma Chemical Co.) as previously described (Walsh et al., 1982). CM-Sephadex, DEAE-Sephacel, PMSF, EGTA, and benzamidine were purchased from Sigma, and DTT was from Boehringer-Mannheim. Leupeptin and pepstatin A were purchased from ICN Biomedicals and imidazole and poly(ethylene glycol) 20 000 from British Drug Houses. Electrophoresis reagents and AG-1-X2 resin were purchased from BioRad Laboratories. General labora-

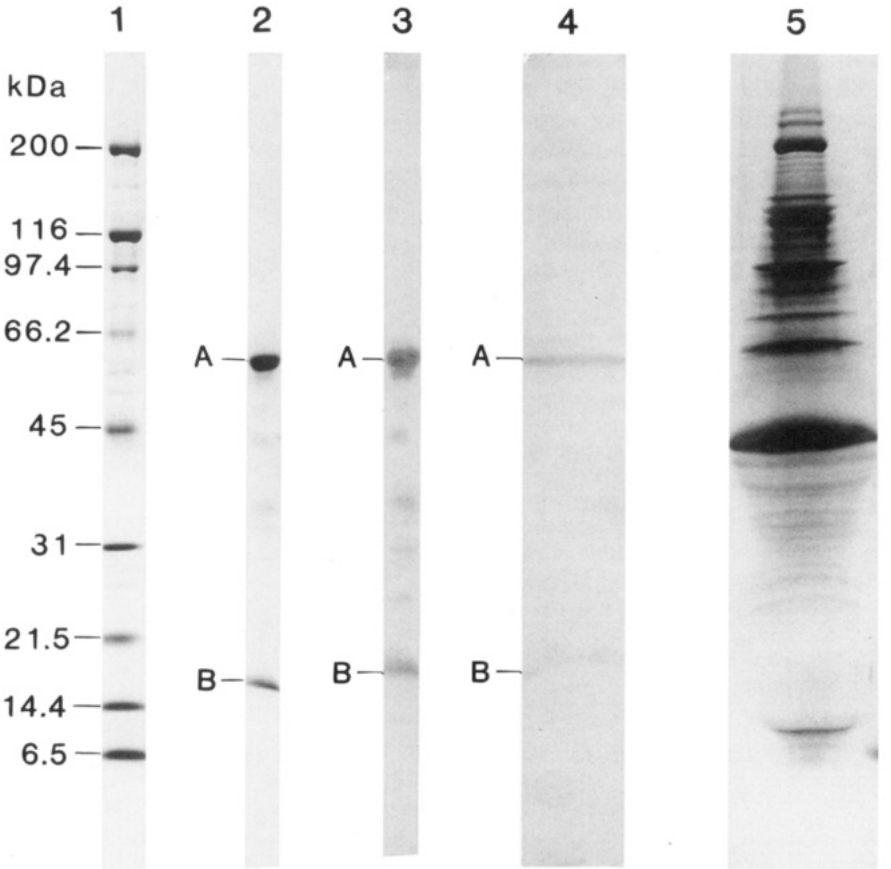

FIGURE 2: Identification of type 2B protein phosphatase (calcineurin) in chicken gizzard smooth muscle. Lanes: 1, molecular mass markers (myosin heavy chain, 200 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa; bovine pancreatic aprotinin, 6.5 kDa); 2 and 3, purified bovine brain calcineurin (2.5 μg/lane); 4 and 5, partially-purified chicken gizzard calcineurin (120 μL/lane). Lanes 1, 2, and 5 are Coomassie Brilliant Blue-stained gels, and lanes 3 and 4 are Western blots with anti-(bovine brain calcineurin).

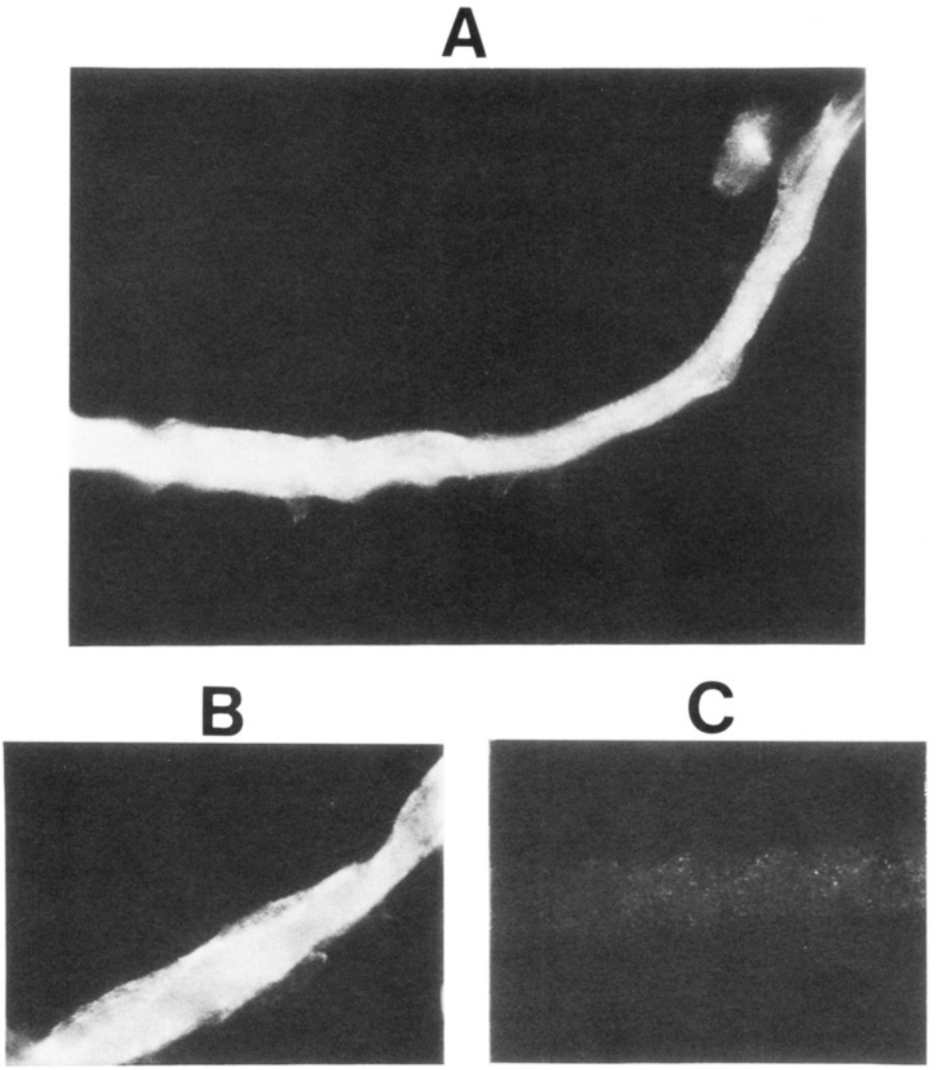

FIGURE 3: Immunofluorescence microscopy showing the presence of calcineurin in rabbit stomach smooth muscle cells. Isolated rabbit stomach smooth muscle cells were fixed and permeabilized as described under Materials and Methods prior to incubation without (C) or with (A and B) anti-calcineurin. Cells were then incubated with rhodamine-labeled secondary antibody and observed with an epifluorescence microscope. Cells in panels B and C were treated and photographed under identical conditions except for omission of the primary antibody from panel C.

tory reagents used were of analytical grade or better and were purchased from CanLab.

Protein Purification. The following proteins were purified by previously-described methods: bovine brain CaM (Walsh et al., 1984), rat brain PKC (Wolf et al., 1985), chicken gizzard calponin (Winder & Walsh, 1990), actin (Ngai et al., 1986), myosin (Persechini & Hartshorne, 1981), tropomyosin (Smillie, 1982), myosin light chain kinase (Ngai et al., 1984), and CaM kinase II (Scott-Woo & Walsh, 1988). Bovine brain calcineurin, purified as described by Sharma et al. (1983), and rabbit anti-(bovine brain calcineurin) were generously provided by Dr. Jerry Wang, University of Calgary. Type 2B protein phosphatase was partially purified from chicken gizzard smooth muscle as follows. Chicken gizzard (50 g) was minced and homogenized for 3 × 30 s in a Waring blender in 4 volumes of 20 mM Tris-HCl (pH 7.5), 2 mM EDTA. The homogenate was centrifuged for 30 min at 15300*g*, the supernatant was filtered through glass wool, and the filtrate was kept on ice. The pellet was resuspended in 2 volumes of homogenization buffer and centrifuged as before. The two supernatants were combined, and DTT and EGTA were added to final concentrations of 1 mM and 0.1 mM, respectively. The sample was applied to a column (1.6 × 20 cm) of DEAE-Sephacel previously equilibrated with buffer A [20 mM Tris-HCl (pH 7.0), 1 mM magnesium acetate, 1 mM imidazole, 1 mM DTT, 0.1 mM EGTA, 0.1 mg/mL PMSF, 0.2 mg/mL benzamidine, 1 mg/L leupeptin, and 1 mg/L pepstatin A] at a flow rate of 30 mL/h. The column was washed overnight with buffer A containing 50 mM NaCl, and bound proteins were eluted with a linear 200-mL [NaCl] gradient (50−300 mM), collecting 2-mL fractions. Phosphatase-containing fractions were identified as follows, using phosphorylated calponin as a substrate. Calponin (5.9 μM) was phosphorylated by incubation at 30 °C for 60 min with 0.5 μg/mL PKC in 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.37 mM L-α-phosphatidyl-L-serine, 0.093 mM 1,2-diolein, 0.03% (w/v) Triton X-100, and 0.2 mM [γ-^{32}P]ATP (152 cpm/pmol) and dialyzed *vs* 20 mM Tris-HCl (pH 7.0). Type 2B phosphatase in column fractions was activated by incubation of individual fractions (20 μL) at 30 °C for 20 min in 27 mM Tris-HCl (pH 7.0), 0.33 mM $MnCl_2$, and 1.0 μM CaM. [Pretreatment of calcineurin with Mn^{2+} (or Ni^{2+}) enhances the activation of calcineurin by Ca^{2+}/calmodulin and may be a physiological mechanism by which calcineurin activity is regulated by Ca^{2+} (Pallen & Wang, 1984).] Phosphorylated calponin (2 μg) was added to the reaction mixture and incubated at 30 °C for 20 min, and the reaction was quenched

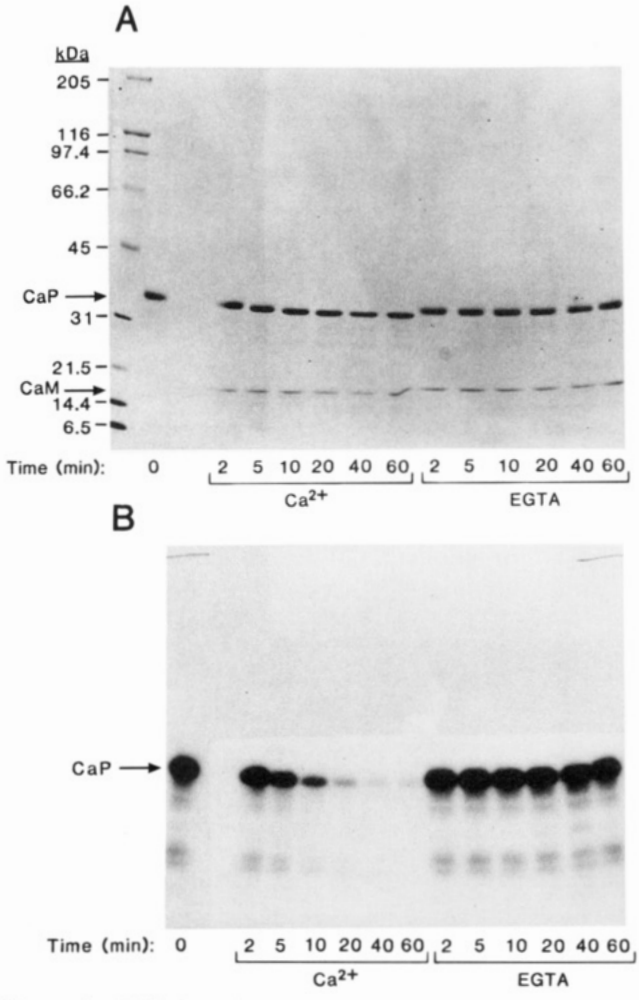

FIGURE 4: Ca^{2+}-dependent dephosphorylation by calcineurin of PKC-phosphorylated calponin. Calponin was phosphorylated by PKC and incubated with activated calcineurin in the absence (EGTA) and presence (Ca^{2+}) of Ca^{2+} as described under Materials and Methods. Samples were withdrawn at the indicated times for SDS−PAGE and autoradiography. (A) Coomassie Blue-stained gel; (B) autoradiogram. In panel A note the Ca^{2+}-dependent electrophoretic mobility shift of CaM.

by addition of SDS−gel sample buffer and boiling. Samples were analyzed for calponin dephosphorylation by mini-SDS−PAGE and autoradiography. Phosphatase-containing fractions were combined, dialyzed *vs* buffer B [20 mM Tris-HCl (pH 7.5), 1 mM magnesium acetate, 1 mM imidazole, 1 mM DTT, and 0.25 mM $CaCl_2$], and applied, at a flow rate of 12 mL/h, to a column (1 × 10 cm) of CaM−Sepharose previously equilibrated with buffer B. The column was washed with 20 mM Tris-HCl (pH 7.5), 1 mM magnesium acetate, 1 mM imidazole, 1 mM DTT, 0.2 M NaCl, 0.01 mM $CaCl_2$, 0.2 mg/mL benzamidine, and 0.1 mg/mL PMSF. Proteins interacting with CaM in a Ca^{2+}-dependent manner were eluted with 20 mM Tris-HCl (pH 7.5), 1 mM magnesium acetate, 1 mM imidazole, 1 mM DTT, 0.5 M NaCl, 0.5 mM EGTA, 0.2 mg/mL benzamidine, and 0.1 mg/mL PMSF, and concentrated from 8 to 3 mL by dialysis *vs* poly(ethylene glycol) 20 000.

Phosphorylation and Dephosphorylation of Calponin. Calponin (5.9 μM) was phosphorylated by PKC (2 μg/mL) at 30 °C for 60 min in 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.37 mM L-α-phosphatidyl-L-serine, 0.093 mM 1,2-diolein, 0.03% (w/v) Triton X-100, 10 μg/mL pepstatin A, 0.5 mM PMSF, 100 μM leupeptin, and 0.2 mM [γ-^{32}P]ATP (≈180 cpm/pmol) in a reaction volume of 0.3 mL. Alternatively, calponin (5.9 μM) was phosphorylated by CaM kinase II (123 μL/mL) at 30 °C for 60 min in 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.2 mM $CaCl_2$, 2.5 μM CaM, 10 μg/mL pepstatin A, 0.5 mM PMSF, 100 μM leupeptin, and 0.2 mM [γ-^{32}P]ATP (≈180 cpm/pmol) in a reaction volume of 0.3 mL. Calponin phosphorylation levels were quantified as previously described (Walsh et al., 1983). Phosphorylated calponin samples were dialyzed *vs* 20 mM Tris-HCl (pH 7.0). Calcineurin (6 μg/mL) was activated by incubation at 30 °C for 20 min in 80 mM Tris-HCl (pH 7.0), 1 mM $MnCl_2$, and 2.5 μM CaM. EGTA (5 mM final concentration) or an equal volume of H_2O was added to [^{32}P]calponin followed by an equal volume of activated calcineurin. Samples (40 μL) were removed at selected times, added to an equal volume of SDS−gel sample buffer, and boiled prior to SDS−PAGE and autoradiography.

Actin-Binding Assay. Calponin (5.9 μM) was incubated under phosphorylating conditions (see above) in the absence of protease inhibitors and in the absence and presence of PKC (2 μg/mL) in a reaction volume of 1.5 mL. Reaction mixtures were then dialyzed *vs* 20 mM Tris-HCl (pH 7.0), 0.1 mM $CaCl_2$, and 1 mM DTT and divided into 2 × 0.7 mL samples. To one sample of each pair was added an equal volume of activated calcineurin; to the other was added an equal volume of buffer [80 mM Tris-HCl (pH 7.0), 1 mM $MnCl_2$, and 2.5 μM CaM] and incubated at 30 °C for 60 min. Reactions were stopped by heating at 85 °C for 2 min, and denatured proteins were removed by centrifugation at 100000*g* for 30 min. Calponin samples (3 μM) were incubated for 1 h at 20 °C with 11 μM actin in 20 mM Tris-HCl (pH 7.5), 0.1 M KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM DTT, and 1 mM ATP in a total volume of 0.18 mL. F-Actin with bound calponin was then sedimented by centrifugation at 100000*g* for 1 h at 4 °C. Separated supernatants and pellets were analyzed by SDS−PAGE and autoradiography.

Actin-Activated Myosin MgATPase. Calponin (11.8 μM) was incubated under phosphorylating conditions (see above) in the absence of protease inhibitors and in the absence and presence of PKC (4 μg/mL) in a reaction volume of 2.2 mL (Figure 1). Reactions were stopped by heating the reaction mixtures at 80 °C for 3 min, and denatured proteins were removed by centrifugation at 100000*g* for 20 min. Supernatants were divided into 2 × 0.95 mL samples. One sample of each pair was treated with activated calcineurin, and the other served as the buffer control, as described above (Figure 1). Samples of reaction mixtures were removed 5, 10, 20, 40, and 60 min after the addition of calcineurin for quantification of protein-bound phosphate (20 μL) and SDS−PAGE and autoradiography (10 μL). The remainder of each reaction mixture was applied at 20 °C to a 1.5-mL column of CM-Sephadex previously equilibrated with 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, and 6 M urea (deionized by prior treatment with AG-1-X2 resin). The columns were washed with equilibration buffer until the radioactivity ([γ-^{32}P]ATP) in the 0.25-mL fractions reached base line. Calponin was then eluted with 20 mM Tris-HCl (pH 7.5), 0.2 M KCl, 1 mM DTT, and 6 M urea. Samples (10 μL) of column fractions were analyzed by SDS−PAGE; calponin-containing fractions were combined and dialyzed *vs* 20 mM Tris-HCl (pH 7.5), 0.1 M KCl, 2.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 1 mM DTT. The protein concentrations of the four calponin samples were determined by coelectrophoresis with known amounts (0.4, 0.8, and 1.2 μg) of BSA

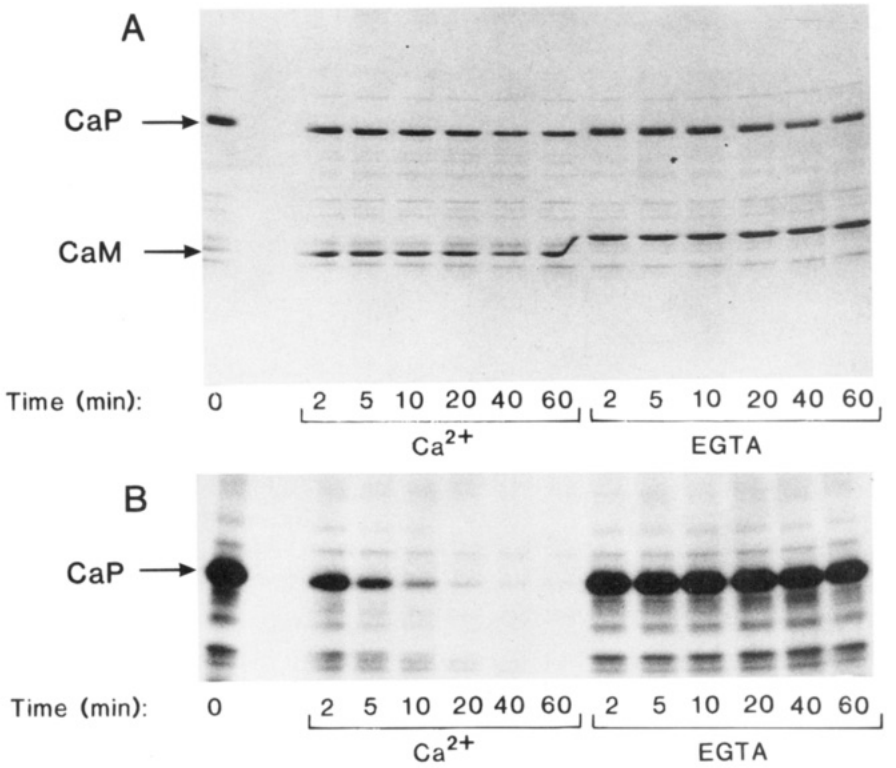

FIGURE 5: Ca^{2+}-dependent dephosphorylation by calcineurin of CaM kinase II-phosphorylated calponin. Calponin was phosphorylated by CaM kinase II and incubated with activated calcineurin in the absence (EGTA) and presence (Ca^{2+}) of Ca^{2+} as described under Materials and Methods. Samples were withdrawn at the indicated times for SDS−PAGE and autoradiography. (A) Coomassie Blue-stained gel; (B) autoradiogram.

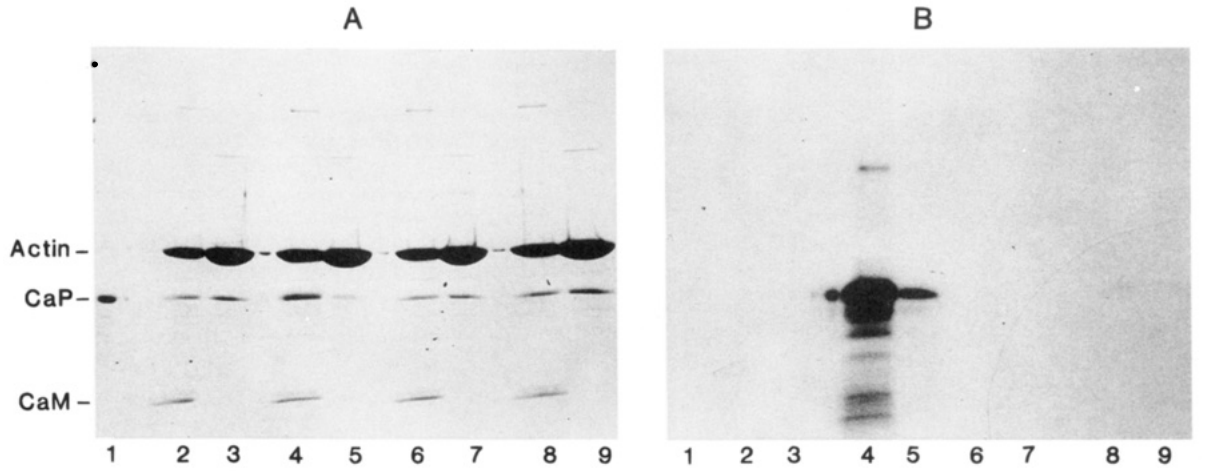

FIGURE 6: Dephosphorylation by calcineurin restores the actin-binding capacity of calponin. Calponin was incubated in the absence and presence of PKC, and samples of both were then incubated in the absence and presence of activated calcineurin as described under Materials and Methods and Figure 1. Actin binding was then assessed by sedimentation analysis. Lanes: 1, calponin marker; 2 and 3, unphosphorylated calponin; 4 and 5, phosphorylated calponin; 6 and 7, dephosphorylated calponin; 8 and 9, phosphorylated/dephosphorylated calponin. Lanes 2, 4, 6, and 8, supernatants; lanes 3, 5, 7, and 9 pellets. (A) Coomassie Blue-stained gel; (B) autoradiogram. The presence of some calponin in the supernatants in lanes 2, 6, and 8 is largely due to the presence of Ca^{2+} and calmodulin which cause partial dissociation of calponin from F-actin (Winder et al., 1993b).

and densitometric scanning of the Coomassie Blue-stained gel; the concentrations varied from 0.15 to 0.21 mg/mL. The effects of the four calponins (unphosphorylated, phosphorylated, dephosphorylated, and phosphorylated/dephosphorylated) on the actin-activated myosin MgATPase were analyzed under the following conditions: 3 μM calponin (where present), 6 μM actin, 2 μM tropomyosin, 1 μM myosin, 0.6 μM CaM, 80 nM myosin light chain kinase, 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 60 mM KCl, 0.1 mM $CaCl_2$, and 1 mM [γ-^{32}P]ATP (8.2 cpm/pmol) at 30 °C in a reaction volume of 1 mL. Samples (0.1 mL) were removed 1, 2, 3, 4, 5, 6, 7, 8, and 9 min after starting the reaction with ATP for quantification of [^{32}P]P_i released (Ikebe & Hartshorne, 1985). SDS−gel sample buffer (0.1 mL) was added to the remainder of each reaction mixture and boiled prior to SDS−PAGE (0.1 mL applied/lane) and autoradiography.

Electrophoresis and Western Blotting. SDS−PAGE, Coomassie Blue staining, and autoradiography were carried out as previously described (Sutherland et al., 1994) using either full-sized 7.5−20% polyacrylamide gradient gels (Figures 2, 4, 5, 6, and 8) or 12.5% polyacrylamide mini gels (Figure 7). Western blotting was carried out as previously described (Sutherland et al., 1994) using rabbit

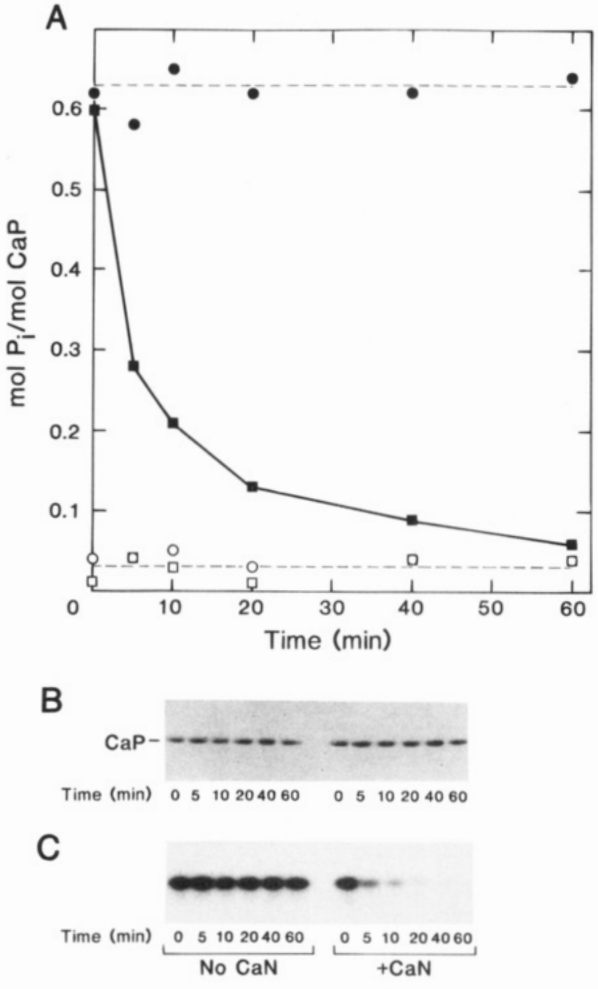

FIGURE 7: Quantification of calponin dephosphorylation by calcineurin. Calponin was incubated in the absence and presence of PKC, and samples of both were then incubated in the absence and presence of activated calcineurin as described under Materials and Methods. (A) Protein-bound phosphate was quantified as previously described (Walsh et al., 1983) at the indicated times following addition of calcineurin or buffer. Symbols: (○) calponin preincubated in the absence of PKC, buffer added at time zero; (●) calponin preincubated in the presence of PKC, buffer added at time zero; (□) calponin preincubated in the absence of PKC, calcineurin added at time zero; (■) calponin preincubated in the presence of PKC, calcineurin added at time zero. (B) Coomassie Blue-stained gel of phosphorylated calponin incubated without or with calcineurin for the indicated lengths of time. (C) Autoradiogram of the gel in (B).

anti-(bovine brain calcineurin) as primary antibody.

Immunofluorescence Microscopy. Rabbit stomach smooth muscle cells were isolated as previously described for uterine cells (Inoue & Sperelakis, 1991). Toad stomach smooth muscle cells were isolated as described by Fay et al. (1982). Cells were fixed with 1% formaldehyde (Sigma) and permeabilized with 1% formaldehyde, 0.1% Triton X-100 as described by Harlow and Lane (1988). Fixed and permeabilized cells were incubated overnight at 4 °C with anti-calcineurin (1:25 dilution) in Hank's balanced salt solution (pH 7.0) containing 0.5% BSA and then for 1 h at 22 °C with secondary antibody [goat anti-rabbit IgG (Sigma) conjugated to tetramethylrhodamine isothiocyanate isomer R diluted 1:100 in Hank's balanced salt solution (pH 7.0) containing 0.5% BSA]. In control experiments, the primary antibody was omitted from the overnight incubation. Wet mounts of cells were prepared by pipetting 6 μL of cells into a drop of 95% glycerol, 5% H_2O on a glass slide. A coverslip was placed over the drop and cemented onto the slide with clear nail polish. An Olympus BHT2 epifluorescence microscope and a Nikon 100× oil immersion objective (NA 1.25) were used for observation of the labeled cells.

RESULTS AND DISCUSSION

Identification of Type 2B Protein Phosphatase in Smooth Muscle. Prior to investigating the dephosphorylation of calponin by calcineurin, it was necessary to determine whether or not the type 2B protein phosphatase is expressed in smooth muscle. CaM-binding proteins in the cytosolic fraction of chicken gizzard smooth muscle were, therefore, partially purified by ion-exchange and CaM affinity chromatography (stained gel shown in Figure 2, lane 5), and the presence of type 2B phosphatase was confirmed by Western blotting (Figure 2, lane 4). A stained gel (Figure 2, lane 2) and Western blot (Figure 2, lane 3) of purified bovine brain calcineurin are shown for comparison. The A subunit (61 kDa) was clearly evident in the chicken gizzard CaM-binding protein preparation; weak immunoreactivity corresponding to the B subunit (19 kDa) was also apparent on the original Western blot but was too weak to obtain a clear photograph. To confirm that type 2B phosphatase is indeed expressed in smooth muscle, immunofluorescence microscopy was used with isolated smooth muscle cells. Figure 3A shows an immunofluorescent image of a single rabbit stomach smooth muscle cell fixed, permeabilized, and treated with anti-calcineurin and a rhodamine-labeled secondary antibody. Immunofluorescence is apparent throughout the sarcoplasm. Panel B shows another cell similarly treated and panel C the corresponding control treated identically but without exposure to the primary antibody. Background fluorescence is clearly very low. Similar results were obtained with toad stomach smooth muscle cells (data not shown). We conclude, therefore, that type 2B phosphatase is expressed in smooth muscle.

Dephosphorylation of Calponin by Type 2B Phosphatase. To determine whether or not phosphorylated calponin is a substrate for type 2B protein phosphatase, calponin was phosphorylated by PKC or CaM kinase II and incubated with purified bovine brain calcineurin in the absence and presence of Ca^{2+}. Calponin dephosphorylation was assessed by SDS–PAGE and autoradiography at selected times following addition of the phosphatase. Calcineurin completely dephosphorylated calponin phosphorylated by PKC (Figure 4) or CaM kinase II (Figure 5) in a Ca^{2+}-dependent manner. From quantitative analyses of time-courses of dephosphorylation of calponin phosphorylated by PKC to a stoichiometry (mean ±SD) of 1.06 ± 0.34 mol of P_i (mol of calponin)$^{-1}$ ($n = 8$), the initial rate of dephosphorylation was determined to be 216 ± 83 nmol of P_i min^{-1} (mg of calcineurin)$^{-1}$ ($n = 4$). For comparison, the specific activity of calcineurin using phosphorylated smooth muscle myosin light chains as substrate was reported to be >400 nmol of P_i min^{-1} (mg of enzyme)$^{-1}$ (Klee et al., 1983). Phosphorylated calponin is, therefore, a good substrate *in vitro* of type 2B phosphatase.

Functional Effects of Calponin Dephosphorylation. Phosphorylation of calponin by PKC or CaM kinase II markedly lowers its affinity for F-actin and thereby alleviates its inhibition of actin-activated myosin MgATPase activity (Winder & Walsh, 1990; Winder et al., 1993a). Dephosphorylation of phosphorylated calponin by calcineurin would

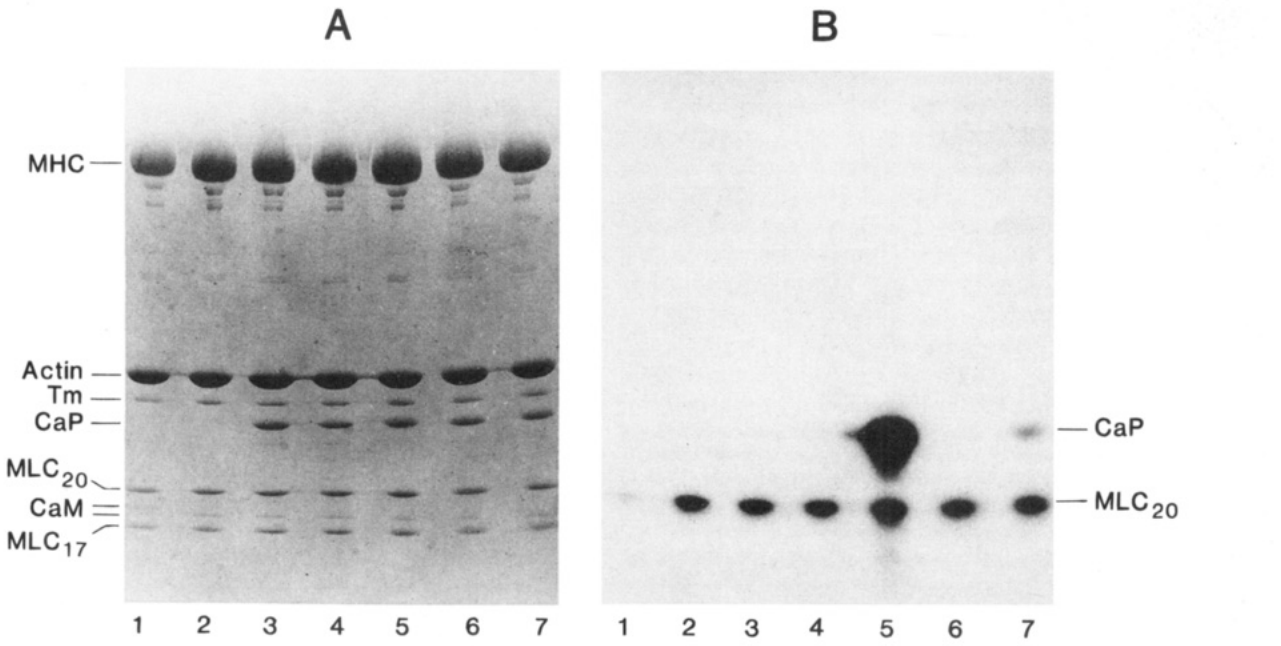

FIGURE 8: Phosphorylated and dephosphorylated forms of calponin have no effect on myosin phosphorylation. Reaction mixtures at the end of the ATPase reactions indicated in Table 1 were subjected to SDS−PAGE and autoradiography. Lanes: 1, control (EGTA, no calponin); 2, control (Ca^{2+}, no calponin); 3, untreated calponin; 4, unphosphorylated calponin; 5, phosphorylated calponin; 6, dephosphorylated calponin; 7, phosphorylated/dephosphorylated calponin. (A) Coomassie Blue-stained gel; (B) autoradiogram. MHC, myosin heavy chain; Tm, the α subunit of tropomyosin (the β subunit comigrates with actin); CaP, calponin; CaM, calmodulin; MLC_{20} and MLC_{17}, the 20 and 17 kDa light chains of myosin.

be expected to restore its capacity to bind to F-actin and inhibit the actomyosin ATPase. Figure 6 demonstrates that high-affinity F-actin binding is indeed restored following dephosphorylation by calcineurin. Scanning densitometry of the gel in Figure 6A indicated the following percentages of calponin bound to F-actin: 64.9% of unphosphorylated calponin; 15.7% of phosphorylated calponin; 52.4% of dephosphorylated calponin; 56.4% of phosphorylated/dephosphorylated calponin. It is also evident from Figure 6B that almost all the phosphorylated calponin was recovered in the supernatant, indicating that most of the 15.7% of the bound fraction of phosphorylated calponin was actually unphosphorylated, reflecting the stoichiometry of phosphorylation of 0.74 ± 0.08 mol of P_i (mol of calponin)$^{-1}$ (n = 7).

To evaluate the effect of dephosphorylation of calponin by calcineurin on its ability to inhibit the actin-activated myosin MgATPase, calponin was first phosphorylated by PKC, in this case to a lower stoichiometry of 0.62 ± 0.02 mol of P_i (mol of calponin)$^{-1}$ (n = 7) (Figure 7A). A control sample incubated in the absence of PKC was not significantly phosphorylated [0.03 ± 0.01 mol of P_i (mol of calponin)$^{-1}$ (n = 12); Figure 7A]. When incubated in the presence of calcineurin, phosphorylated calponin was completely dephosphorylated within 60 min (Figure 7A−C); control phosphorylated calponin incubated in the absence of calcineurin showed no dephosphorylation over the 60-min reaction time (Figure 7A−C). The four calponin species depicted in Figures 1 and 7A were purified by cation-exchange chromatography and their concentrations determined by coelectrophoresis with BSA as described under Materials and Methods. Actin binding was examined as described above (Figure 6) but at 3.5 μM calponin and in the presence of 2 μM tropomyosin. The following percentages of calponin bound to F-actin were determined: 83.9% of unphosphorylated calponin; 67.8% of phosphorylated calponin; 85.6% of dephosphorylated calponin; 83.7% of phosphorylated/dephosphorylated calponin. Finally, the effects of the four calponin species on the actin-activated myosin MgATPase were investigated (Table 1). Basal activity (in the absence of Ca^{2+}) was activated 10-fold by Ca^{2+}. This correlated with the expected phosphorylation of the 20 kDa light chain of myosin (Figure 8B, lanes 1 and 2). The Ca^{2+}-stimulated ATPase was inhibited 77.1% by untreated calponin, 68.1% by unphosphorylated calponin, 42.3% by phosphorylated calponin, 74.6% by dephosphorylated calponin, and 70.9% by phosphorylated/dephosphorylated calponin. None of the calponin species had any effect on myosin light chain phosphorylation (Figure 8). The degree to which phosphorylation of calponin alleviated actomyosin ATPase inhibition was consistent with the level of phosphorylation [0.62 mol of P_i (mol of calponin)$^{-1}$] and the decreased binding to F-actin.

Table 1: Dephosphorylation of Calponin by Calcineurin Alleviates Its Inhibition of the Actin-Activated Myosin MgATPase

calponin	Ca^{2+}	actin-activated myosin MgATPase [nmol of P_i (mg of myosin)$^{-1}$ min^{-1}]
none	+	97.2
none	−[b]	9.8
untreated[a]	+	29.8
unphosphorylated	+	37.7
phosphorylated	+	60.2
dephosphorylated	+	32.0
phosphorylated/dephosphorylated	+	35.2

[a] Calponin purified from chicken gizzard smooth muscle (Winder & Walsh, 1990) without further treatment. [b] 1 mM EGTA replaced 0.1 mM $CaCl_2$ in the ATPase reaction mixture.

We conclude, therefore, that: (1) type 2B protein serine/threonine phosphatase (calcineurin) is expressed in smooth muscle; (2) calcineurin dephosphorylates calponin (phosphorylated by either PKC or CaM kinase II) in a Ca^{2+}/CaM-dependent manner; and (3) dephosphorylation of calponin

by calcineurin restores its ability to bind to F-actin and thereby inhibit the actin-activated myosin MgATPase. These observations raise the possibility that calponin phosphorylation and dephosphorylation may both be regulated by Ca^{2+} and CaM. It would still be possible to regulate the phosphorylation and dephosphorylation of calponin independently since (1) CaM kinase II and calcineurin have different sensitivities to Ca^{2+} and affinities for CaM [the k_{diss} for CaM is ≈0.1 nM for calcineurin and 20−100 nM for CaM kinase II (Klee, 1991)] and (2) CaM kinase II can be rendered Ca^{2+}/CaM-independent by autophosphorylation (Hashimoto et al., 1987).

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