Phosphorylation of Calmodulin Alters Its Potency as an Activator of Target Enzymes

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ABSTRACT: Previous work has shown that calmodulin (CaM) is constitutively phosphorylated in rat liver, probably by casein kinase II [Quadroni, M., James, P., and Carafoli, E. (1994) *J. Biol. Chem.* 269, 16116−16122]. A procedure is now described for the isolation of the phosphorylated forms of calmodulin (PCaM) free from CaM, since in vitro phosphorylation experiments yield a 50:50 mixture of 3−4 times phosphorylated CaM and native CaM. The activation of six target enzymes by PCaM was tested: myosin light chain kinase, 3′,5′-cyclic nucleotide phosphodiesterase, plasma membrane Ca^{2+}-ATPase, Ca^{2+}-CaM-dependent protein phosphatase 2B (calcineurin), neuronal nitric oxide synthase, and CaM-kinase II. In general, the phosphorylation of CaM caused a decrease in enzyme binding affinity, increasing the K_{act} by 2−4-fold for MLCK, PDE, PM Ca^{2+}-ATPase, and calcineurin. The V_{max} at saturating concentrations of PCaM was less affected, with the exception of CaM-kinase II, which was only minimally activated by PCaM and NOS whose V_{max} was increased 2.6 times by PCaM with respect to CaM. Phosphorylation of calmodulin had very little effect on the binding of calcium to the enzyme despite the fact that Ser 101 which is phosphorylated is located in the third calcium binding loop. CD measurements performed on CaM and PCaM indicated that phosphorylation causes a marked decrease in the α-helical content of the protein. Phosphorylated CaM is very prone to dephosphorylation and was thus tested as a substrate for several phosphatases. It was unaffected by calcineurin (PP2B), but was a reasonable substrate for the pleiotropic phosphatases PP1γ and PP2A.

Calmodulin (CaM)[1] is the central protein mediator of the Ca^{2+} signal in the cell. Following the increase in intracellular Ca^{2+}, CaM binds Ca^{2+}, exposes hydrophobic patches on its surface, and binds to a number of enzymes stimulating their activity. Many of the target enzymes are involved in cell signaling cascades; e.g., CaM stimulates certain protein kinases (MLCK, CaM-kinase II, and phosphorylase kinase) and phosphatases (calcineurin), enzymes involved in cyclic nucleotide metabolism (adenylyl cyclase and cAMP phosphodiesterase), and regulators of intracellular Ca^{2+} (plasma membrane Ca^{2+} pump and, indirectly, the SR Ca^{2+} pump) as well as neurotransmission (nitric oxide synthase). Previous in vitro phosphorylation studies have shown that several serine/threonine and tyrosine kinases can phosphorylate CaM (*1*−*3*), and we have shown (*4*) that a fraction of CaM is constitutively phosphorylated in normal rat liver. The consensus sequence around the phosphorylation sites Ser 81, Thr 79, and Ser 101 indicates that casein kinase II (CKII) may be the enzyme responsible. Previous studies by Sacks and co-workers (*5*, *6*), as well as by ourselves, have indicated that crude mixtures of CaM and PCaM were less effective in target enzyme activation than CaM.

The work described in this contribution shows that a "purified" PCaM fraction (i.e., containing no native CaM) displays a reduced ability to activate five of the six CaM targets tested. In contrast, phosphorylation augments the ability of CaM to activate NOS. The magnitude of the change is target-specific: whereas the difference between CaM and PCaM is minor in four of the six enzymes tested,

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[1] Abbreviations: CaM, calmodulin; PCaM, phosphorylated calmodulin; CKII, casein kinase II; MLCK, myosin light chain kinase; PDE, 3′,5′-cyclic nucleotide phosphodiesterase; CaM-kinase II, type II Ca^{2+}-calmodulin-dependent protein kinase; PP1, protein serine/threonine phosphatase 1; PP2Ac, protein serine/threonine phosphatase 2A, catalytic subunit purified from bovine heart; PP2A1, protein serine/threonine phosphatase 2A, holoenzyme purified from rabbit muscle; calcineurin, Ca^{2+}-CaM-dependent protein serine/threonine phosphatase 2B; MBP, myelin basic protein; NOS, neuronal nitric oxide synthase; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); DTT, 1,4-dithio-DL-threitol; EGTA, ethylene glycol bis(aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; TFA, trifluoroacetic acid; Brij 35, poly(ethylene glycol) dodecyl ether; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine 5′-triphosphate; NADPH, nicotinamide adenine dinucleotide phosphate; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; ESI-MS; electrospray ionization mass spectrometry; HPLC-MS, high-pressure liquid chromatography coupled to a mass spectrometer by an electrospray interface; *m*/*z*, mass-to-charge ratio; MH^+, singly protonated species; SR, sarcoplasmic reticulum.

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the activation by PCaM is significantly greater than that by CaM in the case of NOS and lower in the case of CaM-kinase II, which is only marginally activated by PCaM. In the case of CaM-kinase II, the phosphorylation of CaM may play a role in the down-regulation of kinase activity, which may be important when long intracellular Ca^{2+} transients are produced (e.g., during lymphocyte activation). CaM phosphorylation may thus be crucial where pairs of antagonistic enzymes [e.g., kinases/phosphatases; for a review, see (*7*)] exist, specifically affecting only one of the pair.

MATERIALS AND METHODS

Materials

Casein kinase II (CKII) was purified from bovine testis according to (*8*). CaM was purified from bovine brain as described by Guerini et al. (*9*); alternatively, human recombinant CaM was expressed in *Escherichia coli* as described by Rhyner et al. (*10*), and purified by anion exchange and hydrophobic interaction chromatography. Poly-L-lysine (average molecular mass 68 kDa), CaM-depleted bovine PDE, snake venom 5′-nucleotidase, and MBP were purchased from Sigma Chemicals Co. (St. Louis, MO). Chicken gizzard (smooth muscle) MLCK and recombinant myosin light chain (rLC20) were a kind gift of Prof. M. Ikebe (Cleveland, OH). Rat brain CaM-kinase II was a kind gift from Prof. H. Schulman (Stanford, CA). Erythrocyte plasma membrane Ca^{2+}-ATPase was isolated as described by Niggli et al. (*11*, *12*). Calcineurin was isolated as described by Klee et al. (*13*). γ-^{32}P -labeled adenosine triphosphate with a specific radioactivity of 3.7 Mbq/mmol was purchased from Amersham International, Little Chalphout, U.K. Protein phosphatase PP1γ was a kind gift from Dr. Patricia Cohen (Dundee, Scotland), while PP2A1 and PP2Ac were gifts from Dr. Greg Moorehead and Dr. Robert W. MacKintosh, respectively [Dundee, Scotland (*14*)]. CaM-dependent nitric oxide synthase was prepared from porcine brain as described in (*15*). For the NOS measurements, L-[2,3,4,5-^{3}H]arginine (specific activity, 63 Ci/mmol) was purchased from Amersham International. All other reagents were of the highest purity level commercially available. All the remaining synthetic calmodulin binding peptides were synthesized using a 431A Applied Biosystems peptide synthesizer.

Methods

In Vitro Phosphorylation of Calmodulin. CaM was phosphorylated in vitro by incubating it under the following conditions: 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 μM poly-L-lysine, 100 mM NaCl, 1 mM EGTA, 30−50 μg/mL CKII, and 0.1−0.2 mg/mL CaM (6−12 μM). The mixture was incubated at 37 °C with stirring for 18 h. Under these conditions, turbid solutions were observed due to the aggregation of CaM with polylysine; the turbidity could be avoided by adding sodium chloride to a final concentration of 400 mM. PCaM and CaM were purified from the phosphorylation mixture by hydrophobic interaction chromatography on a phenyl-Sepharose column (0.7 × 8 cm) as described for the isolation of CaM from liver (*4*). Two modifications were made: first, the phosphorylation mixture was adjusted to 0.8 M NaCl and 2 mM $CaCl_2$ and stirred for 15 min before loading; second, the column was washed with 15−20 bed volumes of a high-salt buffer (buffer B′: 50 mM Tris, 5 mM $CaCl_2$, 2 M NaCl) to eliminate polylysine bound to CaM. The column was then washed with the same buffer without sodium chloride. Fractions eluted with the EGTA buffer were concentrated by lyophilization, and the buffer was exchanged by gel filtration on a Sephadex G-50 column (1.2 × 80 cm) run in 20 mM ammonium bicarbonate, pH 8.5.

The phosphorylation of CaM in the presence of calmodulin binding domains was carried out as follows: human recombinant CaM (7 μM) was incubated in 50 mM Tris-HCl buffer, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA (or 10 μM $CaCl_2$ when indicated) in the presence of either 0.5 μM polylysine (4 kDa) or equimolar amounts of the various synthetic CaM binding domain analogues (C20W, C24W, CaMKII, CN, PDE, RS20, and NO30). The samples were incubated at 37 °C for 10 min before the addition of 0.5 μg of human recombinant CKII and 0.2 μCi of [γ-^{32}]-ATP and incubation for a further 30 min. The reactions were stopped by heating the samples at 95 °C for 3 min in Laemmli sample loading buffer. Each fraction was loaded onto a 12% SDS−PAGE gel containing 1 mM EGTA in all buffers. The gels were analyzed using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Preparative Nondenaturing Electrophoresis. Desalted and lyophilized PCaM/CaM samples were redissolved in a minimal amount (typically 50−200 μL) of electrophoresis sample buffer (50 mM Tris-HCl, pH 6.8, 1 mM EGTA, 20% glycerol), sonicated for 5 min in a bath sonicator, and spun at 13000*g* in an Eppendorf centrifuge for 3 min. The supernatant was loaded onto several lanes of a preparative 15% polyacrylamide gel (12 cm long, 2 mm thick, pH 8.8) with a 5 cm stacking gel (pH 6.8). All gel solutions as well as the electrode buffer contained 1 mM EGTA to avoid Ca^{2+}-induced band-shifting. Up to 0.5 mg total protein per gel lane could be loaded without affecting the separation. The gels were run at a constant voltage of 90 V for 6 h, until the dye front reached the end of the gel. One lane was cut from the gel and quickly stained in 0.025% Serva Blue G in 10% acetic acid (5−10 min). The measured migration distances were used to cut the two bands out of the rest of the untreated gel.

Protein Recovery after Electrophoresis. Gel slices corresponding to upper (PCaM) and lower (CaM) bands on the nondenaturing gel were cut into small pieces, and the protein was extracted by passive elution in bidistilled water using a 3-fold volume excess of water over gel slice volume at room temperature for 36 h (3 × 12 h). The eluates were concentrated by lyophilization, and the resulting sample was desalted using a Sephadex G-50 gel filtration column.

Determination of the Calcium Binding Affinity. CaM and PCaM purified by native PAGE were precipitated by 3% TCA, pelleted, and redissolved in 50 mM Tris-HCl, pH 7.5, and 150 mM KCl. Calcium binding was measured at 25 °C by flow dialysis as described in (*16*). Protein concentrations used were in the range 18−25 μM. Treatment of the raw data and evaluation of the intrinsic metal binding constants were as described by Cox (*17*).

Mass Spectrometry. Lyophilized CaM or PCaM samples after phosphorylation or preparative electrophoresis were dissolved in 20% (v/v) formic acid and loaded on a reversed phase Poros R2 HPLC column (Perseptive Biosystems,

Framingham, MA). The protein was eluted with a gradient from 0 to 100% buffer B over 20 min (A: 100% water, 0.1% TFA; B: 80% acetonitrile, 0.08% TFA in water) at a flow rate of 20 μL/min. The HPLC system was connected on-line with a Finnigan MAT TSQ700 triple stage quadrupole mass spectrometer (San Jose, CA) equipped with an electrospray source. The protein digestions were separated on a LC-Packings (Zürich, Switzerland) C_{18} column (0.32 × 250 mm) at a flow rate of 3 μL/min, eluting with a linear gradient of 0−60% solvent B over 60 min (solvent A: 0.1% v/v TFA in water; and solvent B: 80% acetonitrile, 0.085% TFA in water) with a coaxial flow of 3 μL/min methoxyethanol as a sheath liquid. The peptides eluting were subject to on-line automated collision-activated dissociation (CAD) tandem quadrupole mass spectrometry (MS/MS) using an instrument control language procedure. Typically, peptides were sequenced using a collision energy of between 10 and 30 eV and 1.8 mtorr argon with a parent ion window of 2−3 mass units (50% half-height) and a daughter ion resolution of 1−2 mass units. Data were analyzed with software provided from the manufacturer.

Circular Dichroism Measurements. CD spectra were recorded using a Dicograph Model CD6 (Jobin Yvons, Paris, France). The measurements were carried out in far-UV jacketed 0.1 mm quartz cells at 15 °C. Spectra were run at a scanning speed of 20 nm/min with a time constant of 1.0 s between 190 and 250 nm. For each sample, four spectra were recorded and then averaged, and corrected by subtraction of the buffer spectrum. Samples of CaM (17.6 μM) and PCaM (30 μM) were prepared in 50 mM Tris-HCl, pH 7.4, containing either 1 mM $CaCl_2$ or 1 mM EGTA. The calculation of the percentages of the various secondary structures was carried with the CONTIN program (*18*, *19*).

Concentration Measurements of CaM/PCaM Stock Solutions. Lyophilized CaM or PCaM samples after native gel electrophoresis were redissolved in 20 mM Hepes, pH 7.4, 20 μM EGTA and subsequently centrifuged for 1 h at 13000*g* to eliminate any high molecular weight aggregates. The concentrations of the two samples were adjusted by adding buffer until the UV absorption spectra intensities (from 240 to 300 nm) were identical. The concentration of the samples used were 0.7 mg/mL. Identical dilutions for each assay were prepared in the appropriate buffer from these stock solutions. For the CD measurements, the concentrations of PCaM and CaM were determined by their molar adsorption coefficient at 276 nm [$\epsilon = 3740\ M^{-1}\ cm^{-1}$; Wolff et al. (*20*)].

Activation of Myosin Light Chain Kinase. Activation of MLCK by CaM was assayed in 30 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 50 mM KCl, 4.44 nM [γ -^{32}P]-ATP (specific activity 3000 Ci/mmol), and 23 nM MLCK. The concentration of CaM ranged from 0 to 0.6 μM. The reaction mixtures were incubated for 15 min at 37 °C, and phosphate incorporation was determined as described below.

Activation of 3′,5′-Cyclic Nucleotide Phosphodiesterase. 3′,5′-Cyclic nucleotide phosphodiesterase (PDE) activity measurements were carried out by a coupled enzyme assay as described by Teo et al. (*21*). The final concentrations and activities of cAMP, 5′-nucleotidase, and PDE in the assay buffer were 0.5 mM, 0.25 milliunit/μL, and 0.02 milliunit/μL (9 nM), respectively. The reaction mixture (50 μL) was incubated for 20 min at 37 °C. The release of inorganic phosphate was measured by the method of Lanzetta (*22*), whereby 1 mL of dye reagent was added to each of the tubes and the absorbance was read at 660 nm.

Activation of Plasma Membrane Calcium Pump. Ca^{2+}-ATPase activity measurements were performed with the coupled enzyme assay described by Niggli et al. (*11*, *12*) using a final Ca^{2+} concentration of 10 μM in the assay medium. The final concentration of the plasma membrane Ca^{2+}-pump in the assay buffer was 16 nM.

Activation of CaM-Kinase II. Type II Ca^{2+}-CaM-dependent protein kinase activity was measured in the presence of the substrate myelin basic protein (MBP). The data presented are based on measurements of total phosphate incorporation, i.e., including kinase autophosphorylation. A set of representative phosphorylation mixtures were also analyzed by gel electrophoresis and radioactivity quantitation on a PhosphorImager scanner (Molecular Dynamics, Sunnyvale, CA). The data obtained for both autophosphorylation and substrate phosphorylation were qualitatively in very good agreement with the measurements of total phosphate incorporation (data not shown), confirming that kinase activation by autophosphorylation was proportional to substrate phosphorylation. The assay conditions were as follows: 50 mM Pipes, pH 7.0, 10 mM $MgCl_2$, 0.2 mM EGTA, 0.3 mM $CaCl_2$, 15 nM kinase, 10 μM MBP, 6 nM [γ -^{32}P]ATP with an incubation time of 30 min at 37 °C. The CaM concentrations ranged from 0 to 18 μM.

Activation of Calcineurin. Protein phosphatase activity was measured by the method of Hubbard and Klee (*23*). The incubation mixture (60 μL) was 40 mM Tris-HCl, pH 8.0, containing 100 mM KCl, 6 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.5 mg/mL bovine serum albumin, 0.5 mM DTT, 30 nM calcineurin, and the concentration of CaM indicated in the legend. After a 5 min incubation at 30 °C, the reaction was started by the addition of the phosphorylated substrate peptide (DLDVPIPGR FDRRVpSVAAE, 400−4000 cpm/pmol) to a final concentration of 1 μM, and the mixture was incubated for 7 min. The reaction was stopped by the addition of 0.5 mL of 5% trichloroacetic acid/0.1 M KH_2PO_4, and the inorganic phosphate released was isolated by chromatography on Dowex AG50W-X8. Initial rates were calculated as described by Klee (*13*).

Activation of Nitric Oxide Synthase. NOS activity was determined by the formation of L-[^{3}H]citrulline from L-[^{3}H]-arginine as previously described (*15*), in a final volume of 140 μL of 50 mM triethanolamine hydrochloride buffer, pH 7.0, containing 0.16 μM L-[^{3}H]arginine (70 000 cpm), 500 μM calcium, 5 μM FMN, 5 μM FAD, 10 μM H_4-biopterin, 200 μM NADPH, and 100 μM L-arginine; 0.35 μg of purified NOS was used per reaction. The reaction was started by the addition of CaM to various final concentrations and stopped after incubation at 37 °C by the addition of 1 mL of chilled 20 mM sodium acetate (pH 5.0) containing 2 mM EDTA. Citrulline was then separated from arginine by cation exchange chromatography (Dowex 50W, 8% cross-linked, 200−400 mesh, H^+ form). The amount of [^{3}H]citrulline produced was determined by liquid scintillation counting (values were corrected by subtraction of the reference sample from which CaM had been omitted).

Phosphate Incorporation Measurements. All phosphate incorporation measurements were performed as described in (*24*). Scintillation counting of radioactivity bound to the

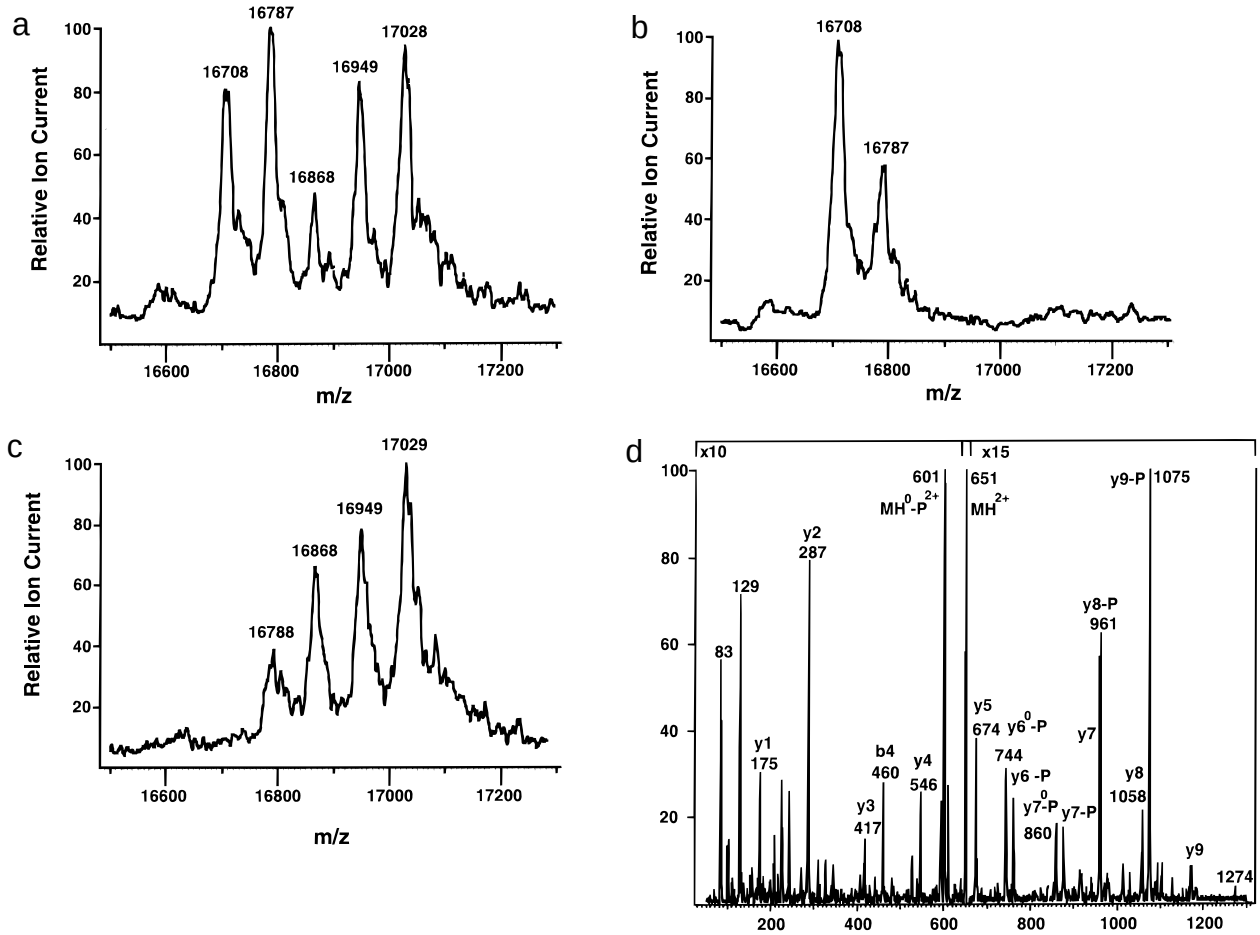

FIGURE 1: Molecular mass measurements of crude phosphorylation mixtures and fractions separated by nondenaturing gel electrophoresis. Deconvoluted electrospray ionization mass spectra of (a) the total calmodulin fraction recovered after phosphorylation by CKII and purification by hydrophobic interaction chromatography, (b) the fraction obtained by passive elution in water from the lower band of nondenaturing polyacrylamide gels (see Figure 2), and (c) the fraction recovered from the upper gel band. The molecular mass expected for recombinant CaM on the basis of the amino acid sequence is 16 708 Da. The addition of one phosphate group causes an average mass increase of 79.97 amu. So the expected masses of multiply phosphorylated CaM, 16 788, 16 868, 16 948, and 17 028 Da for mono-, di-, tri-, and tetraphosphocalmodulin, respectively, are in good agreement with the values found. The site of phosphorylation of the monophosphorylated CaM species running in the lower band on the gel was determined by on-line HPLC-MS/MS. The PCaM was digested first with cyanogen bromide and then with trypsin, and the digest was separated by reversed phase HPLC coupled to the mass spectrometer. The phosphopeptide (assigned by weight only) was subjected to collisionally activated dissociation using 2.5 mtorr of argon and a collision energy of 20 eV (d). The spectrum was recorded by selecting the $MH2^+$ parent ion (mass 651.6, peak 3−4 mass units wide at half-height) and scanning from 50 to 2000 mass units in 4 s (daughter resolution 2−3 mass units at half-height) in the centroid mode. The spectrum is labeled to show the most important sequence ions. The superscript 0 indicated the loss of water from an ion, -P the loss of PO_3 and 2+ that the ion is doubly charged (all others are singly charged). Areas blown up by magnification factors are indicated above the spectrum by × Factor. Ions are named according to Roepstorff and Fohlman (*42*).

filter paper was carried out using 2 mL of scintillation cocktail with a Beckman (Palo Alto, CA) LS 1801 scintillation counter. Alternatively, the incorporation of phosphate into calmodulin in the presence of peptide or polylysine was carried out by densitometry of the dried gels using a Molecular Dynamics Phosphorimager and the manufacturers software.

Dephosphorylation of PCaM by Protein Ser/Thr Phosphatases. Solutions of purified PCaM (0.2 mg/mL) were incubated for 30 min at 30 °C with 50 milliunits/mL PP1γ, PP2Ac, or PP2A1 in the following buffer: 50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1% β-mercaptoethanol, and 0.03% Brij 35. The reaction was stopped by addition of the phosphatase inhibitor microcystin to a final concentration of 1 μM. The dephosphorylation of PCaM by calcineurin was assayed in the same manner as described for the peptide substrate used for activity measurements. The reaction mixtures were then analyzed by nondenaturing gel electrophoresis, and the Coomassie Blue-stained protein bands were quantitated on a Molecular Dynamics laser scanning densitometer.

RESULTS

To obtain a conclusive assesment of the effects of phosphorylation on the functional properties of CaM, it was necessary to produce a sample of "pure" PCaM (i.e., containing no native CaM) phosphorylated at the same sites found in vivo. It was previously shown that CKII can phosphorylate purified CaM to very high phosphorylation stoichiometries (*4*) and that the main phosphorylation sites are identical to those found in vivo. Therefore, CKII was used to extensively phosphorylate CaM, and a procedure was developed to separate the phosphorylated species from native CaM.

In Vitro Phosphorylation and Separation of CaM from PCaM. CaM was phosphorylated as described previously

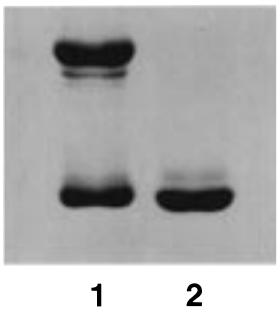

FIGURE 2: Analysis of phosphocalmodulin samples by nondenaturing gel electrophoresis. Purified and desalted CaM samples were analyzed by nondenaturing gel electrophoresis on an SDS-free 15% polyacrylamide gel. The figure shows a Coomassie Brilliant Blue-stained gel with the total CaM fraction after phosphorylation in lane 1 and standard recombinant CaM in lane 2. The molecular masses of the proteins eluted from the upper and lower bands are shown in Figure 1.

(see Materials and Methods) using bovine testis CKII. Both bovine and recombinant CaM were used since control experiments showed that there was no difference between the proteins from different sources in the activity assays. A mass spectrum of a typical CaM sample after extensive phosphorylation is shown in Figure 1a. While it was possible to change the total stoichiometry of phosphate incorporation by varying the enzyme:substrate ratio and the incubation time, it proved impossible to direct the reaction to produce one predominant phosphorylated species. After purification by hydrophobic affinity chromatography and gel filtration, the purified CaM fraction was analyzed by nondenaturing polyacrylamide gel electrophoresis. The phosphorylation mixture gave rise to two well resolved bands on analytical as well as on preparative 15% polyacrylamide gels (Figure 2, lane 1), the lower band running at the same position as native CaM (lane 2). The electrophoretic properties of the two CaM fractions were conserved and reproducible; e.g., proteins purified either from the upper or from the lower bands and re-run on the same gel system retained their characteristic electrophoretic mobilities and gave rise to one single homogeneous band. All electrophoresis experiments were carried out in the presence of 1 mM EGTA in the gel and in all buffers, to ensure removal of free Ca^{2+} and to avoid Ca^{2+}-induced changes in the mobility of calmodulin.

Mass spectrometric analysis of proteins recovered from the lower ($R_f = 0.677$) and upper ($R_f = 0.49$) bands of native gels is shown in Figure 1. As is evident from Figure 1b, native CaM ($MH^+ = 17\,608$) was only detectable in the fraction extracted from lower gel bands, while CaM species carrying two ($MH^+ = 16\,868$) or more ($MH^+ = 16\,948$ and 17 028 for tri- and tetraphosphocalmodulin, respectively) phosphate groups were exclusively migrating with the upper band (Figure 1c). Monophosphocalmodulin ($MH^+ = 16\,787$), on the other hand, appeared to be equally distributed between the slow and the fast migrating pools. However, the monophosphorylated species that migrated with the lower band (Figure 1b) may carry the phosphate in a different position than the monophosphocalmodulin migrating in the upper gel band. Mass spectrometric analysis of the lower band showed that only Ser 81 was phosphorylated since phosphopeptides corresponding to the other potential phosphorylation sites were not detected. Unfortunately, it was impossible to isolate the singly phosphorylated CaM species that migrated in the upper band position to determine the site of phosphorylation.

Thanks to the mild procedure that did not expose the protein to denaturants, organic solvents, or ampholytes, CaM and PCaM were recovered after the desalting step as clean and active fractions. Circular dichroism measurements showed that there was a slight loss of secondary structure after gel electrophoresis and extraction. The difference was the same between native bovine CaM and native recombinant CaM and can thus be regarded as negligible. There were no signs of degradation, oxidation, or other modifications, as assessed by electrospray mass spectrometric analysis of the intact protein (Figure 1) or trypsin digests of it (data not shown).

Activation of Casein Kinase Phosphorylation of CaM by CaM Binding Domain Peptide Analogues. Casein kinase II requires a polybasic activator such as spermine or polylysine to express activity. However, we noticed that a synthetic peptide corresponding to the calmodulin binding domain of the plasma membrane calcium pump can substitute for polylysine. We compared the efficiency of CKII phosphorylation of CaM using polylysine (4 kDa) and the peptide C28Y (Figure 3, top panel) since they are roughly equivalent in molecular mass. Both supported the phosphorylation of CaM but probably in different ways. Quantitation of the autoradiograms (Figure 3, middle panel) showed that the activation of CKII CaM phosphorylation by polylysine is concentration-independent while C28Y correlates well with the concentration of CaM, suggesting that the peptide binds to CaM and alters its conformation, allowing CKII to phosphorylate it without the need for a polybasic activator.

The phosphorylation of CaM by CKII in the presence of CaM binding domains from various proteins is shown in Figure 3 (lower panel). In all cases, the phosphorylation is more efficient in the absence of calcium as previously described in the literature (*1*). The rate of phosphate incorporation varies according to the peptide used in the incubation and follows the order C24W > CaMKII > NO30 > C20W > PDE > CN > RS20 > PolyK. The intensity of the autophosphorylated band corresponding to the β subunit of CKII appears independent of the peptide used.

Calcium Binding Affinity. Flow dialysis experiments carried out in triplicate on CaM and PCaM showed that the two proteins display very similar calcium binding properties (Figure 4). An analysis of the isotherms by the Adair equation yielded the intrinsic constants presented in Table 1. Both proteins possess three high-affinity calcium binding sites displaying slight positive cooperativity and one site with a 10-fold lower affinity. PCaM displays a slightly ($\times 1.4$) lower affinity for the three high-affinity sites than CaM. Duplicate flow dialysis measurements using CaM purified from a phenyl-Sepharose column yielded the same results as CaM purified by native PAGE electrophoresis.

Secondary Structure of Calmodulin and Phosphocalmodulin. The CD spectra of CaM and PCaM showed a marked difference in the amount of α-helical and β-sheet content in the presence (Figure 5) and in the absence of calcium (data not shown). Both CaM and PCaM showed the usual increase in the amount of α-helix upon the addition of calcium (data not shown). However, both in the presence and in the absence of calcium, PCaM showed a decreased α-helical content with respect to the nonphosphorylated

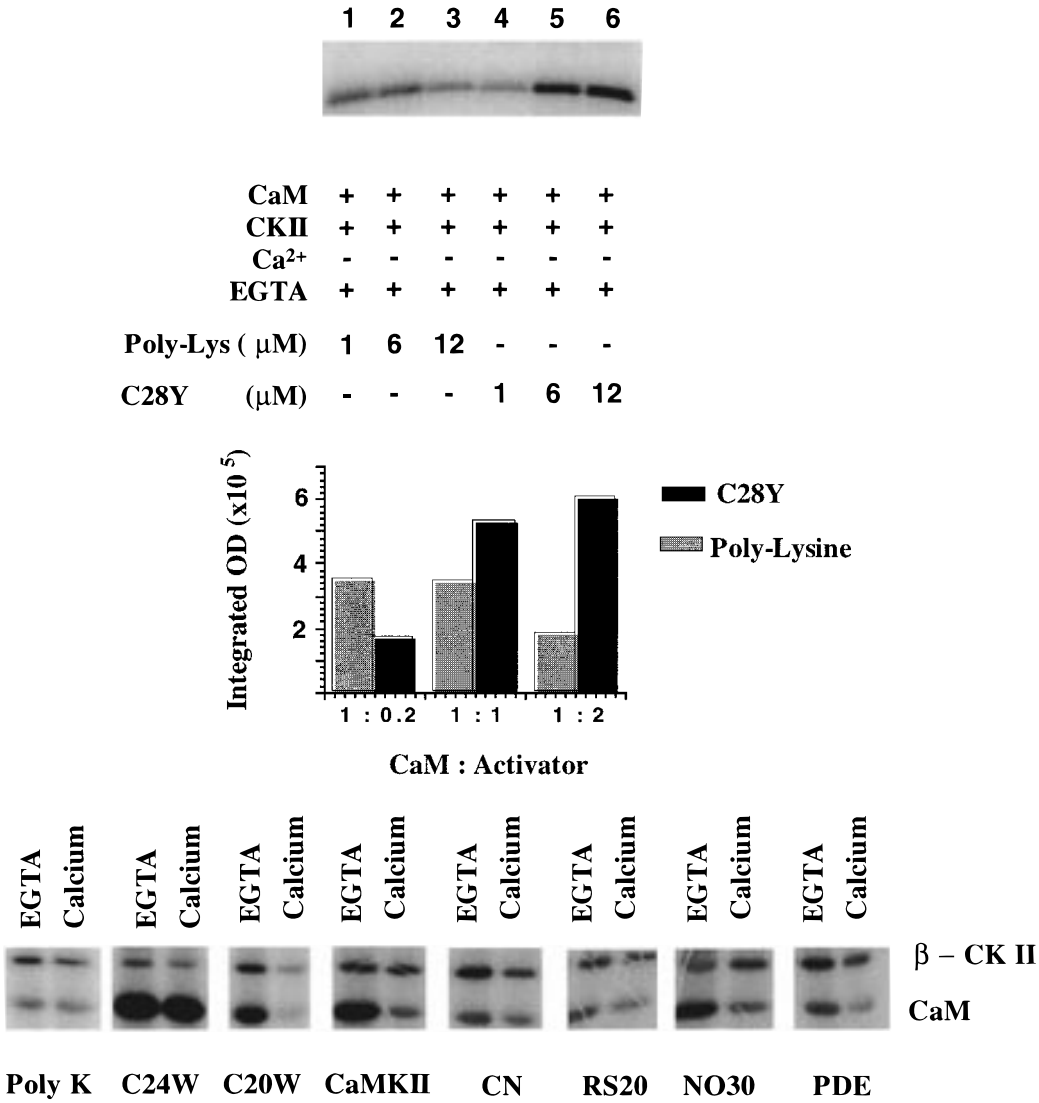

FIGURE 3: Activation of casein kinase II CaM phosphorylation by CaM binding domain peptide analogues. The upper panel shows an autoradiogram of CaM phosphorylated under various conditions. The concentration of CaM was 6 μM, and the amounts of poly-L-lysine (average molecular mass 4 kDa) and the CaM binding peptide C28Y were varied as shown. The middle panel shows the integrated optical densities of the bands determined using a phosphorimager. The lower panel shows the results obtained using different synthetic CaM binding domains to activate casein kinase II in the presence or absence of calcium. Poly K, polylysine; C24W, plasma membrane calcium pump; C20W, plasma membrane calcium pump; CaMKII, calcium-dependent type II protein kinase; CN, calcineurin; RS20, myosin light chain kinase; NO30, nitric oxide synthase; PDE, phosphodiesterase.

protein. These results suggest that the presence of the phosphate groups destabilizes the secondary structure, lowering the percentage of α-helix.

Enzyme Assays. The enzymes selected for testing the activatory properties of PCaM were chosen from various classes of CaM targets: MLCK, calcineurin, and CaM-kinase II are representative of the role of CaM in regulation of protein phosphorylation; PDE is an example of its ability to modulate cyclic nucleotide metabolism, while the plasma membrane Ca^{2+}-ATPase is one of the enzymes whose activation by CaM terminates the intracellular Ca^{2+} signal. NOS is unique in providing a point for cross-talk between the two intracellular messengers, calcium and NO. The PCaM used for the assays was isolated from the top band of the native gels. Native CaM which had been treated in exactly the same way as the PCaM but without incubation with the kinase was used as the control in all of the enzyme assays. The activities of the CaM samples isolated from either source were found to be identical to that of pure native CaM.

As is evident from Figure 6a−e, phosphorylation generally resulted in a diminished ability of CaM to activate targets. In four cases (MLCK, PDE, calcineurin, and the Ca-ATPase), a 3−5-fold increase in the amount of CaM required for half-maximal stimulation (referred to as K_{act}, the amount of protein required for half-maximal activation) was observed, while the effect on the V_{max} of the enzymes was relatively small if not absent. One should mention here that calcineurin could not dephosphorylate PCaM under the assay conditions used. In one case, however, the observed effect was much more pronounced: CaM-kinase II could be stimulated by PCaM only up to 22% of the V_{max} obtained with CaM. The K_{act} of CaM-kinase II for PCaM was also increased by a factor of 3, in parallel to what was observed for the other enzymes (Table 2). The results with NOS, however, were completly different. Although the K_{act} was only slightly

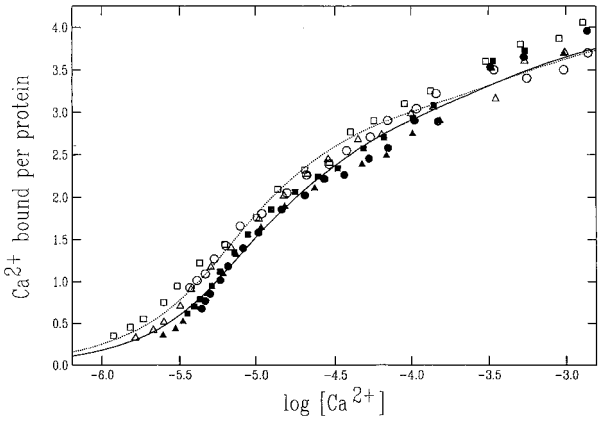

FIGURE 4: Calcium binding to phosphorylated and nonphosphorylated CaM. Calcium binding was measured by flow dialysis at 25 °C to CaM (open symbols) and PCaM (closed symbols) in triplicate (each experiment is shown by a different symbol) in 50 mM Tris, pH 7.5, 150 mM KCl. The dotted lines indicate the theoretical curves generated using the Adair equation and the constants listed in Table 1 [after conversion to stoichiometric constants as described in (*17*).

Table 1: Calcium Affinities of the Various Binding Sites on Calmodulin and Phosphocalmodulin

	CaM	PCaM
K'_1	6×10^4	4×10^4
K'_2	1×10^5	1×10^5
K'_3	1×10^5	5×10^4
K'_4	7×10^3	7×10^3
average K'_{1-3}[a]	8.4×10^4	5.8×10^4

[a] $K'_{1-3} = (K'_1 \times K'_2 \times K'_3)^{1/3}$.

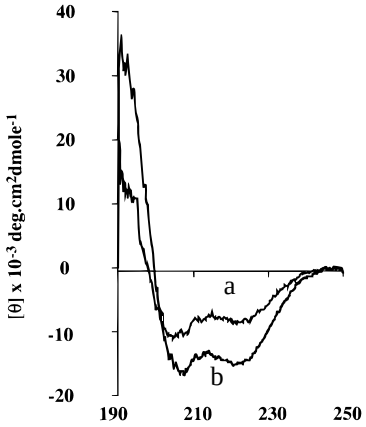

FIGURE 5: CD spectra of PCaM and CaM. The CD spectra of (a) PCaM and (b) CaM in 50 mM Tris-HCl, pH 7.4, containing 1 mM $CaCl_2$ are shown. For each sample, three spectra were recorded and averaged, and the spectrum of the buffer alone was subtracted.

increased (K_{act} for CaM is 1 nM and for PCaM is 2 nM), the V_{max} was increased by a factor of 2.6. This is the only reported case where the Ser/Thr phosphorylation of PCaM produced an increase in the activity of a target protein. Most K_{act} and V_{max} values found for the control CaM samples were in good agreement with those from the literature. Only the absolute values for the K_{act} for PDE and CaM-kinase II were higher than those reported in other studies, but also in these two cases the differences between CaM and PCaM were consistent with the rest of the assays.

CaM Dephosphorylation. One of the reasons for the difficulty in detecting PCaM in vivo is its lability to dephosphorylation (*4*). We therefore systematically tested the sensitivity of PCaM to various protein phosphatases (PP1, PP2A, and PP2B). PCaM samples were incubated with calcineurin under standard assay conditions, and the protein was analyzed by nondenaturing gel electrophoresis and electrospray mass spectrometry (data not shown). It was found that calcineurin was unable to dephosphorylate PCaM, so that one could rule out any direct involvement of calcineurin in the termination of the CaM phosphorylation event. PCaM was a better substrate (although not a very good one) for the pleiotropic phosphatases PP1 and 2A (Figure 7): optical densitometry of Coomassie Blue-stained gels revealed that the amount of PCaM converted to the monophosphorylated or the native state, and thus migrating with the lower band, corresponded to about 10%, 12%, and 15% of the total amount of protein for PP1γ, PP2Ac, and PP2A1, respectively. Since the differences lie within the experimental error, it cannot be concluded that PP2A was more active toward PCaM that PP1. Thus, PCaM dephosphorylation under these conditions was low, corresponding to around 2% of the activity displayed by these enzymes toward model phosphopeptides. In all cases, the dephosphorylation reaction was unaffected by the prescence or absence of calcium or of the CaM binding peptides.

DISCUSSION

The transduction of the Ca^{2+} signal by CaM as a point of cross-talk with other second messenger systems has emerged in the past few years, when CaM was shown to be a good in vitro substrate for several kinases (*1*) such as casein kinase II and the tyrosine kinases *fgr* and *fyn* as well as the epidermal growth factor and insulin receptors (*2*, *3*). PCaM has subsequently been isolated in vivo and characterized (*4*), and activity measurements have been performed with crude mixtures containing native CaM together with the various phosphorylated forms (*4*, *5*). In this work, we have studied the activation of several target enzymes by a defined species of purified PCaM.

Some of the discrepancies between the work presented here and previous work are in all likelihood due to the different isoforms of the target enzyme used (for example, using smooth muscle MLCK from chicken gizzard, the K_{act} is lowered slightly and the V_{max} remains unchanged while when platelet MLCK is used the V_{max} is greatly decreased, to less than 24% residual activity). At variance with Sacks et al. (*6*), who observed an unchanged V_{max}, we have observed an increase in the K_{act} of CaM-kinase II for PCaM and a drastic drop in V_{max} to 22%. This may reflect the use of an unpurified mixture of PCaM/CaM by Sacks et al. since the authors report that their PCaM samples contained up to 15% CaM. Furthermore, mass spectrometric analysis of PCaM samples which have the same phosphate incorporation stoichiometry as Sacks et al. (2 mol of phosphate/mol of CaM) shows that the mixture consists of CaM and 4× phosphorylated CaM in an approximate ratio of 1:1. The factor(s) responsible for this stoichiometry is (are) not known, although it could be speculated that CaM becomes inaccessible to the kinase by forming complexes with either PCaM or the poly-L-lysine polymer. The role of the latter in the reaction and in CKII activation remains obscure. CKII is a heterodimer consisting of two regulatory β and two catalytic α subunits. The presence of the β subunit is necessary for

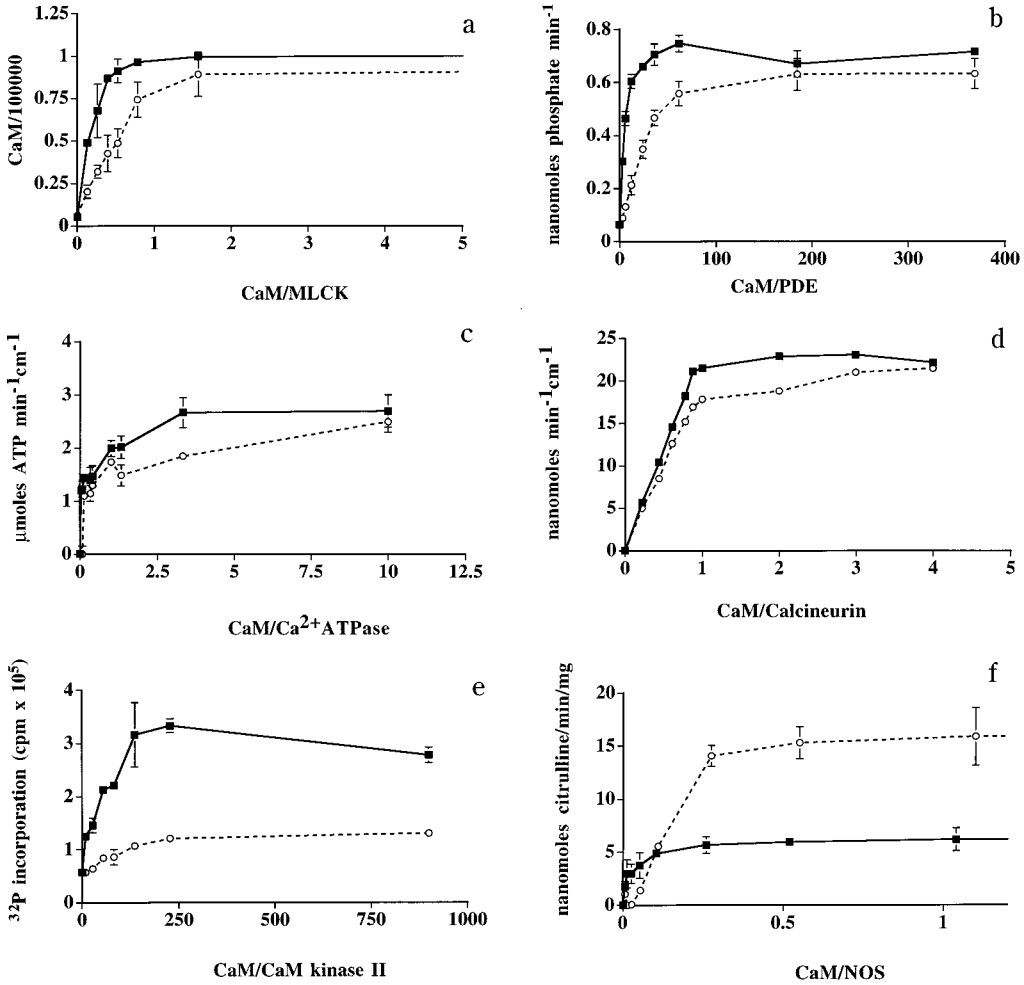

FIGURE 6: Activation of CaM-dependent enzymes by CaM and PCaM. The ability of purified CaM (filled squares) and purified PCaM (open circles) to activate various target enzymes was tested in vitro. Calmodulin titration experiments were performed on (a) myosin light chain kinase, (b) 3′,5′-cyclic nucleotide phosphodiesterase, (c) the plasma membrane Ca^{2+}-pump, (d) calcineurin, (e) type II Ca^{2+}-CaM-dependent protein kinase, and (f) the neuronal nitric oxide synthase. Protein substrates used for the kinases as well as all details of the assays are described under Materials and Methods. CaM/enzyme ratios are expressed as molar ratio of calmodulin over the catalytic subunit of the enzyme. All values are averages of three or more measurements. Standard error bars smaller than the symbols are not shown.

Table 2: Effect of Calmodulin Phosphorylation on the Activation of Target Enzymes[a]

enzyme	K_{act}(CaM) (nM)	K_{act}(PCaM) (nM)	V_{max}(CaM)	V_{max}(PCaM)
calcineurin	0.09	0.53	100	100
CaM-kinase II	375	1050	100	22
MLCK	3.1	11.9	100	98.8
NO synthase	1	2	100	260
PDE	45	225	100	93
PM ATPase	10	30	100	85

[a] The K_{act} is the CaM or PCaM concentration required for half-maximal activation of the target enzymes. V_{max} for PCaM is expressed as a percentage of the V_{max} (=100%) found for CaM. PDE, phosphodiesterase; MLCK, myosin light chain kinase; PM, plasma membrane.

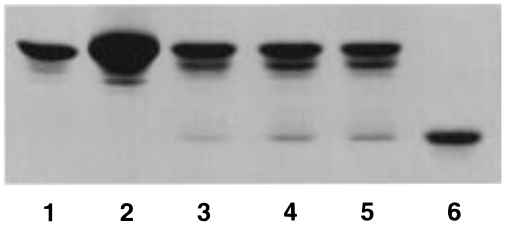

FIGURE 7: Analysis of phosphocalmodulin dephosphorylation. The figure shows the analysis by native gel electrophoresis using the same gel system of samples of purified PCaM (upper gel band) treated with protein phosphatases: lane 1, sample treated with calcineurin (PP2B); lane 2, purified PCaM (lane was overloaded to detect any weak band) before any phosphatase treatment; lane 3, sample treated with PP1γ; lane 4, sample treated with PP2Ac; lane 5, sample treated with PP2A1; lane 6, control, native CaM. The appearance of the lower band suggests partial or complete dephosphorylation of a fraction of the sample. The minor band visible below the upper band on the gel was not observed on all gels and could not be unambiguously correlated with any phosphorylated form of CaM.

the stabilization of the tetramer and increases the activity of the catalytic subunit toward most of its substrates, with the exception of CaM (*25*). In the case of CaM, the β subunit acts as an inhibitor of the phosphorylation reaction, and polybasic compounds such as polylysine or spermine are required for activation (*1*). An acidic region in the β subunit of CKII located between residues 55 and 64 has been shown to be responsible for the inhibition of CaM phosphorylation, and release of inhibition by polybasic compounds is probably due to binding at this site (*26*). A similar activation is obtained using a peptide corresponding to the heparin binding domain of the α subunit (*27*). The observation that synthetic peptides corresponding to the CaM binding domains of several enzymes can effectively substitute for polylysine as in vitro activators of CaM phosphorylation by CKII suggests

that in vivo a basic domain of a third protein may be activating CKII. This finding may be relevant to the problem of a natural modulator of the activity of CKII, whose absence has been a paradox to the protein kinase field.

The magnitude of the change in activation by PCaM with respect to CaM is strongly enzyme-dependent, possibly because of the great differences in CaM-target binding affinities and/or the variety of possible binding modes. Contrary to the view of phosphorylation as a means to invariably lower CaM activity, we have shown that NOS is significantly more activated by PCaM than by CaM. The binding of CaM to the neuronal NOS has been shown to modulate the transfer of electrons from NADPH through the flavins to the heme (*28*, *29*). The higher activity of NOS in the presence of PCaM could reflect a change in the orientation and the distance between the flavin and heme groups caused by the modified binding of PCaM in comparison to CaM, producing a higher rate of electron transfer. In fact, a duality of the effects of phosphorylation (inhibition/activation) had been previously observed with CaM phosphorylated on tyrosine residues by the insulin receptor kinase, which lowers the activation of MLCK but increases the activation of CaM-kinase II (*6*). Since it proved impossible to separate the various phosphorylated forms of CaM, it could not be established which phosphorylation sites were responsible for the changes in the properties of CaM. However, since the mixture of PCaM and CaM in the lower band of the nondenaturing gels had properties identical to calmodulin, one can conclude that phosphorylation at Ser 81 does not cause any change in the activity of CaM toward any of its targets.

Phosphorylation of Ser 101 in the third Ca^{2+} binding loop would have been thought likely to change the overall Ca^{2+} binding affinity of CaM since this loop forms one of the two high-affinity Ca^{2+} sites in the C-terminal half of CaM which are responsible for the conformational changes which expose hydrophobic patches for target binding. Although the oxygen of the side chain of Ser 101 is not directly involved in Ca^{2+} binding, the introduction of a bulky charged group in the loop may distort its structure so as to lower Ca^{2+} binding affinity. The affinity measurements show that this is not the case; i.e., the affinity remains almost unchanged.

Phosphorylation of Ser 81 and Thr 79 may influence the overall shape of CaM in solution by modifying the structure of the central linker region. The central helix of CaM has been a subject for discussion since the first crystal structure was published (*30*) since isolated α-helices are known to be only marginally stable when completely exposed to the solvent environment. Later studies by nuclear magnetic resonance and small-angle X-ray scattering showed that in fact the two lobes of CaM can tumble relatively independently from each other (*31*) and that CaM in solution is in a dynamic equilibrium between an extended and a more compact globular conformation (*32*−*34*). To bind target peptides, CaM folds around the axis of the central helix, allowing it to envelop them. From NMR studies of Ca^{2+}-free CaM, it has become clear that, on a time-averaged basis, the helix is disrupted in the region between Asp 78 and Ser 81 (*35*, *36*), allowing a relatively independent rotation of the two lobes. The central region of CaM is characterized by a peculiar distribution of charged residues, with three basic amino acids in the N-terminal part (Arg 74, Lys 75, and Lys 77) followed by a cluster of acidic residues (Asp 78, Asp 80, Glu 82, Glu 82, and Glu 83). The two phosphorylated residues Thr 79 and Ser 81 are located between these oppositely charged stretches and build the core of the hinge that allows CaM to bend. The two phosphorylated side chains could thus either form salt bridges with all three basic residues located upstream as well as hydrogen bonds with some of the side chains of the carboxylic acids located C-terminally or induce a destabilization of the secondary structure of CaM by repulsion of the negatively charged groups. The CD results clearly show a decrease in the percentage of α-helix, both in the presence and in the absence of calcium. However, one cannot say whether the central helix is in an extended or bent conformation.

The physiological role of CaM phosphorylation requires investigation to define under which circumstances cells may need to inhibit their intracellular responses to a Ca^{2+} transient by lowering the affinity of CaM for most of its targets (NOS being the exception). For instance, CaM phosphorylation could well play a role in the prolongation of calcium signals observed in some neurones (*37*, *38*) and B lymphocytes (*39*): i.e., the long time required for restoring normal intracellular Ca^{2+} concentrations could be explained by the reduced ability of PCaM to stimulate the plasma membrane and, possibly, the endoplasmic reticulum Ca^{2+}-pumps, in the tissues where the CaM-dependent protein kinase-phospholamban cascade is active. Furthermore, under conditions of sustained high calcium, it may become necessary to "switch off" CaM to avoid uncontrolled activation of protein kinases or phosphatases. While this may be the prevailing rule in CaM phosphorylation since the effect of phosphorylation in most cases is the down-regulation of CaM action, alternative, opposite effects must also be considered and rationalized, e.g., the significant up-regulation of nitric oxide synthase.

The difficulties encountered in the past in detecting PCaM in liver in vivo strongly indicate that CaM phosphorylation is under strict control by protein Ser/Thr phosphatases. We could rule out the direct involvement of calcineurin (PP2B), while PCaM was a better substrate for the pleiotropic kinases PP1γ and PP2A. This last finding agrees with the established observation that PP2A is rather abundant and active in liver tissue (*40*), indicating that both putative elements regulating CaM phosphorylation (the kinase and the phosphatase) are present and active in that tissue. The relatively low efficiency of PCaM dephosphorylation by PP1 and PP2A might be explained by the absence of a targeting subunit (*41*) which is usually essential for the full activation of the phosphatases toward targets. To determine the physiological role of CaM phosphorylation, attempts should be made to correlate it with known cell signaling events, a difficult task since the mechanism of activation of casein kinase II is unknown in vivo. By monitoring CaM phosphorylation following cell stimulation and correlating it to the phosphorylation state of the β subunit of casein kinase, one may be able to establish a causal relationship between the two and define stimulants for this pathway. Finally, it must be stressed that the enzyme assays shown here only give indirect data on both Ca^{2+} and target binding to CaM: measurements of binding affinities will be necessary to assess the magnitude of these changes.

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