Phosphorylation of the 61-kDa Calmodulin-Stimulated Cyclic Nucleotide Phosphodiesterase at Serine 120 Reduces Its Affinity for Calmodulin†

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Received March 3, 1994; Revised Manuscript Received May 10, 1994®

ABSTRACT: Phosphorylation of the 61-kDa isoform of bovine calmodulin (CaM)-stimulated cyclic nucleotide phosphodiesterase (CaM-PDE) by the catalytic subunit of cyclic AMP-dependent protein kinase A (PKA) results in a decrease in the affinity of the enzyme for calmodulin [Sharma, R. K., & Wang, J. H. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 2603–2607]. In the present study, purified 61-kDa CaM-PDE was phosphorylated in the presence of [γ-^{32}P]ATP and cleaved with a Lys-C endoproteinase. The resultant phosphopeptides were resolved by reverse-phase HPLC and analyzed by electrospray mass spectrometry and Edman sequencing. Serine residues 120 and 138 were identified as the principal sites of phosphorylation. A cDNA encoding the 61-kDa CaM-PDE [Sonnenburg, W. K., Seger, D., & Beavo, J. A. (1993) *J. Biol. Chem. 268*, 645–652] was used to generate point mutants in which either or both of these serines were replaced with alanine. The mutants were expressed in COS-7 cells, purified, and phosphorylated. Phosphorylation of the mutant Ser 138 → Ala resulted in a decrease in affinity for CaM that was comparable to that seen with the wild-type enzyme. In contrast, phosphorylation of the mutant Ser 120 → Ala had virtually no effect on CaM affinity. We conclude that phosphorylation of serine 120 by PKA is responsible for the reduction in affinity of the 61-kDa CaM-PDE for CaM.

The principal mechanism for the termination of intracellular signaling by cyclic AMP (cAMP) and cyclic GMP (cGMP) is their hydrolysis to 5′-nucleotides by cyclic nucleotide phosphodiesterases (PDEs).[1] Biochemical and genetic analysis of these enzymes has revealed the existence of multiple isozyme families, which differ in their substrate specificity, tissue distribution, and regulation [for reviews, see Beavo and Houslay (1990) and Bentley and Beavo (1992)]. Recent studies from several laboratories have suggested that covalent phosphorylation may be an important mechanism for the regulation of PDE activity. Phosphorylation appears to be a mode of activation for the cGMP-inhibited (type III) PDEs (Macphee et al., 1988; Degerman et al., 1990; Manganiello et al., 1991; Lopez-Aparicio et al., 1992). Houslay and coworkers have reported the phosphorylation and activation of a cyclic AMP-specific (type IV) PDE after the treatment of hepatocyte plasma membranes with insulin (Houslay & Kilgour, 1990). The phosphorylation in vitro of cyclic GMP-specific (type V) PDE by cyclic GMP-dependent protein kinase has been reported by Corbin and collaborators (Thomas et al., 1990), and more recently, Burns and associates reported phosphorylation of this isozyme by PKA (Burns et al., 1992). It has also recently been reported that the cGMP-specific rod photoreceptor PDE (type VI) could be phosphorylated by a phosphatidylinositol-stimulated protein kinase activity from frog rod outer segments (Hayashi et al., 1991).

The work of Sharma, Wang, (Sharma & Wang, 1985, 1986; Sharma, 1991), and others (Hashimoto et al., 1989) has demonstrated that the phosphorylation of CaM-stimulated (type I) PDEs in vitro increases the concentration of Ca^{2+}-CaM needed for their activation. The 63-kDa isoform of CaM-PDE is phosphorylated by Ca^{2+}/CaM kinase II (Sharma & Wang, 1986; Hashimoto et al., 1989) but not by protein kinase A (PKA), while the reverse is true for the 59- and 61-kDa CaM-PDE enzymes (Sharma & Wang, 1985; Sharma, 1991). Sharma and Wang reported that PKA could catalyze the incorporation of 2 mol of phosphate/mol of bovine 61-kDa CaM-PDE monomer and that phosphorylation was accompanied by an increase of almost 20-fold in the half-maximal activating concentration (EC-50) of CaM (Sharma & Wang, 1985). Work with the highly homologous 59-kDa isozyme from bovine heart (Sharma, 1991) showed that its phosphorylation by PKA resulted in a 20-fold increase in the EC-50s of both CaM and Ca^{2+}. Furthermore, phosphorylation of either enzyme was completely inhibited in the presence of a 2-fold molar excess of Ca^{2+}-CaM and could be reversed by incubation with calcineurin. After calcineurin treatment, the EC-50 for Ca^{2+}-CaM returned to control values.

The 61-kDa form of CaM-PDE has been purified (Sharma et al., 1980; Hansen & Beavo, 1982), sequenced (Charbonneau et al., 1991), and cloned (Sonnenburg et al., 1993). The native enzyme is a homodimer that hydrolyzes both cAMP and cGMP with relatively high K_m values. Its sequence contains a central region that shares significant homology with a putative catalytic domain that is conserved across isozyme families (Charbonneau et al., 1986). The amino-terminal portion of

† Supported by NIH Grants DK 221723 and HL44948 to J.A.B. and GM15731 to K.A.W.

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® Abstract published in *Advance ACS Abstracts,* July 1, 1994.

[1] Abbreviations: DTT, dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; MOPS, 3-morpholinopropanesulfonic acid; PDE, phosphodiesterase; PMSF, phenylmethanesulfonyl fluoride; PKA, protein kinase A (cyclic AMP-dependent); AMP, cyclic AMP; PNPP, *p*-nitrophenyl phosphate; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; Lys-C, achromobacter protease I; EC-50, the concentration required to produce 50% of the maximal effect; CaM, calmodulin; CaM-PDE, CaM-stimulated cyclic nucleotide phosphodiesterase.

[2] The numbering of amino acid residues in this manuscript follows that of the deduced amino acid sequence of the cDNA .

[3] A unit is defined as micromoles of cAMP or cGMP hydrolyzed per minute at 30 °C.

0006-2960/94/0433-8948$04.50/0

the molecule contains at least two potential binding sites for CaM: one very near the amino terminus [residues 23–41; see Charbonneau et al. (1991)] and a second site closer to the catalytic region (Kincaid et al., 1985). Limited proteolysis with trypsin or chymotrypsin removes both of these potential sites and yields an active species that is unaffected by CaM (Kincaid et al., 1985; Novack et al., 1991).

We report here the identification of serine 120 and serine 138 as the two residues at which the 61-kDa CaM-PDE is phosphorylated by PKA. *Mutation of serine 120 to alanine* virtually eliminates the effect of phosphorylation on the EC-50 for CaM, while the same mutation at serine 138 has little effect. These results suggest an important function for the putative second CaM-binding site in the regulation of this enzyme.

MATERIALS AND METHODS

Materials. The following materials were obtained from Sigma Chemical: CaM–sepharose, protein A–agarose, calmodulin, ATP, MOPS, Tris base, phenylmethanesulfonyl fluoride, iodoacetamide, benzamidine, *p*-nitrophenyl phosphate, and 2-mercaptoethanol. Staphylococcal V8 protease, pepstatin A, leupeptin, and dithiothreitol were from Boehringer Mannheim, DE-52 was from Whatman, and CNBr-activated Sepharose 4B and Sephacryl S-200 were from Pharmacia. Trifluoroacetic acid was from Pierce Chemical. [γ-^{32}P]ATP and [^{3}H]cAMP were obtained from New England Nuclear. WIPTIDE was from Peninsula Laboratories. Achromobacter protease I was a gift from Dr. T. Masaki (Ibaraki University, Japan).

Purification of 61-kDa CaM-PDE from Bovine Brain. The 61-kDa isozyme was purified from 2000 g of frozen bovine brain (Pelfreez), as described previously (Hansen et al., 1988), through the initial DE-52 step with the following modifications: (1) in addition to 0.5 mM PMSF, the homogenization buffer contained 1 μM pepstatin A and 15 mM benzamidine; (2) the DE-52 gradient solutions and all subsequent buffers contained 0.5 mM PMSF and 1 μM leupeptin. The DE-52 pool (200 mL) was incubated with 13 mL of ACAP-Sepharose [ACAP is a monoclonal antibody directed against 61-kDa PDE; see Hansen et al. (1988)] overnight at 4 °C. After the column was washed with 200 mL of buffer A (20 mM Tris-HCl, pH 8.0/1 mM $MgCl_2$/0.1% 2-mercaptoethanol/0.1 mM $CaCl_2$/0.1 M NaCl/0.5 mM PMSF/1 μM leupeptin), 11 mL of buffer B was applied (20 mM Tris-HCl, pH 8.0/2 M $MgCl_2$/0.1% 2-mercaptoethanol/2 mM EDTA/0.5 mM PMSF/1 μM leupeptin), and the column was incubated for 10 min before elution. The eluate was dialyzed immediately against buffer A containing 20 μM $CaCl_2$ and frozen in liquid nitrogen. Yields were approximately 1 mg/kg of wet weight with a specific activity of 250–400 units/mg.

Phosphorylation of 61-kDa CaM-PDE. Purified PDE from bovine brain was thawed and concentrated 5-fold by centrifugation in Centriprep-10 concentrators (Amicon) prior to phosphorylation (0.3 mg/mL final concentration). CaM-PDE (61 kDa) expressed in COS-7 cells was phosphorylated immediately after purification (see below). In all cases, phosphorylation was carried out overnight at 4 °C in the presence of 50 mM MOPS, pH 7.0/1 mM DTT/6 mM $MgCl_2$ and the indicated concentrations of ATP and PKA catalytic subunit. For experiments designed to identify phosphorylated residues, 0.5 mCi of [γ-^{32}P]ATP was also included.

Achromobacter Protease I Digestion of Phosphorylated PDE. Phosphorylated 61-kDa CaM-PDE was applied to a column (2.4 × 18 cm) containing Sephadex G-150 equilibrated with 20 mM Tris-HCl, pH 7.5/0.1 mM EGTA/150 mM NaCl/1 mM DTT and eluted at 0.4 mL/min. The initial peak of ^{32}P was pooled and dialyzed against 200 vol of 2.5% formic acid containing 0.1% 2-mercaptoethanol for 24 h with three changes. The PDE was dried in presiliconized polypropylene tubes in a SpeedVac concentrator and resuspended in 50 μL of 50 mM Tris-HCl, pH 8.0 containing 8 M urea. Reduction and carboxymethylation were carried out as described (Stone et al., 1989). A total of 200 μL of 55 mM Tris-HCl (pH 9.2) was added, and digestion was initiated by the addition of achromobacter protease I, which cleaves carboxy terminal to lysine residues, at a weight ratio of 1:100. The mixture was incubated at 37 °C for 6 h and stored at −20 °C.

HPLC Analysis of Digests. Digests of phosphorylated PDE were fractionated on a microbore C-18 column (Spheri-10 RP-18; 30 × 2.1 mm, Pierce Chemical) using a Hewlett-Packard 1090 Chemstation. The flow rate was 0.3 mL/min, and the gradient of 0–60% acetonitrile containing 0.08% TFA was run at 1%/min.

Mass Spectral Analysis. Electrospray mass spectra (Fenn et al., 1989; Covey et al., 1991) were acquired on a Sciex API-III triple-quadrupole mass spectrometer equipped with a nebulization-assisted electrospray ion source (PE Sciex, Thornhill, ON, Canada). Samples dissolved in 1:1 water/methanol containing 0.1% formic acid were continuously infused into the mass spectrometer at a flow rate of 2 μL/min. Typically, 3–5 scans were summed using a mass step of 0.1 Da with a dwell time of 1 ms/step; the instrument was operated at unit resolution.

Oligonucleotide-Directed Mutagenesis. The 715-base *Hin*dIII/*Xba*I restriction endonuclease cleavage fragment of the cDNA clone pCAMPDE-40 was subcloned into M13mp18 cloning vector for the generation of single-stranded DNA (Ausubel et al., 1987–1993; Sambrook et al., 1989). This fragment encodes the first 208 residues of the 61-kDa CaM-PDE isozyme (Sonnenburg et al., 1993). Antisense oligonucleotides designed to change serine 120 (oligomer S120A: 5′-CATGCACAATGGCCCGAAATCTTG-3′) or serine-138 (oligomer S138A: 5′-CCATGTGATAGGCCTTT-CTGTACA-3′) to alanine of the 61-kDa CaM-PDE were synthesized using an Applied Biosystems Model 381A DNA synthesizer (the nucleotide changes are underlined). Site-specific mutagenesis was carried out using the Amersham oligonucleotide-directed in vitro mutagenesis system (version 2), adapted from the method of Eckstein and co-workers (Sayers et al., 1988). The mutagenized DNA was used to transform TG1 competent cells prepared by the calcium chloride method (Ausubel et al., 1987–1993; Sambrook et al., 1989). Single-stranded or replicative-form DNA isolated from putative mutant phage was screened by sequencing performed using the Applied Biosystems Taq DyeDeoxy Terminator Cycle Sequencing Kit and an Applied Biosystems Model 373 DNA sequencer (McCombie et al., 1992). Confirmed mutants were completely sequenced in both directions to ensure that no unwanted "second-site" mutations were introduced.

Construction of Expression Plasmids. The entire 2.3-kb pCAMPDE-40 cDNA encoding the 61-kDa CaM-PDE was ligated into the *Hin*dIII- and *Not*I-digested expression vector, pCDM8, and propagated in MC1061/p3 cells (Seed, 1987). This plasmid (designated pCAMPDE-40/CDM8) was subsequently used to generate mutant constructs for expression studies. Mutant cDNA inserts were removed from M13mp18 RF DNA by cleavage using *Hin*dIII and *Xba*I restriction endonucleases and subcloned into the corresponding site in the pCAMPDE-40/CDM8 plasmid. These constructs were

sequenced to ensure that the mutant inserts were successfully introduced into the expression plasmid.

COS Cell Transfections. Expression plasmid DNA was prepared for transfections as described previously (Sonnenburg et al., 1993). Either parent or mutant plasmids were introduced into COS-7 cells by electroporation (Chu et al., 1987). Briefly, COS-7 cells were cultured in complete medium (composition: 90% Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum/2 mM glutamine/50 mL penicillin/50 μg/mL streptomycin) at 37 °C in a water-saturated, 7% CO_2 atmosphere. After reaching 50–80% confluency, the cells were detached from the culture plates by treatment with 0.1% trypsin/0.5 mM EDTA at 37 °C for 5–10 min. Trypsinized COS-7 cells were immediately diluted in 2 vol of complete medium and collected by centrifugation (200*g* for 5 min). The cell pellet was resuspended in PBS (20 mL per 4 plates) and counted. The cells were collected by centrifugation and resuspended in DMEM at a density of (2–4) × 10^6 cells/mL. Cells (0.75 mL) and DNA (10 μg) were placed into electroporation cuvettes (width = 0.4 cm) and incubated on ice for 10 min. The electroporator (Invitrogen) was set at 250 μF capacitance and charged at 300 V for 2 min. Immediately following discharge, the cuvettes were removed from the electroporator and placed on ice for 5 min. The cells from each cuvette were subsequently transferred to a 100-mm cell culture dish containing 10 mL of complete medium. After 2 days, the cells were harvested as described below.

Purification of 61-kDa CaM-PDE Expressed in COS-7 Cells. Seven plates (100-mm diameter) of cells were rinsed once in PBS. To each plate was added 0.5 mL of 40 mM Tris-HCl, pH 7.5/15 mM benzamidine/1 μg/mL leupeptin/1 μg/mL pepstatin A, and the cells were removed by scraping. The cells were then homogenized with 10 strokes in a Dounce homogenizer and centrifuged for 15 min at 10000*g*. The supernatant was incubated with 1.0 mL of ACAP-Sepharose (Hansen et al., 1988) for 2 h at 4 °C. The resin was loaded into a column and washed with 20 mL of buffer A and eluted with buffer B, collecting 0.4-mL fractions. The peak of PDE activity was immediately applied to a column (1.6 × 15 cm^2) containing Sephadex G-150 equilibrated with 20 mM Tris-HCl, pH 7.5/0.15 M NaCl/1.0 mM EGTA/1 mM DTT. The column was eluted at 0.3 mL/min, and fractions of 0.6 mL were collected and assayed for PDE activity. The four most active fractions were pooled for phosphorylation. Reaction conditions for phosphorylation were the same as those described for the purified bovine enzyme.

Other Procedures. Calcineurin was purified by chromatography on DEAE-Sepharose, calmodulin–Sepharose, and Sephacryl S-200, essentially as described by Sharma (Sharma et al., 1983). Phosphatase activity was assayed as calmodulin-stimulated PNPP phosphatase, using a modification of the phosphate release assay (Gillespie & Beavo, 1989). Activity pooled from the S-200 column contained no detectable PDE activity. The PKA catalytic subunit was purified as described by Lai and colleagues (Lai et al., 1990). This procedure is an adaptation of the original procedure of Krebs and co-workers (Beavo et al., 1974), as modified by Greengard and associates (Kaczmarek et al., 1980). Protein sequence was acquired on an Applied Biosystems Model 470A gas-phase sequencer as described (Charbonneau et al., 1991). Phosphodiesterase activity was measured as described using 30 μM cGMP as substrate and either radiometric (Sonnenburg et al., 1993) or phosphate release (Gillespie & Beavo, 1989) assays. SDS–polyacrylamide gel electrophoresis was carried out using established procedures (Ausubel et al., 1987–1993). Protein was assayed by the method of Bradford (1976).

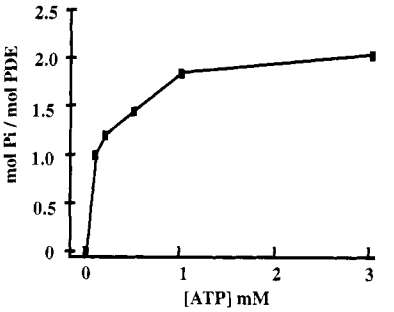

FIGURE 1: Phosphorylation of 61-kDa CaM-PDE by PKA. Reactions contained 0.3 μg of PDE (immunoaffinity purified from bovine brain) and 0.2 μg of the catalytic subunit of PKA. Incubations were conducted under the conditions described above (see Materials and Methods) in a final volume of 65 μL. Reactions were terminated by the addition of 40 μL of 3× SDS–polyacrylamide gel electrophoresis sample buffer, 12 μL of 0.1 M EDTA, and 2.5 μL of 1 M DTT, followed by incubation at 90 °C for 2 min. After electrophoresis on 9% polyacrylamide gels, the PDE was visualized by autoradiography, and the bands were excised and counted.

RESULTS

As reported by Sharma and Wang, incubation of the 61-kDa CaM-PDE with Mg-ATP and the catalytic subunit of PKA resulted in phosphorylation of the enzyme, as well as a decrease in its apparent affinity for Ca^{2+}-CaM (Sharma & Wang, 1985). Figure 1 shows the incorporation of ^{32}P into the purified 61-kDa enzyme with increasing concentrations of [γ-^{32}P]ATP. Phosphorylation was completely dependent on the addition of PKA and was abolished by WIPTIDE (Cheng et al., 1986), a specific PKA inhibitor (data not shown). Maximal phosphorylation was observed at 0.4 μM PKA, a concentration similar to the value of 0.78 μM reported by Krebs and co-workers (Hofmann et al., 1977) for whole brain tissue. The stoichiometry of phosphorylation was generally 1–2 mol of phosphate/mol of enzyme monomer.

Identification of Phosphorylation Sites. In order to identify the sites of phosphorylation on the 61-kDa CaM-PDE, it was first necessary to remove the PKA catalytic subunit. Initial experiments in which the reaction mixture was digested directly with achromobacter protease I (Lys-C) indicated that autophosphorylation of PKA generated a [^{32}P]Lys-C fragment that comigrated with one of the CaM-PDE phosphopeptides on the C-18 reverse-phase column. However, chromatography on Sephadex G-150 completely separated the dimeric PDE from PKA.

When Lys-C digests (Charbonneau et al., 1991) of the isolated PDE were chromatographed on C-18 reverse-phase HPLC, greater than 90% of the total ^{32}P was bound to the column, and overall recoveries were 75–100%. Elution with 0–60% acetonitrile in 0.08% TFA separated two major peaks and one minor peak of radioactivity (Figure 2). In different experiments in which the overall stoichiometry of phosphorylation varied between 0.3 and 1 mol of ^{32}P/mol of enzyme, these two peaks were always roughly equal in size and constituted >90% of the ^{32}P recovered in the gradient. The mass spectral and Edman sequence data shown here are from different digests performed under identical conditions.

The first major peak (fraction 28) yields two sequences by gas-phase sequencing (Table 1), which correspond to two predicted Lys-C peptides beginning at residues 138 and 156 of the enzyme (Sonnenburg et al., 1993). Analysis of this

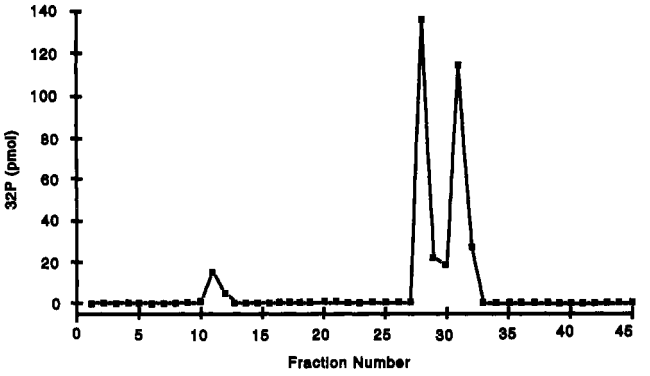

FIGURE 2: Chromatography of the 61-kDa CaM-PDE Lys-C digest. Methods for purification, phosphorylation, recovery, digestion, and reverse-phase chromatography are described in Materials and Methods. Absorbance peaks (wavelength = 210 nm) were collected manually, and aliquots were counted in water. Radioactive peaks were concentrated by vacuum centrifugation for Edman sequencing or mass spectral analysis.

Table 1: Identification of 61-kDa CaM-PDE Peptides Obtained from HPLC Fractions Containing ^{32}P[a]

fraction	Edman sequence	MS peptide	peptide designation	mol of phosphate
28	SYHMV	T	138–155	0
	S'YHMV	T'	138–155	1
	DVDKW	U	156–177	0
31	PRFRS'IVHVV	V	116–137	1
11	P-YGASRR	W	452–464	1
	MGMMK	X	105–109	0
	ELA-QGEPDP	Y	503–514	0
	NSDLVNA	Z	515–524	0

[a] Aliquots of HPLC fractions were prepared for Edman sequencing or electrospray mass spectrometry as described in Materials and Methods. For each fraction, the direct Edman sequences obtained are listed, followed by the corresponding peptide notations shown in the mass spectra of Figure 3 (MS peptide). Peptide designations are based on the predicted amino acid residue number deduced from the bovine 61-kDa CaM-PDE cDNA (Sonnenburg et al., 1993). Moles of phosphate per mole peptide are determined from the molecular masses of the peptides as determined in the mass spectral analysis. S' denotes dehydroalanine, and – indicates an unidentified amino acid residue.

peak by electrospray mass spectrometry (Figure 3A) indicates that the peptide 138–155 is phosphorylated since its mass, on the basis of the mass to charge ratio of its doubly and triply charged ions, is 80 mass units greater than that predicted for the unmodified peptide [note that in this experiment both the phospho (peak T') and dephospho (peak T) forms of the peptide are present]. This result is consistent with Edman sequencing data that indicated the presence of dehydroalanine (S', see Table 1) due to the phosphorylation of serine 138. In contrast, the mass of the peptide 156–177 (peak U) is identical to its predicted mass, indicating that it is not phosphorylated. The peptide 138–155 contains one serine and one threonine; the serine is placed at a favorable site (Bairoch, 1993) for PKA phosphorylation (RKS), while the threonine is in an unfavorable position (VIVT). To confirm that the serine was in fact the site of phosphorylation, the peptide was subdigested with staphylococcal V8 protease (endoproteinase Glu-C), and the products were purified by C-18 reverse-phase chromatography. A portion of the ^{32}P ran ahead of the undigested peptide in a fraction that contained the unique sequence S'YHMV, corresponding to the Glu-C peptide S'YHMVGLAYPE (residues 138–148). This result, in combination with the mass spectral data indicating only one phosphorylated residue on peptide T', confirms that serine 138 is phosphorylated by PKA.

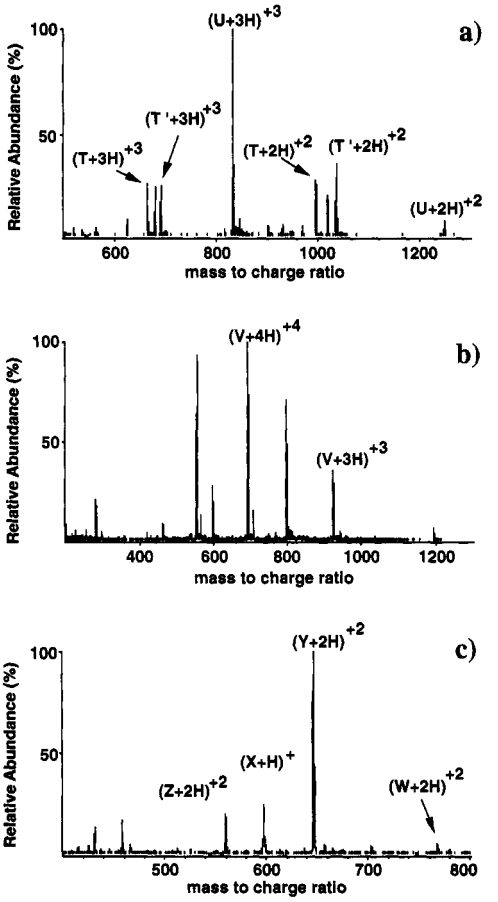

FIGURE 3: Mass spectra of ^{32}P-containing fractions. Mass spectra from HPLC fractions containing ^{32}P were acquired as described in Materials and Methods. The peptide designations for each peak are given in Table 1. (a) Fraction 28: The doubly and triply charged ions of peptide T, the corresponding phosphopeptide T', and U are denoted. (b) Fraction 31: The triply and quadruply charged ions of phosphopeptide V are observed. (c) Fraction 11: Four different peptides from 61-kDa CaM-PDE are identified, but only one (W) is phosphorylated.

Edman degradation of the second major peak (fraction 31) yields the sequence (116–137) shown in Table 1, and mass spectral analysis (Figure 3B) shows the presence of the full-length phosphorylated Lys-C peptide containing that sequence (peak V). The only residue that is capable of accepting phosphate is the serine in the fifth position of this peptide. This residue is denoted S' because the actual amino acid observed is dehydroalanine, generated by dephosphorylation during the Edman cycle (Wang et al., 1988). It should be noted that other Lys-C peptides from the PDE are sometimes found in this fraction (Figure 3B) but are never phosphorylated.

In several experiments, a minor peak of radioactivity was detected in fraction 12. Edman degradation of this fraction yielded four partial sequences from 61-kDa CaM-PDE (Table 1), including one (452–464) that contains the PKA consensus sequence (Bairoch, 1991) RRS at its carboxy terminus. Mass spectral analysis confirms the presence in this fraction of the phosphorylated Lys-C peptide 452–464 (Figure 3C), indicating that in vitro phosphorylation can also occur at serine 461.

Mutagenesis of Phosphorylation Sites. As described above (Materials and Methods), oligonucleotide-directed mutagenesis was employed to generate constructs of the 61-kDa CaM-PDE gene in which either serine 120, serine 138, or both were replaced with alanine. These plasmids were transfected into COS-7 cells, and the expressed CaM-PDEs were purified using the monoclonal antibody ACAP coupled to agarose. The

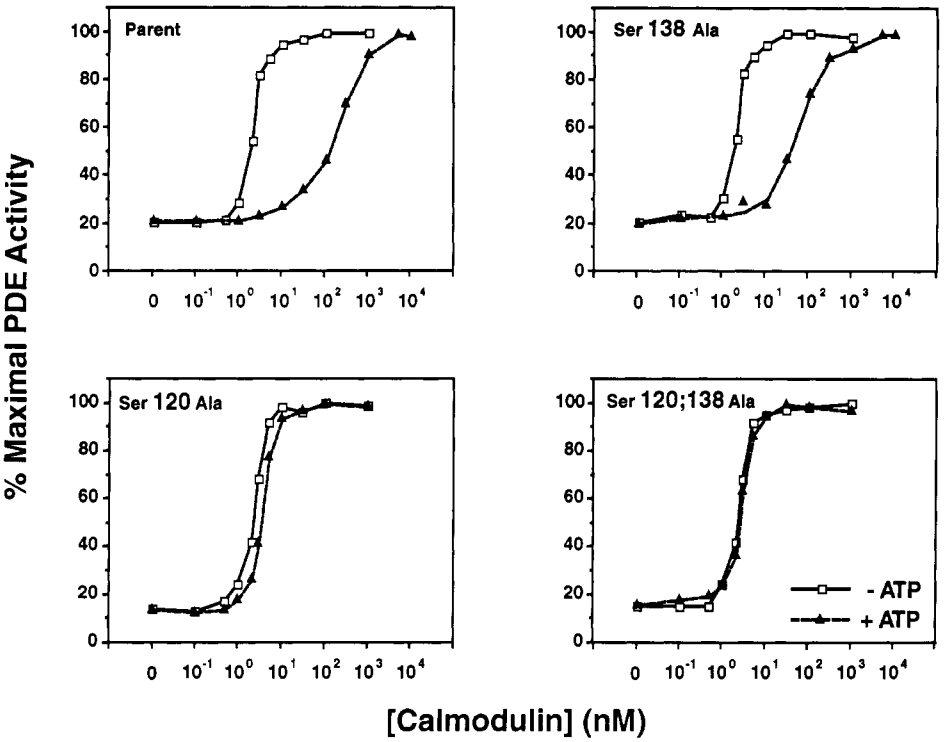

FIGURE 4: Mutational analysis of phosphorylation-induced desensitization of CaM-PDE activation by CaM. CaM-PDE activities affinity purified from extracts of COS cells transfected with either parent or mutated CaM-PDE expression plasmids were treated with PKA catalytic subunit in the absence (□) or presence (▲) of ATP. Samples were subsequently assayed for PDE activity using varying concentrations of CaM (see Materials and Methods for details). Activity is expressed as a percent of the maximally stimulated enzyme for each treatment pair. The actual values for the CaM-PDE activities (expressed as pmol/min/mL) are as follows: parent, 2181 (–ATP) and 1918 (+ATP); Ser 120 Ala, 7134 (–ATP) and 6864 (+ATP); Ser 138 Ala, 2385 (–ATP) and 2247 (+ATP); Ser 120, 138 Ala, 7212 (–ATP) and 6876 (+ATP). The variability in the concentration of partially purified CaM-PDE activity for each construct was due to differences in transfection efficiency. The data presented in this figure exhibit representative results of three or more different experiments. The labels in the upper left corner of each panel indicate the mutations made in each expression plasmid and are designated as follows: (1) top left panel, nonmutagenized construct; (2) bottom left panel, serine residue 120 mutated to alanine; (3) right top panel, serine residue 138 mutated to alanine; (4) bottom right panel, serine residues 120 and 138 mutated to alanine.

purified enzymes were incubated with the catalytic subunit of PKA in the presence or absence of ATP, and the effect of calmodulin on PDE activity was determined.

Figure 4 shows the effect of these amino acid replacements on the response of 61-kDa CaM-PDE to phosphorylating conditions. Phosphorylation of the parent molecule results in a substantial (100-fold) decrease in the EC-50 of CaM. When serine 138 is replaced with alanine, the effect of phosphorylation is slightly attenuated—the decrease in the affinity of the enzyme for CaM is reduced to approximately 40-fold. However, replacement of serine 120 with alanine results in almost total abolition of the effect of phosphorylation. The double mutant is similarly unaffected by incubation under phosphorylating conditions. Thus, phosphorylation of serine 120 clearly results in a significant decrease in the affinity of CaM for the 61-kDa CaM-PDE, while phosphorylation of serine 138 has little or no effect.

DISCUSSION

Phosphorylation of the 61-kDa CaM-PDE by PKA in vitro was originally demonstrated by Sharma and co-workers (Sharma & Wang, 1985). The development of techniques for the purification of this enzyme (Hansen et al., 1988; Charbonneau et al., 1991) has now allowed the direct demonstration that serine 120 and serine 138 are the sites at which this phosphorylation occurs. Furthermore, the isolation of cDNAs that encode the sequence of this PDE (Sonnenburg et al., 1993) has enabled us to modify these two sites individually and in combination, in order to assess their relevance to the biochemical effects of phosphorylation. We have found that while the replacement of serine 138 with alanine (Ser 138 → Ala) causes only a slight diminution in the effect of phosphorylation on CaM affinity, the replacement of serine 120 with alanine (Ser 120 → Ala) abolishes this effect almost completely. We conclude that the phosphorylation of serine 120 is primarily responsible for the change in CaM affinity observed after the incubation of 61-kDa CaM-PDE with PKA and ATP. It should be noted that this result was somewhat surprising, inasmuch as serine 138 appears to be a more favorable site for phosphorylation by PKA (RKS) than serine 120 (RFRS).

Identification of the sites of phosphorylation was achieved in this study by a combination of Edman amino-terminal sequencing and electrospray mass spectrometry. These techniques are complementary in several respects: (1) Phosphoserine cannot be directly identified by sequencing; it must be inferred [see Wang et al. (1988)]. Therefore, an exact molecular weight of the putative phosphopeptide is significant confirmatory evidence. (2) In radioactive HPLC peaks that contain ^{32}P but consist of more than one peptide, a mass spectrum can identify which peptide is phosphorylated. (3) Mass spectral analysis indicates the number of moles of phosphate present on each phosphopeptide. (4) In the case of the minor phosphorylation site (serine 461), the peptide was found in low abundance in a fraction containing several other peptides. However, Edman degradation in combination with mass spectral analysis was sufficiently sensitive to identify each peptide and confirmed that the peptide containing the

consensus sequence RRS (serine 461) was the phosphorylated species.

Phosphorylation of CaM-binding proteins is a widespread phenomenon that can result in stimulation (James et al., 1989) or inhibition (Nishikawa et al., 1984) of the target protein. In some cases, notably myosin light-chain kinase (MLCK; Nishikawa et al., 1984) and the 61- and 63-kDa CaM-PDEs (Sharma & Wang, 1986; Sharma, 1991), inhibition by phosphorylation has been shown to be the result of a decreased affinity of the enzyme for CaM. In the case of MLCK, phosphorylation places a negative charge in the basic amphipathic helical domain, which functions as the CaM-binding site (Lukas et al., 1986). In the case of the 61-kDa CaM-PDE, evidence from peptide inhibition and secondary structure predictions initially suggested that the CaM-binding domain was located in an amino-terminal region (residues 23–41) with a high probability of amphipathic helix formation (Charbonneau et al., 1991). However, this region does not contain any candidate sites for phosphorylation by PKA, and in fact we did not detect any phosphorylated residues in this domain. If the CaM-binding domain of the 61-kDa CaM-PDE is near the amino terminus, the phosphorylation of serine 120 must modulate its affinity for CaM by a mechanism different from that observed for MLCK.

It remains possible, however, that a different or additional CaM-binding site exists on this enzyme. Kincaid et al. (1985) observed that limited digestion of 61-kDa CaM-PDE with chymotrypsin produced a stable 47-kDa fragment in the presence of CaM and that dissociation of CaM by the addition of EGTA caused the molecular mass to shift to 45 kDa. They therefore speculated that a CaM-binding site was located approximately 14 kDa from the amino terminus. It is of interest to note that the region near serine 120 probably would have been contained within this 14-kDa amino-terminal fragment. Further mutational studies with the recombinant protein will be necessary to define the existence and role of this second potential CaM-binding site.

In summary, we have identified the amino acid residues of the 61-kDa CaM-PDE that are phosphorylated by cAMP-dependent protein kinase. Phosphorylation of serine 120 increases the concentration of calmodulin required for activation of the enzyme. The fact that this serine is located nearly 100 residues carboxy terminal to the previously identified CaM-binding site (Charbonneau et al., 1991) suggests either that phosphorylation utilizes a mechanism for decreasing calmodulin affinity in the PDE different from that in other CaM-dependent enzymes or that a second CaM-binding site is present on the PDEs nearer to position 120. Since in vitro phosphorylation of the 61-kDa CaM-PDE inhibits activation of the enzyme by calmodulin, a likely consequence of phosphorylation in the intact cell would be to prolong and potentiate cAMP or cGMP accumulation in response to the activation of adenylyl or guanylyl cyclase (Wang et al., 1990; Beltman et al., 1993). Moreover, modulation of cyclic nucleotide degradation via this mechanism may also contribute to synaptic plasticity in certain regions of the brain (Beltman et al., 1993; Yan et al., 1993).

ACKNOWLEDGMENT

The authors gratefully acknowledge the assistance of the Molecular Pharmacology Facility at the University of Washington for oligonucleotide synthesis and DNA sequencing. In addition, the expert assistance of Maria Harrylock in protein sequencing is greatly appreciated.

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