Phosphorylation of neuronal nitric-oxide synthase (nNOS) by Ca\textsuperscript{2+}/calmodulin (CaM)-dependent protein kinases (CaM kinases) including CaM kinase I (CaM-K I), CaM kinase I\(\alpha\) (CaM-K I\(\alpha\)), and CaM kinase IV (CaM-K IV), was studied. It was found that purified recombinant nNOS was phosphorylated by CaM-K I, CaM-K I\(\alpha\), and CaM-K IV at Ser\textsuperscript{847} \textit{in vitro}. Replacement of Ser\textsuperscript{847} with Ala (S847A) prevented phosphorylation by CaM kinases. Phosphorylated recombinant wild-type nNOS at Ser\textsuperscript{847} (~0.5 mol of phosphate incorporation into nNOS) exhibited a 30% decrease of \(V_{\text{max}}\) with little change of \(K_m\) for \(\text{L-arginine}\) and \(K_{\text{act}}\) for CaM relative to unphosphorylated enzyme. The activity of mutant S847D was decreased to a level 50–60% as much as the wild-type enzyme. The decreased NOS enzyme activity of phosphorylated nNOS at Ser\textsuperscript{847} and mutant S847D was partially due to suppression of CaM binding, but not to impairment of dimer formation which is thought to be essential for enzyme activation. Inactive nNOS lacking CaM-binding ability was generated by mutation of Lys\textsuperscript{782}-Lys-Leu to Asp\textsuperscript{782}-Asp-Glu (Watanabe, Y., Hu, Y., and Hidaka, H. (1997) FEBS Lett. 403, 75–78). It was phosphorylated by CaM kinases, as was the wild-type enzyme, indicating that CaM-nNOS binding was not required for the phosphorylation reaction. We developed antibody NP847, which specifically recognizes nNOS in its phosphorylated state at Ser\textsuperscript{847}. Using the antibody NP847, we obtained evidence that nNOS is phosphorylated at Ser\textsuperscript{847} in rat brain. Thus, our results suggest that CaM kinase-induced phosphorylation of nNOS at Ser\textsuperscript{847} alters the activity control of this enzyme.

Calcium ion acts as a ubiquitous second messenger, especially in neural tissues where many of its physiological responses are mediated by the Ca\textsuperscript{2+}-binding protein calmodulin (CaM).\textsuperscript{1} The actions of CaM are mediated by its association with specific target proteins, some of which are known as CaM-binding proteins, which include kinases such as CaM kinase I (CaM-K I), CaM kinase II (CaM-K II), and CaM kinase IV (CaM-K IV) (1, 2). CaM also associates with flavoproteins such as the nitric-oxide synthases (NOSs), which catalyze the formation of nitric oxide (NO) and L-citrulline from L-arginine (3, 4). NO is a major cellular signaling molecule and has been implicated in synaptic plasticity in the brain (5, 6). These enzymatic forms are involved in biological processes as diverse as neurotransmission (CaM-dependent nitric-oxide synthase (nNOS)), blood pressure homeostasis (endothelial NOS (eNOS)), and cytotoxicity (inducible NOS (iNOS)). These three NOS isoforms are the products of distinct genes located on different chromosomes and show distinct tissue-specific patterns of expression (7).

nNOS is found in neurons and contains anNH\textsubscript{2}-terminal PDZ domain that links the enzyme to PSD-95 (8, 9), as well as CAPON (10), which controls the subcellular localization and sensitivity to Ca\textsuperscript{2+}-dependent activation of the enzyme in some neurons. It has been confirmed that not only cyclic AMP-dependent protein kinase (PKA) but also cyclic GMP-dependent protein kinase, protein kinase C (PKC), and CaM kinase can phosphorylate nNOS, although the physiological significance of this phosphorylation remains uncertain, as there is either no detectable effect on enzyme activity or else the effect is controversial (11–14). More recently, it was determined that Ca\textsuperscript{2+}/CaM-dependent NOSs (cNOSs) contain a unique polypeptide phosphorylation site for CaM-K II. Since a synthetic peptide derived from the insertion of eNOS interferes with CaM binding to cNOSs, while a peptide derived from S847 of nNOS does not (15), phosphorylation/dephosphorylation of the serine and threonine residues found at amino acids 831–872 of nNOS would seem to influence its activity. Thus, we suspected that one of the three serine residues in this element of nNOS may be the phosphorylation site for CaM-K II.

Here, we report that nNOS is phosphorylated at Ser\textsuperscript{847} by CaM kinases, including CaM-K I, CaM-K I\(\alpha\), and CaM-K IV \textit{in vitro}. The effects of phosphorylation at Ser\textsuperscript{847} on activation of the enzyme activity are also documented. Furthermore, we present evidence that CaM kinases phosphorylate nNOS at Ser\textsuperscript{847} \textit{in vivo}. These findings demonstrate a novel regulatory mechanism for nNOS in neural cells via Ser\textsuperscript{847} phosphorylation by CaM kinases.
**EXPERIMENTAL PROCEDURES**

**Materials**—CaM-K I, CaM-K II, CaM-K IV, and CaM kinase (CaMK-K) were obtained from a rat brain cDNA library. The cDNA for rat brain nNOS was a generous gift from Dr. Solomon H. Snyder (Johns Hopkins University School of Medicine, Baltimore, MD). pGroESL, containing groEL and groES cDNAs, was from DuPont. pC-Wnt, was provided by Dr. Michael Waterman (Vanderbilt University, Nashville, TN). t(3)Jarginine, (γ-32P)ATP (6,000 Ci/mmol), and ECL Western blotting detection reagents were purchased from Amersham Pharmacia Biotech. Restriction enzymes and DNA-modifying enzymes were obtained from Takara Shuzo. Electrophoresis reagents and Bradford protein dye reagents were products of Bio-Rad. All other materials and reagents were of the highest quality available from commercial suppliers.

**cDNA Construction and Mutagenesis**—The cDNA for an inactive mutant nNOS was generated by mutation of Lys792-Lys-Leu to Asp792-Asp-Glu, as described previously (16). pCWNOS, the plasmid for the expression of nNOS in Escherichia coli, was constructed as described previously (17). The EspITxO1 fragment of the cDNA for nNOS was inserted into M13mp18 to produce single-stranded DNA for mutagenesis. Four different oligonucleotides (5′-ATCAGGAAGAATCTGATGTTAAGGAGGA-3′, 5′-GTGGAGAGAATGTTGATGTTAAGGAGGA-3′, 5′-TCCTGACCGTCGTCGTCGTCGTCGACCTTCTTGG-GA-3′) were synthesized to provide mutations of Ser847 to Ala, Ser847 to Asp, Ser847 to Ala, and Ser847 to Ala (underlined codons), respectively. The fragment was cut out using the Sculptron in vitro mutagenesis system (Amersham Pharmacia Biotech). Mutant clones were isolated, and the presence of site-directed mutations was confirmed by DNA sequencing. The appropriate cDNAs were ligated into pCWnNOS, pGroESL were co-transformed into the protease-deficient E. coli strain BL21 (Strategen).

The CaM kinase Iα–293 fragment was constructed by polymerase chain reaction from rat CaM kinase Iα with BamHI and EcoRI restriction sites. This fragment was cloned into the pGEX-2T vector containing GST (Amersham Pharmacia Biotech), and the fusion protein (GST/CaM kinase Iα–293) was expressed in E. coli (BL21 cells, Strategen) and purified by affinity chromatography on glutathione-Sepharose (Amersham Pharmacia Biotech) (18).

**Purification of Expressed Proteins**—Recombinant wild-type and mutants rat nNOS were expressed in E. coli or Sf9 cells and purified by 2′,5′-ADP-agarose (Sigma), as described previously (17, 19). Recombinant rat CaM-K Iα, CaM-K IIα, and CaM-K IVα were expressed in Sf9 cells and purified by CaM-Sepharose chromatography. Recombinant CaM-KK was expressed in E. coli and purified by CaM-Sepharose chromatography. Protein concentrations were determined by the method of Bradford using BSA as the standard (20).

**In Vitro Activation of CaM-K Iα and CaM-K IV and Phosphorylation of nNOS**—Recombinant CaM-K Iα and CaM-K IV (1 μM each) were preincubated with recombinant CaM-KK (350 nM) at 30 °C for 10 min in 50 mM HEPES (pH 7.0), 10 mM MgCl2, 0.4 mM dithiothreitol, 1 mM CaCl2, 1 μM CaM, 100 μM ATP, and 100 μg/ml BSA before phosphorylation of nNOS. The standard assay conditions were 40 mM HEPES (pH 7.0), 10 mM MgCl2, 0.4 mM dithiothreitol, 1 mM CaCl2, 1 μM CaM, 100 μM (γ-32P)ATP, 100 μg/ml BSA, 53 μg/ml nNOS, and 100 μM CaM kinase at 30 °C in a final volume of 30 μl. The final concentrations of protein kinases were 6.8, 4, and 6.6 μM for activated CaM-K Iα, CaM-K IIα, and activated CaM-K IV, respectively. Reactions were stopped by the addition of sample buffer, followed by electrophoresis on 6–12.5% sodium dodecyl sulfate-polyacrylamide gel (21). Gels were stained with Coomassie Brilliant Blue, dried, and subjected to autoradiography. Quantitation of 32P incorporation into nNOS was achieved by using appropriate gels and determining their radioactivity by liquid scintillation counting.

**Assay of nNOS Activity**—Activity was determined by measuring the conversion of l-[3H]arginine to l-[3H]citrulline as described previously (17, 19). Briefly, incubation was performed for 2 min at 25 °C (E. coli-expressed enzyme) or 3 min at 30 °C (Sf9 cell-expressed enzyme) (over which time the reaction was linear) in 50 μl of 40 mM Tris-HCl buffer containing 30 mM (E. coli-expressed enzyme) or 100 mM (Sf9 cell-expressed enzyme) NOS, 1 μM CaM, 100 μM l-[3H]arginine, 0.1 mM NADPH, 30 μM BSA, 4 μM FAD, and 4 μM FMN. The reaction was terminated by adding 100 μl of acetate buffer (pH 5.5) containing 1 mM L-citrulline, 2 mM EDTA, and 0.2 mM EGTA, and then the mixture was passed through a column of AG 50W-X8 resin (Bio-Rad). The eluted [3H]citrulline was quantified by liquid scintillation counting.

**Reductase Activities of nNOS**—nNOS-catalyzed reduction of cytochrome c and DCPIP was determined photometrically in 0.2 ml of a 50 mM triethanolamine/HCl buffer, pH 7.0, containing 0.1 mM NADPH, 4 μM FAD, 4 μM FMN, and 30 nM nNOS either in the presence (cytochrome c) or absence (DCPIP) of CaM. The concentrations of the electron acceptors in the incubations were 100 μM, and the initial rates of their reduction were calculated using the following extinction coefficients, given as mm−1 cm−1: cytochrome c, 21 at 550 nm; and DCPIP, 20.6 at 600 nm, respectively.

**CaM Overlays**—Wild-type and mutant nNOS proteins were resolved by 7.5% SDS-PAGE and electrophoretically transferred onto PVDF membranes as described by Towbin et al. (22). The membranes were blocked with 100 μl NaCl, 50 mM Tris-HCl (pH 7.5), 1% nonfat milk, and 0.05% Tween 20 in the presence of 1 mM CaCl2 for 30 min at room temperature. Biotinylated CaM was then added at a final concentration of 0.1 μg/ml in the same buffer for 1 h. After washing with the buffer, membranes were incubated with avidin-biotin-peroxidase (Vector Laboratories, Inc.), washed extensively, and then developed with the chemiluminescence reagent (Amersham Pharmacia Biotech).

In Vitro Phosphorylation of nNOS by PKA and PKC—Recombinant nNOS (0.5 μg) expressed in E. coli was incubated for 10 min at 30 °C in 40 mM HEPES (pH 7.0), 10 mM MgCl2, and 1 mM ATP. The final concentrations of PKA and PKC were 100 nM, respectively. For phosphorylation by PKC, 100 μg/ml phosphatidylserine and 1 mM CaCl2 were added. Reactions were stopped by the addition of sample buffer followed by 6% SDS-PAGE and electrophoretically transferred onto PVDF membranes. For immunoprecipitation of the transfected proteins, the procedure of Burnette (23) was used, except that the second antibody was linked to hors eradise peroxidase.

**Preparation of Antiphosphopeptide Antibodies**—We developed antibody recognizing the phosphorylation at Ser847 on nNOS as described previously (24). Briefly, 1 μg of the phosphorylated synthetic peptide, CKVRFNS(phosphoS)VSSYS, coupled with keyhole limpet hemocyanin, emulsified in Freund's complete adjuvant, was injected into a rabbit subcutaneously. Every 2 weeks, the same portion of the antigen solution emulsified in Freund's incomplete adjuvant was boosted. The rabbit was bled 5 weeks after the initial injection. Immunoglobulin G was affinity-purified by tandem column chromatography using columns coupled to phosphorylated synthetic peptide and non-phosphorylated synthetic peptide, CKVRFNSSVSSYS.

**RESULTS AND DISCUSSION**

**Phosphorylation of nNOS by CaM Kinases**—We first determined whether CaM kinases could phosphorylate recombinant nNOS expressed in the E. coli system and found that nNOS was phosphorylated by these kinases in the presence of Ca2+ in vitro (Fig. 1). CaM-K IIα caused the most rapid phosphorylation with half-maximal phosphorylation apparent at 3 min and a plateau level at 10 min. CaM-K Iα and CaM-K IV activated by CaM-KK also phosphorylated nNOS, but the plateau level was not reached until 100 min (−0.4 and −0.7 mol of 32P/mol of nNOS at 100 min, respectively). nNOS phosphorylation was not observed if Ca2+/CaM or the CaM kinase was omitted from the reaction mixture and CaM-KK (140 μM) could not phosphorylate nNOS (data not shown). The maximal phosphorylation of nNOS by CaM-K Iα was observed at −0.4 mol of 32P/mol of nNOS under the conditions employed. To clarify whether sites of phosphorylation on the CaM kinase were the same, nNOS was sequentially phosphorylated by each of the CaM kinases. nNOS was initially phosphorylated by CaM-K IIα for 30 min and was then exposed to activated CaM-K IV or CaM-K Iα for an additional 30 min. No significant additional phosphate was incorporated into nNOS by addition of activated CaM-K IV or CaM-K Iα (data not shown), indicating that CaM-K IIα, CaM-K IV, and CaM-K Iα phosphorylated the same amino acid residue of nNOS.

In order to determine whether Ca2+/CaM binding to CaM kinases, nNOS, or both, was essential for the phosphorylation reaction, we needed a substrate of CaM kinases that did not bind Ca2+/CaM. We have previously shown that mutation to Glu or Asp of several residues (Lys792-Lys-Leu) of nNOS blocks the binding of CaM and activation of nNOS. Therefore, it was of interest to determine whether inactive nNOS lacking CaM
Phosphorylation of Neuronal NOS by CaM Kinases

Phosphorylation of nNOS by CaM kinases. A, 1 µg of recombinant wild-type nNOS expressed in E. coli was incubated at 30 °C with 100 nM activated CaM-K I (panel 1), CaM-K IIa (panel 2), or activated CaM-K IV (panel 3), 100 µg/ml BSA, and 100 µM \([γ^32P]ATP\) at 30 °C for the indicated time. The reaction was stopped by addition of sample buffer, and samples were analyzed by SDS-PAGE on 6% gel followed by autoradiography. B, radioactivity incorporated by nNOS was quantified by scintillation counting of the excised gel as described under “Experimental Procedures.” The relative position of phosphorylated nNOS is marked. These data are representative of an experiment repeated twice.

Binding ability, generated with mutation of Lys732-Lys-Leu to Asp732-Asp-Glu could be phosphorylated by CaM kinases. As shown in Fig. 2, mutant nNOS could be phosphorylated by CaM kinases like the wild-type enzyme in the presence of Ca\(^{2+}\)/CaM. We also constructed a fusion protein of GST and a fragment of CaM kinase Iα (residues 1–293) (GST/CaM kinase I α1–293), which did not contain the autoinhibitory domain or the CaM-binding domain (18). GST/CaM kinase I α1–293 phosphorylated wild-type nNOS either in the presence or absence of Ca\(^{2+}\)/CaM (data not shown). These results demonstrated that the binding of Ca\(^{2+}\)/CaM to nNOS was not required for the phosphorylation of nNOS by CaM kinases.

Construction and Expression of nNOS Mutants—Analysis of a series of truncated mutants of nNOS suggested that the residues phosphorylated by CaM kinases are included within residues 825–884 (data not shown). It is known that CaM kinase II recognizes sites that have an Arg located three residues toward the NH\(_2\) terminus of the phosphorylated Ser or Thr. Since phosphoamino acid analysis revealed that all three CaM kinases phosphorylated nNOS at amino acids that co-migrated with phosphoserine (data not shown), we made single mutants by introducing Ala residues in place of Ser847 (S847A) and Ser858 (S858A), which were at the P-O positions on the COOH-terminal sides of Arg844 and Arg855, respectively. We also wanted to place a Ser at three residues toward the NH\(_2\) terminus of Arg550 (S852A), because the orientation of the consensus phosphorylation sequence for CaM-K II (25) may be opposite to that of the normal substrate. Wild-type and single mutants were expressed using the E. coli or the baculovirus/Sf9 cell system and purified on 2–5′-ADP-agarose as described under “Experimental Procedures.” All of the recombinant nNOSs were at least 90% pure and gave a major band at 160 kDa on SDS-PAGE with Coomassie Brilliant Blue staining (Fig. 3A). Mutants S852A and S858A were phosphorylated by CaM kinases as was the wild-type enzyme, but the S847A mutant was not phosphorylated by activated CaM-K Iα or CaM-K IV. In contrast, CaM-K IIα did phosphorylate this mutant, but only very slightly (Fig. 3B).

Effect of Phosphorylation on nNOS Activity—The above results demonstrated that Ser847 was a major phosphorylation site for CaM kinases. Mutant S847D was also constructed and expressed in the E. coli system (Fig. 3A). We tested the ability of Ser847 residue phosphorylation to affect NOS enzyme activity using the mutant S847D. Enzyme activity was determined from the rate of conversion of L-arginine to L-citrulline. The mutant S847D exhibited NOS enzyme activity that was approximately 40% of the wild-type level in the presence of Ca\(^{2+}\)/CaM (Fig. 4A). Since pretreatment of the nNOS preparation from E. coli caused instability of NOS enzyme activity (data not shown), wild-type nNOS was also expressed in the baculovirus/Sf9 system (Fig. 3A).
Phosphorylation of Neuronal NOS by CaM Kinases

Effect of phosphorylation on nNOS activity. A, equivalent amounts (30 nm) of wild-type (WT) and mutant (S847D) nNOSs expressed in E. coli were used for the NOS enzyme activity assay by measuring the conversion of L-[3H]arginine to L-[3H]citrulline with 100 μM L-arginine, 100 nM CaM, 20 μM BH4, 100 μM NADPH, and 4 μM FAD, and 4 μM FMN either in the presence of 1 mM CaCl2 (-) or 1 mM EGTA (+) for 2 min at 25 °C. B, wild-type nNOS expressed in Sf9 cells was incubated either with (P+) (-0.5 μM of phosphate incorporation into nNOS) or without (P-) activated GST/CaM kinase I α1-293 at 30 °C with 100 μM ATP and 100 μg/ml BSA in the presence of EGTA. Equivalent amounts (100 nm) of unphosphorylated (P-) and phosphorylated (P+) nNOSs were used for the NOS enzyme activity assay with 100 μM L-arginine, 1 μM CaM, 20 μM BH4, 100 μM NADPH, 4 μM FAD, and 4 μM FMN either in the presence of 1 mM CaCl2 (-) or 1 mM EGTA (+) for 5 min at 30 °C. The means ± S.E. of two or three experiments is shown.

Sf9 cell system (Fig. 3A). Most properties of the purified overexpressed enzyme were identical to those previously reported, including the specific activity (195 nmol/min/mg) (19). However, preincubation of Ca2+/CaM with nNOS from the Sf9 cells for 10 min markedly attenuated NOS enzyme activity to less than 10% of that without preincubation, and we did not detect any changes in NOS activity following in vitro phosphorylation by CaM kinases in the presence of Ca2+/CaM (data not shown). We employed a constitutive active form of CaM-K Iα (GST/CaM kinase I α1-293) for phosphorylating nNOS. Wild-type nNOS was incubated either with (~0.5 μM of phosphate incorporation into nNOS) or without activated GST/CaM kinase I α1-293 at 30 °C with 100 μM ATP in the presence of EGTA, and NOS activity was determined from the rate of conversion of L-arginine to L-citrulline. The preincubation under the conditions employed also attenuated NOS enzyme activity to approximately 50% (85.8 ± 1.7 nmol/min/mg) of that without incubation. However, phosphorylated nNOS exhibited NOS enzyme activity that was approximately 70% compared with unphosphorylated enzyme (Fig. 4B). Neither mutant S847D nor nNOS exhibited Ca2+/CaM-independent NOS activity. All NOS isoforms are known to catalyze electron transfer to a variety of artificial electron acceptors (26–28). Phosphorylated nNOS exhibited CaM-dependent reduction of cytochrome c that was approximately 65% compared with unphosphorylated enzyme. Meanwhile, CaM-independent DCPIP reduction of phosphorylated nNOS was essentially indistinguishable from that of the unphosphorylated enzyme (Table I). Interestingly, the reduction activities of preincubated nNOS was similar to that of the enzyme without incubation (data not shown). Thus, Ser847 in nNOS appears to represent an essential determinant for transducing the nNOS-CaM interaction into stimulation of enzyme activity via its phosphorylation.

Effect of Phosphorylation on nNOS Kinetic Parameters of nNOS—It was important to determine how the phosphorylation at Ser847 altered the kinetics of nNOS. Table II summarizes some of the properties of the phosphorylated nNOS compared with the unphosphorylated enzyme. From the results shown, it is clear that the main effect of the phosphorylation at Ser847 was to lower the Vmax of NOS activity, with little change of the Km for L-arginine and Kact for CaM. We also analyzed the kinetics of the mutant S847D and wild-type enzyme. Mutation of Ser847 to Asp resulted in attenuation of the specific activity (Vmax) (304 ± 24.5 versus 158.5 ± 18.5 nmol/min/mg), with little change of the Km for L-arginine and Kact for CaM.

In order to understand how CaM kinases regulate nNOS, the CaM-binding ability of the mutants was analyzed. The CaM binding of mutant S847D or phosphorylated nNOS was significantly decreased compared with that of wild-type or unphosphorylated enzyme, respectively, as assessed by the gel overlay technique (Fig. 5). Previous characterization of NOS has indicated that the native protein is a homodimer, and dimerization has been shown to be necessary for catalytic activity of the enzyme. We therefore explored the possibility that the mutated or phosphorylated residue (Ser847) affected the dimerization of nNOS. It is known that the nNOS homodimer is stabilized by tetrahydrobiopterin and L-arginine during low temperature SDS-PAGE. In this assay, the dimerization of mutant S847D or phosphorylated nNOS was essentially indistinguishable from that of the wild-type or unphosphorylated enzyme, respectively (data not shown). These findings suggest that Ser847 residue influenced the interaction between CaM and nNOS via its phosphorylation.

Identification of the in vivo phosphorylation at Ser847 on nNOS: nNOS is phosphorylated on different serine site of the enzyme by PKA, PKC, and CaM-K II in vitro (13). To confirm in vivo phosphorylation at Ser847 on nNOS, we used a phospo-specific antibody, NPS847. This antibody was specific for Ser(P)847 in nNOS, reacting with phosphorylated nNOS by CaM-K IIb but not with that by PKA or PKC (Fig. 6A). Protein immunoblot analyses with NPS847 of a partially purified rat brain nNOS detected an immunoreactive band corresponding to the 160-kDa nNOS (Fig. 6B). These data demonstrate that phosphorylation at Ser847 on nNOS is catalyzed not only in vitro but also in vivo.

Conclusions—Although it has already been confirmed that
nNOS is phosphorylated by several protein kinases, such as PKA, PKC, and CaM kinase, the easy thermal denaturation and inactivation of NOS has limited our understanding of the activation mechanisms mediated via phosphorylation. By using truncated and site-directed nNOS mutants, we identified the amino acid residue phosphorylated by CaM kinases and found that this phosphorylation caused a decrease in NOS enzyme activity. We also employed a constitutive active form of CaM-K Iα for phosphorylating nNOS, followed by analysis of the phosphorylated enzyme. This finding is consistent with the results of previous in vitro studies demonstrating a dramatic attenuation of nNOS enzyme activity following CaM-K II phosphorylation (12). Such down-regulation of nNOS activity by CaM kinases may represent an important component of the "cross-talk" between kinases and NO. All three tested CaM kinases incorporated 0.4–0.7 mol of $^{32}$P/mol of recombinant nNOS expressed in E. coli, in good agreement with the results using purified rat brain nNOS (13). Nakane et al. reported that 9 mol of $^{32}$P/mol of purified rat brain nNOS were incorporated by CaM-K II (12). We also analyzed the $^{32}$P stoichiometry using partially purified rat brain nNOS as a control experiment, which was similar to the results using recombinant nNOS expressed in E. coli under the conditions employed (data not shown).

It is well known that the activities of several enzymes can be inhibited by phosphorylation of their CaM-binding domains, but the mutant residue (Ser$^{847}$) was not near the putative CaM-binding domain of nNOS and binding of Ca$^{2+}$/CaM to nNOS did not inhibit the phosphorylation of nNOS by CaM kinases (Fig. 2). Furthermore, the replacement of Ser$^{847}$ by Asp and phosphorylated nNOS at Ser$^{847}$ had little effect on the CaM for CaM (Table II). However, the CaM binding of mutant S847D or phosphorylated nNOS at Ser$^{847}$ was significantly decreased compared with that of the wild-type and unphosphorylated enzyme, respectively (Fig. 5). The mutant S847D and phosphorylated nNOS at Ser$^{847}$ also showed loss of NOS enzyme activity in the presence of Ca$^{2+}$/CaM (Fig. 4). Thus, the putative nNOS inhibitory element on nNOS may allosterically perturb the CaM binding domain through phosphorylation of the Ser$^{847}$ residue. Salerno et al. reported that cNOSs contain a unique polypeptide insert in the FMN binding domains that acts as an inhibitor of cNOS enzyme activity (15). The synthetic peptides derived from the insertion of eNOS interfered with CaM binding to cNOSs by interacting with cNOSs but not with CaM. However, nNOS-derived polypeptides had no effect on nNOS. Since the Ser$^{847}$ residue is located at the insertion of nNOS, phosphorylation of the Ser$^{847}$ residue may influence the affinity of the insertion for an internalized binding site on nNOS. It was surprising to find that inactive nNOS lacking CaM-binding ability was phosphorylated by CaM kinases to the same extent as wild-type nNOS and that constitutively active CaM kinase Iα (residues 1–293) phosphorylated nNOS either in the absence or presence of Ca$^{2+}$/CaM (Fig. 2), since CaM displaces the FMN binding domain insert peptide of nNOS that enhances exposure of the insert to trypsinolysis at Lys$^{856}$ (15). It is possible that exposure of Lys$^{856}$ is induced by CaM-nNOS binding, whereas that of Ser$^{847}$ is not.

We expected an Asp mutant but not an Ala one to mimic the negatively charged state of a phosphorylated Ser$^{847}$ on nNOS; however, both mutants exhibited decrease in NOS enzyme activity and similar characteristics (data not shown). It is possible that Ser$^{847}$ is crucial for catalytic activity of nNOS, perhaps through involvement in the binding of CaM. Since the Ser$^{847}$ residue is located in FMN binding domains of nNOS, it was of interest to determine whether the replacement of Ser$^{847}$ interferes with FMN incorporation and therefore results in lower specific activities. However, flavin content of the mutants S847A and S847D was essentially indistinguishable from that of the wild-type enzyme by the measuring fluorescence (data not shown). Since nNOS, CaM-K Iα, CaM-K IIα, CaM-K IV, and CaM-KK have very similar requirements for Ca$^{2+}$/CaM (Table I) (18, 30, 31), it is puzzling why the adjacent steps in kinase and NO signaling should both require the same activator, Ca$^{2+}$/CaM. Phosphorylation of nNOS at Ser$^{847}$ is thought to be physiological, since partially purified rat brain nNOS is phosphorylated at Ser$^{847}$ by a given amount, as assessed by immunoblot analysis with NP847 (Fig. 6). However, the physiological meaning of nNOS phosphorylation by CaM kinases, including which CaM kinase is essential for such phosphorylation in vivo, must be determined in the future.

**FIG. 5.** CaM overlays of mutant S847D or phosphorylated wild-type nNOS at Ser$^{847}$. A, wild-type (WT) and mutant (S847D) nNOSs expressed in E. coli (0.5 μg each) were resolved on SDS-PAGE using 6% gel. B, unphosphorylated (P–) and phosphorylated (P+) nNOSs (0.5 μg each) as in Fig. 4B were resolved on SDS-PAGE using 7.5% gel. These gels were electrophoretically transferred onto PVDF membranes, and analyzed by reaction with 1× biotinylated CA in the presence of 1 mM CaCl$_2$ (right panel) as described under "Experimental Procedures." The same membranes were then stained by Amido Black (left panel). Arrow shows the position of nNOSs. Top panel, illustrative example; bar graph, composite values normalized per microgram of enzymes (mean ± S.E., n = 3; *, p < 0.01).

**FIG. 6.** Identification of the in vivo phosphorylation at Ser$^{847}$ on nNOS. A, 1 μg each of purified unphosphorylated nNOS expressed in E. coli (lane 1), the phosphorylated nNOS by CaM-K Iαa (lane 2), PKA (lane 3), or PKC (lane 4) were separated by 6% SDS-PAGE, and electrophoretically transferred onto PVDF membranes, and analyzed by reaction with phosphospecific antibody, NP847 (right panel), as described under "Experimental Procedures." The same membrane was then stained by Amido Black (left panel). B, partially purified rat brain nNOS was separated by 7.5% SDS-PAGE and stained by Coomassie Brilliant Blue (left panel), and electrophoretically transferred onto PVDF membranes, and analyzed by reaction with nNOS-specific antibody (middle panel) and phosphospecific antibody, NP847 (right panel).
initiating a study to determine whether Ser847 affects intramolecular protein protein interaction using peptide chemistry.

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