J. Biochem. **133**, 773–781 (2003)
DOI: 10.1093/jb/mvg099

Regulation of Endothelial Nitric Oxide Synthase by Protein Kinase C

Mamoru Matsubara*,†, Nobuhiro Hayashi, Tao Jing‡ and Koiti Titani
Division of Biomedical Polymer Science, Institute for Comprehensive Medical Science, Fujita Health University, Toyoake, Aichi 470-1192

Received March 11, 2003; accepted April 1, 2003

Endothelial nitric oxide synthase (eNOS) is a key enzyme in nitric oxide–mediated signal transduction in mammalian cells. Its catalytic activity is regulated both by regulatory proteins, such as calmodulin and caveolin, and by a variety of post-translational modifications including phosphorylation and acylation. We have previously shown that the calmodulin-binding domain peptide is a good substrate for protein kinase C [Matsubara, M., Titani, K., and Taniguchi, H. (1996) *Biochemistry* 35, 14651–14658]. Here we report that bovine eNOS protein is phosphorylated at Thr497 in the calmodulin-binding domain by PKC both *in vitro* and *in vivo*, and that the phosphorylation negatively regulates eNOS activity. A specific antibody that recognizes only the phosphorylated form of the enzyme was raised against a synthetic phosphopeptide corresponding to the phosphorylated domain. The antibody recognized eNOS immunoprecipitated with anti-eNOS antibody from the soluble fraction of bovine aortic endothelial cells, and the immunoreactivity increased markedly when the cells were treated with phorbol 12-myristate 13-acetate. PKC phosphorylated eNOS specifically at Thr497 with a concomitant decrease in the NOS activity. Furthermore, the phosphorylated eNOS showed reduced affinity to calmodulin. Therefore, PKC regulates eNOS activity by changing the binding of calmodulin, an eNOS activator, to the enzyme.

Key words: calmodulin, nitric oxide synthase, phosphorylation, protein kinase C, signal transduction.

Abbreviations: NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; PKC, protein kinase C; NMT, *N*-myristoyl transferase; BAEC, bovine aortic endothelial cells; PMA, phorbol 12-myristate 13-acetate; RP-HPLC, reversed-phase high performance liquid chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

Nitric oxide (NO) is a major messenger molecule that plays key roles in many physiological processes (*1–3*). NO is produced by nitric oxide synthase (NOS), which catalyzes the conversion of L-arginine to L-citrulline and NO. At least three isoforms of NOS have been identified and characterized, namely, neuronal NOS (nNOS) (*4*, *5*), endothelial NOS (eNOS) (*6*) and inducible NOS (iNOS) (*7*, *8*). The fundamental structures of the three isoforms are similar to each other and consist of three well-conserved domains: a cytochrome P-450-like heme protein domain and a cytochrome P-450 reductase-like flavoprotein domain, which are connected by a calmodulin-binding domain (*5*, *9*, *10*).

Since high concentrations of NO are rather toxic to cells, its production must be tightly regulated. eNOS is localized at specialized cell surface signal-transducing domains termed plasmalemmal caveolae through association with an integral-membrane protein, caveolin (*11–15*). The interaction of eNOS with caveolin leads to an inactivation of the catalytic activity, and calmodulin relieves the inhibition (*16–18*). Furthermore, eNOS translocates from the caveolae to other cell compartments upon various stimuli (*3*, *19*, *20*). Although phosphorylation and acylation (myristoylation and palmitoylation) have been implicated in the stimulation-dependent translocation and activation, exact mechanisms are not well understood. We have previously shown that a peptide derived from the calmodulin binding domain of eNOS interacts with acidic phospholipids (*21*). Furthermore, we found that the peptide is stoichiometrically phosphorylated by protein kinase C (PKC) and that the phosphorylated peptide loses its ability to bind phospholipids (*21*). This raises the possibility that the binding to phospholipids and the PKC phosphorylation of the calmodulin-binding domain of eNOS may modulate eNOS activity and its translocation.

NOS isozymes are phosphorylated by several protein kinases *in vitro*. Purified nNOS is a substrate for protein kinase A, PKC, and calmodulin-dependent protein kinases *in vitro* (*22–24*), but the effects of the phosphorylation on the physiological function have not been reported. It has been also demonstrated that iNOS in macrophage cells and eNOS in endothelial cells are phosphorylated at tyrosine residues, and that the phosphorylation seems to affect the enzymatic activities (*25*, *26*). Furthermore, the phosphorylation of eNOS at serine residues increases upon agonist stimulation, which seems to

*Present address: R&D Laboratories, Nippon Organon K.K., 1-5-90 Tomobuchi-cho, Osaka, 534-0016.
†To whom correspondence should be addressed: R&D Laboratories, Nippon Organon K.K., 1-5-90 Tomobuchi-cho, Osaka, 534-0016. Tel: +81-6-6921-9490, Fax: +81-6-6921-9078, E-mail: Mamoru.Matsubara@organon.jp
‡Present address: The Department of Aetiology, Lanzhou Medical College, Lanzhou, China.

be correlated with the increased activity and the translocation (*27*). Several protein kinases have been implicated more directly in eNOS regulation, including kinase Akt (*28–31*), AMP-activated protein kinase (*32*, *33*), cyclic GMP-dependent protein kinase (*32*), and MAP kinase (*34*). It has been established that eNOS is directly phosphorylated in endothelial cells at Ser^{1179} (bovine sequence) by the Akt protein kinase resulting in about a 2-fold increase in eNOS catalytic activity (*28–31*). Therefore, it is very important to identify the precise phosphorylation sites as well as the mechanisms of activation in all cases.

In the present report, we describe the phosphorylation of eNOS by PKC *in vitro* and *in vivo*. Thr^{497} in the calmodulin-binding domain was identified as the major phosphorylation site *in vitro*. A specific antibody that recognizes phosphothreonine 497 was raised against a synthetic phosphopeptide. The antibody recognized eNOS immunoprecipitated from bovine aortic endothelial cells (BAEC), and the immunoreactivity increased markedly when the cells were treated with phorbol 12-myristate 13-acetate (PMA). Treatment with a specific PKC inhibitor, staurosporine, diminished the immunoreactivity. Furthermore, phosphorylated eNOS showed decreased binding to calmodulin with a concomitant reduction in the catalytic activity. These findings suggest that the phosphorylation of eNOS by PKC affects the enzyme activity by inhibiting of the interaction between eNOS and calmodulin.

EXPERIMENTAL PROCEDURES

Materials—2′,5′-ADP Sepharose 4B, protein A Sepharose 4B, γ-[^{32}P]ATP and L-[^{14}C]arginine were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Calmodulin agarose, δ-aminolevulinic acid and riboflavin were obtained from Sigma (St. Louis, MO). Tissue culture reagents were from Life Technologies (Gaithersburg, MD) and Nissui Pharmaceutical (Tokyo). Monoclonal anti-eNOS antibody was purchased from Transduction Laboratories (Lexington, KY). PKC was purified from the cytoplasmic fractions of bovine brain as described previously (*35*). Calmodulin was purified from bovine brain. A 20-residue peptide corresponding to the calmodulin-binding domain of eNOS, RKKTFKEVANAVKISASLMG, was synthesized and purified as described previously (*36*). The bovine eNOS expression plasmid, Bov-eNOSpCW, was a gift from Dr. B.S.S. Masters (the University of Texas Health Science Center). pGroELS was kindly provided by Dr. A. Gatenby (Dupont). pBB131NMT was a gift from Dr. J. Gordon (Washington University). All other common chemicals were of the highest grade commercially available.

Expression and Purification of Recombinant Myristoylated eNOS—The myristoylated eNOS protein was expressed in *E. coli* and purified using a modification of the published protocol for non-myristoylated eNOS (*37*). In order to produce recombinant myristoylated eNOS in *E. coli*, three proteins, eNOS, GroELS, and *N*-myristoyl transferase (NMT) were coexpressed simultaneously. The details of the method will be described elesewhere. Expressed eNOS was purified by successive column chromatographies on 2′, 5′-ADP Sepharose and calmodulin agarose. The purified protein was kept frozen at –80°C until use. Protein concentration was determined by Bio-Rad protein assay.

Production of Site-Specific Phosphopeptide Antibody—A phosphopeptide corresponding to residues 491–503 of eNOS, GITRKKpTFKEVANC where pT is phosphothreonine, was obtained from Research Genetics (Huntsville, AL). The C-terminal cysteine residue was included for coupling to keyhole limpet hemocyanin, and the coupled phosphopeptide was used to immunize rabbits. The obtained serum reacted only with the phosphopeptide, whereas the reaction with the corresponding unphosphorylated peptide was negligible. The site-specific phosphopeptide antibody thus obtained (referred to as PT497) was used for the following experiments without further purification.

Phosphorylation of eNOS In Vitro—Phosphorylation of eNOS (150 nM) by PKC (10 nM) was carried out in 25 mM Tris-HCl, pH 7.4, containing 10 mM $MgCl_2$, 100 μM $CaCl_2$, 100 μM phosphatidylserine, 10 μM dioleoylglycerol, and 250 μM γ-[^{32}P]ATP. After the indicated time of incubation at 25°C, the reaction mixture was boiled in SDS-PAGE sample buffer and resolved by SDS-PAGE. The radioactivity was analyzed by autoradiography using imaging plates (Fujix Bioimage Analyzer BAS 1000 system, Fuji Film, Tokyo). For immunoblotting, phosphorylated and unphosphorylated eNOS were prepared as described above, except that the reaction was carried out for 60 min in the presence of cold ATP. The samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes by electroblotting, which was followed by immunostaining with anti-eNOS antibody or PT497 antibody.

Mass Spectrometry Analysis of Phosphorylation Site by PKC In Vitro—Recombinant eNOS was incubated with PKC-phosphorylation buffer in the presence of γ-[^{32}P]ATP as described above. After 60 min incubation, the reaction mixture was stopped by adding trichloroacetic acid to a final concentration of 10%, and the resulting precipitated sample was digested with L-tosylamido-2-phenylethylchloromethyl ketone–treated trypsin (Promega, Madison, WI) in 100 mM Tris-HCl, pH 8.0, in the presence of 2 M urea at 37°C for 4 h. After stopping the reaction by adding trifluoroacetic acid to a final concentration of 0.1%, the resulting peptide mixture was separated by reversed-phase high performance liquid chromatography (RP-HPLC) using a linear gradient of H_2O-acetonitrile in the presence of 0.1% trifluoroacetic acid. ^{32}P incorporation into peptides was monitored by liquid scintillation counting. The ^{32}P labeled peaks were analyzed on a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (Voyager™-RPF, PerSeptive Biosystems, Foster City, CA). MS/MS analyses of the phosphorylated peptide were performed with a Micromass Q-TOF quadrupole mass analyzer (Micromass, UK) consisting of a nanoflow electrospray source.

Cell Culture, Immunoprecipitation, and Immunoblotting—BAEC obtained from Dainippon Pharmaceutical (Osaka) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum. To activate PKC, confluent BAEC cultures in 100-mm dishes were treated with 100 nM PMA for 15 min. In some experiments, the cells were exposed to 400 nM stau-

rosporine for 15 min prior to PMA treatment. Incubations were terminated by aspirating the medium and washing the cells twice with ice-cold phosphate-buffered saline containing either PMA or staurosporine. For immunoprecipitation experiments, cells were lysed in ice-cold modified RIPA buffer (20 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM sodium ortho-vanadate, 0.1 mM PMSF, 1 μM leupeptin, and 1 μM pepstatin together with experiment reagents, 100 nM PMA, or 400 nM staurosporine). The cell lysates were centrifuged at 15,000 rpm for 15 min to remove insoluble material. The protein concentration of the supernatants was determined by Bio-Rad protein assay and adjusted to 1 mg/ml. Equal amounts of protein were precleared by incubation with protein A Sepharose 4B for 1 h at 4°C. The cleared lysates were incubated with anti-eNOS antibody for 2 h at 4°C. After adding the beads, the mixture was incubated for an additional 1 h. The immune complexes were washed three times with buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100. The immunoprecipitated proteins were eluted from the beads by boiling the samples in SDS-PAGE sample buffer and then separated by SDS-PAGE (7.5% gels), followed by transfer of the proteins to nitrocellulose membranes. The membranes were blocked by incubation in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 5% non-fat dry milk for 1 h, followed by 1.5 h incubation in primary anti-eNOS antibody (1:2,500) or PT497 antibody (1:1,000) diluted in the blocking buffer. The membranes were washed extensively in Tris-buffered saline containing 0.05% Tween 20, and then incubated with goat anti-mouse or donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody. The membranes were then washed and visualized with the ECL Plus Western blotting detection system and an ECL mini-camera (Amersham Pharmacia Biotech, Upsara, Sweden).

eNOS activity—Recombinant eNOS (150 nM) was incubated with PKC-phosphorylation buffer in the absence or presence of PKC (10 nM) as described above. At the indicated times (0, 10, 30, and 60 min), the progress of the reaction was determined by measuring the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline. The samples were added to a total volume of 200 μl assay buffer (50 mM Tris-HCl, pH 7.4, 100 μM L-[^{14}C]arginine, 0.5 mM NADPH, 0.5 mM $CaCl_2$, 60 nM calmodulin, and 10 μM tetrahydrobiopterin). The concentrations of eNOS and PKC in the assay buffer were 15 nM and 1 nM, respectively. After 30 min incubation at 37°C, the reaction was stopped by the addition of 400 μl of cold buffer (100 mM HEPES, pH 5.5, containing 10 mM EDTA). L-[^{14}C]citrulline was separated by passing the reaction mixtures through a Dowex AG50WX-8 (Na^+ form, Bio-Rad) cation exchange column and quantitated by liquid scintillation counting. NOS activity of recombinant NOS before the addition of PKC was expressed as 100% (control).

Interaction of eNOS protein or Peptide with Calmodulin—Recombinant eNOS (150 nM) or the calmodulin-binding domain peptide of eNOS (15 μM) was phosphorylated by PKC (10 nM for protein, 500 nM for peptide) for 60 min as described above. The phosphorylated or unphosphorylated forms were mixed with calmodulin agarose beads in 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM $CaCl_2$ and incubated for 30 min at room temperature. After a short centrifugation in a tabletop centrifuge, the supernatants and pellets were subjected to SDS-PAGE. Tricine buffer system was used for peptides and the peptides were stained with Coomassie Brilliant Blue (*21*). eNOS proteins were detected by immunoblotting using anti-eNOS or PT497 antibodies.

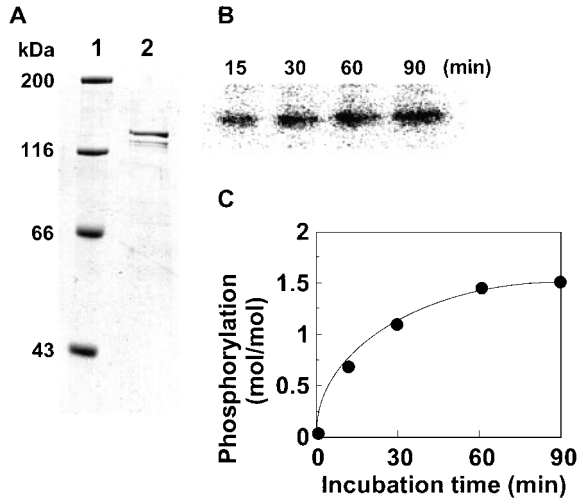

Fig. 1. **Phosphorylation of eNOS by PKC.** A: Recombinant myristoylated eNOS expressed in *E. coli* (lane 2) was separated by 7.5% SDS-PAGE and stained with Coomassie Brilliant Blue. Lane 1 shows molecular mass markers. B: Recombinant eNOS was incubated with PKC reaction buffer for the indicated times. The reaction was stopped by the addition of sample buffer, and the samples were analyzed by SDS-PAGE on 7.5% gels followed by autoradiography. C: The radioactivity was quantified by scintillation counting of the excised gel.

RESULTS

Phosphorylation of eNOS by PKC In Vitro—We have previously shown that PKC phosphorylates a peptide derived from the calmodulin-binding domain of eNOS specifically at Thr^{497} in the bovine sequence (*21*). To examine whether the intact eNOS protein is phosphorylated by PKC, recombinant bovine eNOS was produced in *E. coli*. Since eNOS is a myristoylated protein, a coexpression system with yeast NMT was used to produce myristoylated eNOS. As shown in Fig. 1A, the purified protein preparation showed a major band on SDS-PAGE with a molecular mass of 135 kDa, which is in good agreement with the molecular mass of native or recombinant non-myristoylated eNOS (*37*). Sequencing of the purified protein indicated that the N-terminus of eNOS is blocked, suggesting that the purified eNOS was myristoylated. The authenticity of the protein was further demonstrated by the NOS activity and the immunoreactivity as described below.

When the purified eNOS was incubated with PKC in the presence of γ-[^{32}P]ATP, the incorporation of radioactivity into the eNOS protein was clearly observed (Fig. 1B). As shown in Fig. 1C, the level of the eNOS phosphorylation by PKC increased in a time-dependent manner and reached a plateau at around 1.5 mol phosphate/mol

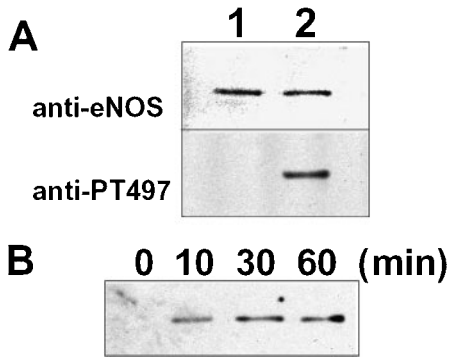

Fig. 2. **Specificity of anti-PT497 in the phosphorylation of eNOS by PKC.** A: Unphosphorylated eNOS (lane1) and eNOS phosphorylated by PKC (lane2) were separated by 7.5% SDS-PAGE, electrophoretically transferred onto nitrocellulose membranes, and analyzed by reaction with the anti-eNOS antibody and phosphospecific antibody, PT497, as described under "EXPERIMENTAL PROCEDURES". B: Recombinant eNOS was incubated with PKC reaction buffer for the indicated times, and electrophoretically transferred onto nitrocellulose membranes. The membranes were analyzed by reaction with PT497.

of protein after 1 h. Therefore, eNOS is a substrate of PKC *in vitro*, and seems to contain one or two major phosphorylation sites. Whether eNOS has one or two PKC phosphorylation sites was shown by mass spectrometric analysis as described in the next Section.

Identification of the PKC Phosphorylation Site on eNOS—To examine whether Thr[497] in intact eNOS is phosphorylated by PKC *in vitro*, we raised a rabbit polyclonal antibody (referred to as PT497) against a synthetic phosphopeptide containing phosphothreonine 497 and surrounding eNOS sequences as described under "EXPERIMENTAL PROCEDURES." The antibody was tested by comparing its reactivity against the phosphorylated and unphosphorylated forms of the immunizing peptide. The antibody PT497 recognized only the phosphopeptide but not the unphosphorylated peptide (data not shown). We then examined the reactivity of eNOS phosphorylated by PKC *in vitro* by Western blotting. As shown Fig. 2A, the anti-phospho-specific antibody PT497 recognized only phosphorylated eNOS but not unphosphorylated eNOS, whereas the anti-eNOS antibody reacted with both unphosphorylated and phosphorylated proteins to a similar extent. The immunoreactivity increased in a time-dependent manner during PKC-phosphorylation (Fig. 2B). These results suggest that Thr[497] is phosphorylated by PKC not only in the synthetic peptide (*21*) but also in the intact eNOS protein.

Our radioisotope experiment demonstrated that approximately 1.5 mol of phosphate/mol of protein is incorporated into eNOS. This suggests that a site other than Thr[497] may be partially phosphorylated by PKC. To identify directly the PKC phosphorylation site, the phosphorylated eNOS was digested with trypsin and the resulting peptides were separated by RP-HPLC followed by MALDI-TOF mass spectrometric analysis. When the tryptic peptides were separated by RP-HPLC, one major radioactive peak was obtained (data not shown). We then analyzed the predominant tryptic peptide mass. As shown in Fig. 3A, the peptide mass included a peak at

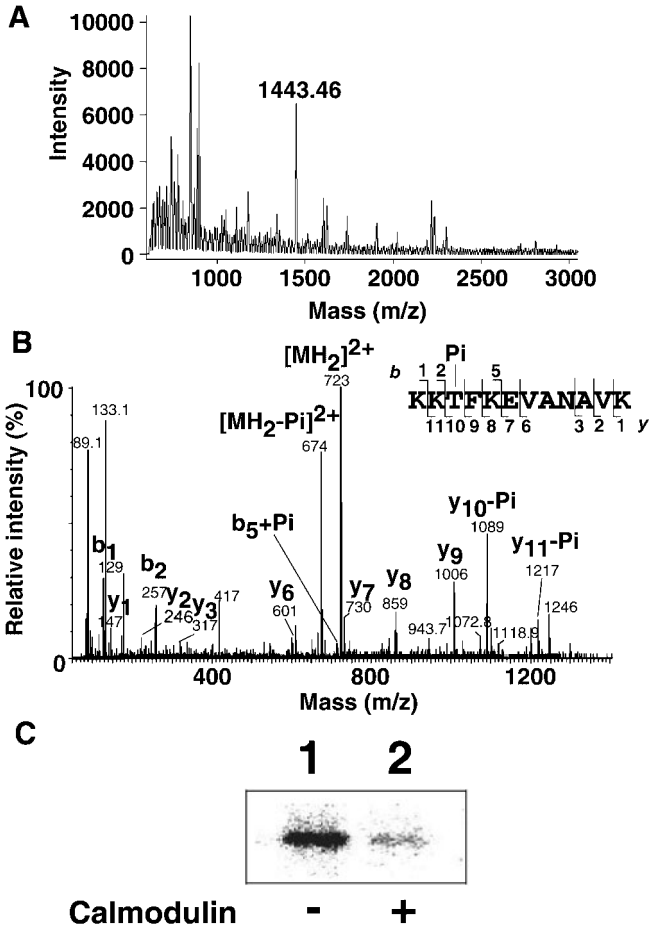

Fig. 3. **Identification of the in vitro phosphorylation site at Thr497 on eNOS.** A: 32P-labeled eNOS was digested with trypsin. The peptides were separated by RP-HPLC as described under "EXPERIMENTAL PROCEDURES." One main peak was analyzed by MALDI-TOF mass spectrometry. The peptide mass included a peak at 1,443.5 Da. This mass is almost identical to the calculated molecular mass of 1,443.6 Da, which corresponds to the mono-phosphorylated form of KKTFKEVANAVK. B, Tandem mass spectra of the phosphorylated peptide allowed the identification of the site of phosphorylation. The doubly-charged ion of the phosphorylated peptide (*m*/*z* 723) was subjected to collision-induced dissociation in the Q-TOF mass analyzer. Fragment ions observed are indicated above and below the peptide sequence. C, Effects of calmodulin on the phosphorylation of eNOS by PKC. eNOS (0.15 μM) was phosphorylated by PKC in the absence or presence of calmodulin (2 μM)for 45 min. The reaction was stopped by the addition of sample buffer, and samples were analyzed by SDS-PAGE on a 7.5% gel followed by autoradiography.

1,443.5 Da. This mass is identical to the calculated molecular mass of 1,443.6 Da, which corresponds to the mono-phosphorylated form of KKTFKEVANAVK. The tandem mass spectra of the tryptic peptide clearly showed that The[497] is the phosphorylation site for PKC (Fig. 3B). Furthermore, the addition of calmodulin to the PKC-phosphorylation reaction buffer for eNOS markedly reduced the phosphorylation of eNOS by PKC (Fig. 3C), suggesting that the binding of calmodulin to the calmodulin-binding domain interferes with the PKC accessibility to Thr[497] on eNOS. Although it is difficult to interpret the incorporation of 1.5 mol phosphate/mole of eNOS

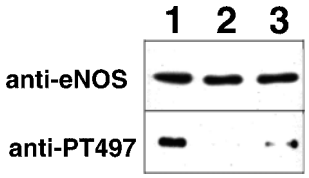

Fig. 4. **Phosphorylation of Thr497 of eNOS in BAECs.** BAECs were stimulated by PMA (lane 1), PMA+staurosporine (lane 2) or received no stimulation (lane 3), and the cell lysates were immunoprecipitated with anti-NOS antibody. The immunoprecipitants were immunoblotted with the PT497 antibody, which recognizes only the phosphorylated eNOS as described under "EXPERIMENTAL PROCEDURES."

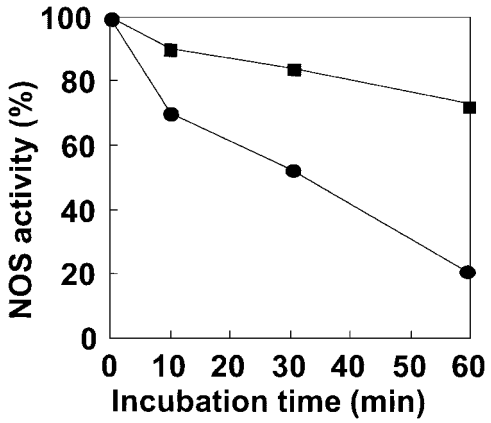

Fig. 5. **Effects of phosphorylation on eNOS activity.** Recombinant eNOS (150 nM) was preincubated with or without PKC (10 nM), and assayed for NOS activity as described under "EXPERIMENTAL PROCEDURES". At the indicated times (0, 10, 30, 60 min), the reaction progress was determined by measuring the conversion of L-[^{14}C]arginine to L-[^{14}C]citrulline. The NOS activity of recombinant NOS before the addition of PKC was expressed as 100%. In the assay buffer, the concentrations of eNOS, PKC, and calmodulin were 15, 1, and 60 nM. The changes in the activity of unphosphorylated eNOS and phosphorylated eNOS are shown by closed rectangles and closed circles, respectively.

obtained by radioisotope experiment, these data show that the primary PKC phosphorylation site on eNOS is Thr^{497}.

Phosphorylation of Thr^{497} of eNOS in Cells—To examine whether eNOS is phosphorylated by PKC *in vivo*, we studied the phosphorylation of eNOS in BAEC. The cell lysate was immunoprecipitated with anti-NOS antibody, and the immunoprecipitant was immunoblotted with the PT497 antibody, which recognizes only phosphorylated eNOS. Weak staining was observed with eNOS immunoprecipitated from the control cells (Fig. 4), suggesting that the eNOS protein is phosphorylated to some extent at Thr^{497} under basal conditions. When the cells were treated with PMA, a potent PKC activator, the immunoreactivity of the immunoprecipitated eNOS increased significantly. In contrast, treatment of the cells with a PKC-specific inhibitor, staurosporine, suppressed the reactivity almost completely. These results suggest that Thr^{497} on eNOS is phosphorylated by PKC even under basal conditions, and that the activation of PKC leads to the increased phosphorylation of eNOS by PKC. Recently, the same results have been shown by Michell and coworkers (*38*). Also, they demonstrated that PMA treatment reduces eNOS activity in BAEC (*38*). The weak phosphorylation of eNOS at Thr^{497} observed with eNOS obtained from the control cells may be in accordance with the recent phosphoamino acid analysis, which showed that eNOS is mostly phosphorylated at serine residues, but that threonine and tyrosine are also phosphorylated weakly in non-stimulated cells (*39*).

Effects of Phosphorylation on the Catalytic Activity of eNOS—To gain insight into the function of eNOS phosphorylation by PKC, we next examined the effects of phosphorylation on the catalytic activity of eNOS. Recombinant eNOS was preincubated with or without PKC, and assayed for the NOS activity. Even in the absence of PKC, the catalytic activity of eNOS decreased gradually to 70% after 60 min of incubation (Fig. 5), reflecting the intrinsic instability of the protein. However, the catalytic activity of eNOS decreased markedly when preincubated with PKC. After 60 min, when eNOS was almost stoichiometrically phosphorylated by PKC under the conditions used, the NOS activity decreased to 20% of the initial value. Since the concentrations of eNOS, PKC and calmodulin were 15, 1 and 60 nM, respectively, in the assay buffer, the competitive inhibition of PKC for calmodulin-binding domain should be negligible. Furthermore, when recombinant eNOS was preincubated with PKC in the absence of ATP, the catalytic activity of eNOS was the same as that of eNOS preincubated without PKC. Therefore, the inhibition of the catalytic activity is due to phosphorylation by PKC, not competitive inhibition of PKC for the calmodulin-binding site. These results suggest that the phosphorylation of eNOS in the calmodulin-binding domain (Thr^{497}) by PKC negatively regulates the eNOS catalytic activity.

Effects of Phosphorylation on the Interaction with Calmodulin—Since the major phosphorylation site, Thr^{497}, is located in the calmodulin-binding domain of eNOS, it is possible that the decrease in the activity results from the reduced affinity of the phosphorylated enzyme for calmodulin. We have previously shown that the PKC phosphorylation affects the binding of the calmodulin-binding domain in eNOS to acidic phospholipids (*21*). To test this possibility, we examined the effects of PKC phosphorylation on the calmodulin-binding ability of the eNOS peptide and protein. The calmodulin-binding domain peptide of eNOS was preincubated with or without PKC for 60 min, and the samples were incubated with calmodulin agarose beads followed by centrifugation. As shown in Fig. 6A, the control eNOS peptide co-sedimented with the calmodulin agarose beads, whereas the phosphorylated eNOS peptide was detected only in the supernatant. Interestingly, the mobility of the phosphorylated peptide in the gel decreased slightly, and it was possible to distinguish the phosphorylated from the unphosphorylated peptide. The phosphopeptide was found only in the unbound fraction, while the unphosphorylated peptide was found only in the bound fraction (Fig. 6A). Therefore, there is a clear correlation between the phosphorylation of the calmodulin-binding domain and the binding to calmodulin. Similar results were obtained for the binding of the intact eNOS protein to calmodulin. As shown in Fig.

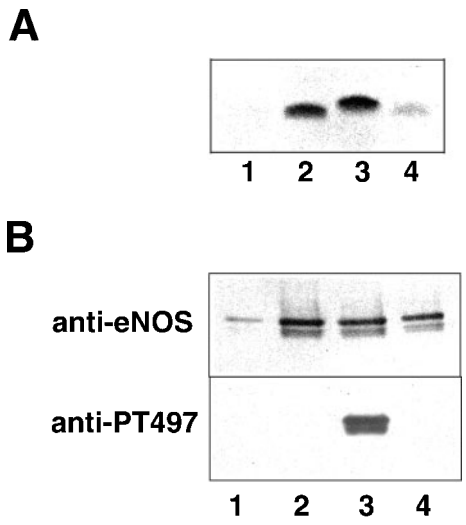

Fig. 6. **Effects of phosphorylation on the binding of eNOS to calmodulin.** Recombinant eNOS (150 nM) and the calmodulin-binding peptide of eNOS (15 μM were phosphorylated by PKC (10 nM for protein, 500 nM for peptide) for 60 min. The phosphorylated or unphosphorylated forms were mixed with calmodulin agarose beads in 20 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM $CaCl_2$, and incubated 30 min at room temperature. After a short centrifugation on a tabletop centrifuge, the supernatants and pellets were subjected to SDS-PAGE. A: The unphosphorylated (lanes 1 and 2) or phosphorylated (lanes 3 and 4) peptides were mixed with calmodulin agarose beads, and then the supernatant (lanes 1 and 3), and pellet (lanes 2 and 4) fractions were resolved in a Tricine gel system and the peptides were stained with Coomassie Brilliant Blue. B: The unphosphorylated (lanes 1 and 2) or phosphorylated (lanes 3 and 4) eNOS proteins were also mixed with calmodulin agarose beads, and the supernatant (lanes 1 and 3) and pellet (lanes 2 and 4) fractions were analyzed by immunoblotting with anti-eNOS (upper panel) or PT497 antibody (lower panel).

6B, the unphosphorylated eNOS bound calmodulin-agarose beads. When eNOS was first incubated with PKC and ATP, and then mixed with the calmodulin-beads, the protein was found in both the unbound and bound fractions (Fig. 6B). Immunoblotting of the same gel with the PT497 antibody clearly showed the eNOS phosphorylated at Thr^{497} to be present only in the unbound fraction, whereas the eNOS protein associated with calmodulin was not phosphorylated at Thr^{497}. In our experiments, PKC did not disturb the binding of eNOS to calmodulin-beads for the same reason as described above (the concentration of PKC was much lower than those of eNOS and calmodulin). These results clearly demonstrate that the phosphorylation of a single amino acid (Thr^{497}) can regulate the affinity of the calmodulin-binding domain of eNOS to calmodulin, and that the dissociation of calmodulin leads to the inactivation of eNOS.

DISCUSSION

The importance of NO as one of the major physiological signal mediators has been well established. However, because of its high reactivity with important cellular components including proteins and nucleic acids, the production of NO must be tightly regulated. The regulatory mechanisms of NOS, therefore, have been the focus of intensive studies. As for the endothelial NOS isozyme, several types of protein phosphorylation have been suggested to play such a role. It is well established that the phosphorylation of eNOS at serine residues increases when endothelial cells are stimulated by receptor-dependent and independent agonists including shear stress (*27*). The phosphorylation is assumed to be associated with the agonist-induced translocation of eNOS from the membrane to the cytosol. Recently, specific sites of phosphorylation in eNOS and specific protein kinases that mediate the phosphorylation have been identified. Several laboratories have shown that serine/threonine kinase Akt can phosphorylate eNOS at Ser^{1179} and activate the enzyme, leading to NO production (*28–31*).

In this report, we present several lines of evidence that suggest the direct involvement of PKC in the regulation of eNOS activity. Our results clearly show that the phosphorylation of eNOS at Thr^{497} by PKC abolishes the calmodulin-binding ability of the domain, which results in the inactivation of eNOS. This suggests that the PKC-dependent phosphorylation of eNOS may play an important role in the negative regulation of eNOS. In fact, the involvement of a PKC-dependent pathway in eNOS regulation has been suggested in several cells and tissues. Many groups have reported that the treatment of endothelial cells with PMA diminishes NO production (*38*, *40–42*) and there is one report that the increased PKC activity leads to decreased NO production in glomeruli isolated from diabetic rats (*43*). In our hands, eNOS was found to be phosphorylated in its calmodulin-binding domain not only in PMA-treated endothelial cells, but also in control BAEC cells. The fact that the addition of the specific PKC inhibitor staurosporine completely abolished the phosphorylation suggests that the basal level phosphorylation is also catalyzed by PKC. Phosphoamino acid analysis of eNOS in endothelial cells revealed that eNOS is phosphorylated mainly at serine residues, but also at threonine residues (*44*). It is conceivable that at least some of the latter is at Thr^{497} in the calmodulin-binding domain. All in all, these results indicate that the phosphorylation of eNOS by PKC is physiologically relevant.

We have previously shown that the calmodulin-binding domain of eNOS is directly involved in the interaction of eNOS with acidic membrane phospholipids and that phosphorylation of the domain by PKC decreases its affinity for phospholipids (*21*). The same domain, therefore, is involved in complex regulation and the interaction of eNOS with other signal transduction pathways. These characteristics are shared by other proteins such as MARCKS and GAP-43, major *in vivo* PKC substrates (*45*, *46*). In these proteins, the calmodulin-binding domains are, at the same time, the domains phosphorylated by PKC. The phosphorylation of the domains by PKC significantly reduces their ability to bind to membrane phospholipids and/or calmodulin (*45*, *46*). Reversible translocation between the membrane and cytosol in response to PKC-dependent phosphorylation and calmodulin-binding plays important roles in the regulation and function of these proteins. Similar mechanisms have also been suggested for other proteins including adducin and AKAP79 (*47*, *48*). Now, we can add eNOS to the collection of PKC substrate proteins whose functions and membrane-targeting are regulated by both PKC-depend-

ent phosphorylation and calmodulin-binding. Since PKC, calmodulin and phospholipids bind to the same domain, and since the bindings are mutually competitive, these domains of basic amphiphilic nature may be one of the important crosstalk points between various signal transduction pathways.

Since the other two NOS isozymes share the overall structural characteristics of eNOS, it is of interest to examine whether the same regulatory mechanism is operative with these isozymes. We have already shown that the calmodulin-binding domains of nNOS and iNOS have the ability to bind to acidic phospholipids (*21*). Furthermore, the binding of acidic phospholipids to the domain results in the inhibition of nNOS activity. As for the phosphorylation by PKC, there are several reports demonstrating the *in vitro* phosphorylation of nNOS by PKC (*22*, *23*). Furthermore, the phosphorylation by PKC is accompanied by reduced NO production (*22*). However, although the calmodulin-binding domain of nNOS contains one potential PKC phosphorylation site (a serine residue), a synthetic peptide derived from the calmodulin-binding domain of nNOS was not phosphorylated by PKC to a significant extent (data not shown). The lack of PKC-dependent phosphorylation of nNOS in cerebellar slices has also been reported (*49*). Taken together, nNOS may be not a substrate of PKC *in vivo* and other regulatory mechanisms may be operative in the regulation of nNOS activity. Since iNOS lacks any potential phosphorylation sites in its calmodulin-binding domain, the regulation of NOS activity by PKC phosphorylation in the calmodulin-binding domain seems to be specific to eNOS.

Recently, other groups have shown that bradykinin activates eNOS in endothelial cells by triggering dephosphorylation at Thr^{497}, and PKC signaling causes eNOS phosphorylation at Thr^{497} as well as promoting dephosphorylation of Ser^{1177} (*38*, *50*). Thus, phosphorylation and dephosphorylation reactions at Thr^{497} may be tightly regulated by multiple protein kinases and phosphatases.

Finally, protein phosphorylation is not the only regulatory mechanism of eNOS. It has been suggested that NOS functions are also regulated by several signaling proteins. Recent publications demonstrate that eNOS is localized in caveolae of endothelial cells through association with an integral membrane protein called caveolin (*11–15*). The direct binding of caveolin to eNOS has inhibitory effects on the eNOS activity, but the inhibition can be relieved by calmodulin (*16–18*). It is of interest to note in this context that the specific localization of PKC to caveolae and the involvement of PKC in the regulation of caveolae functions have been demonstrated very recently (*51*, *52*). PKC interacts directly with caveolin and the activity of PKC is also regulated by the interaction with the caveolin scaffolding domain as in the case of eNOS (*53*). A close physical location between PKC and eNOS in caveolae, therefore, may be significant for facilitating the phosphorylation reaction of eNOS by PKC.

We thank Dr. B.S.S. Masters for providing us with the eNOS expression vector, Dr. A. Gatenby for the GroELS expression vector, and Dr. J. Gordon for the NMT expression vector. We thank Dr. H. Taniguchi for valuable discussion about the manuscript. This work was supported in part by grants-in-aid from the Fujita Health University (to K.T.) and the Asahi Glass Foundation (to N.H.) and by grants-in-aid for Scientific Research on Priority Areas (C) "Genome Information Science" (to N.H.) and Young Scientists (B) (to M.M., to N.H.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

REFERENCES

1. Nathan, C. (1992) Nitric Oxide as a secretory product of mamalian cells. *FASEB J.* **6**, 3051–3064
2. Marletta, M.A. (1993) Nitric oxide synthase structure and mechanism. *J. Biol. Chem.* **268**, 12231–12234
3. Michel, T. and Feron, O. (1997) Nitric oxide synthases: which, where, how, and why? *J. Clin. Invest.* **100**, 2146–2152
4. Schmidt, H.H.H.W., Wilke, P., Evers, B., and Bohme, E. (1989) Enzymatic formation of nitrogen oxides from l-arginine in bovine brain cytosol. *Biocem. Biophys. Res. Commun.* **165**, 813–819
5. Bredt, D.S., Hwang, P.M., Glatt, G.E., Lowenstein, C., Reed, R.R., and Snyder, S.H. (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* **351**, 714–718
6. Pollock, J.S., Forstermann, U., Mitchell, J.A., Warner, T.D., Schmidt, H.H.H.W., Nakane, M., and Murad, F. (1991) Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. *Proc. Natl Acad. Sci. USA* **88**, 10480–10484
7. Stuehr, D.J., Cho, H.J., S., K.N., Weise, M., and Nathan, C. (1991) Purification and characterization of the cytokine-induced macrophage nitric oxide synthase: an FAD- and FMN-containing flavoprotein. *Proc. Natl. Acad. Sci.USA* **88**, 7773–7777
8. Xie, Q.W., Che, H.J., Galaycay, J., Mumford, R.A., Swiderek, K.M., Lee, T.D., Ding, A., Troso, T., and Nathan, C. (1992) Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. *Science* **256**, 225–228
9. MacMillan, M.A., Bredt, D.S., Hirsch, D.J., Snyder, S.H., Clark, J.E., and Masters, B.S.S. (1992) Cloned, expressed rat cerebellar nitric oxide synthase contains stoichiometric amounts of heme, which binds carbon monoxide. *Proc. Natl Acad. Sci. USA* **89**, 11141–11145
10. Klatt, P., Schmidt, K., and Mayer, B. (1992) Brain nitric oxide synthase is a haemoprotein. *Biochem. J.* **288**, 15–17
11. Feron, O., Belhassen, L., Kobzik, L., Smith, T.W., Kelly, R.A., and Michel, T. (1996) Endothelial nitric oxide synthase targeting to caveolae: Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells. *J. Biol. Chem.* **271**, 22810–22814
12. Liu, J., Garcia-Cardena, G., and Sessa, W.C. (1996) Palmitoylation of endothelial nitric oxide synthase is necessary for optimal stimulated release of nitric oxide: implications for caveolae localization. *Biochemistry* **35**, 13277–13281
13. Shaul, P.W., Smart, E.J., Robinson, L.J., German, Z., Yuhanna, I.S., Ying, Y., Anderson, R.G., and Michel, T. (1996) Acylation targets emdothelial nitric-oxide synthase to plasmalemmal caveolae. *J. Biol. Chem.* **271**, 6518–6522
14. Garcia-Cardena, G., Oh, P., Liu, J., Schnitzer, J.E., and Sessa, W.C. (1996) Targeting of nitric oxide synthase to endothelial cell caveolae *via* palmitoylation: implications for nitric oxide signaling. *Proc. Natl Acad. Sci. USA* **93**, 6448–6453
15. Michel, J.B., Feron, O., Sase, K., Prabhakar, P., and Michel, T. (1997) Caveolin versus calmodulin. Counterbalancing allosteric modulators of endothelial nitric oxide synthase. *J. Biol. Chem.* **272**, 25907–25912
16. Ju, H., Zou, R., Venema, V.J., and Venema, R.C. (1997) Direct interaction of endothelial nitric-oxide synthase and caveolin-1 inhibits synthase activity. *J. Biol. Chem.* **272**, 18522–18525
17. Garcia-Cardena, G., Martasek, P., Masters, B.S., Skidd, P.M., Couet, J., Li, S., Lisanti, M.P., and Sessa, W.C. (1997) Dissecting the interaction between nitric oxide synthase (NOS) and

caveolin: Functional significance of the nos caveolin binding domain in vivo. *J. Biol. Chem.* **272**, 25437–25440
18. Michel, J.B., Feron, O., Sacks, D., and Michel, T. (1997) Caveolin versus calmodulin: Counterbalancing allosteric modulators of endothelial nitric oxide synthase. *J. Biol. Chem.* **272**, 15583–15586
19. Feron, O., Saldana, F., Michel, J.B., and Michel, T. (1998) The endothelial nitric-oxide synthase-caveolin regulatory cycle. *J. Biol. Chem.* **273**, 3125–3128
20. Feron, O., Michel, J.B., Sase, K., and Michel, T. (1998) Dynamic regulation of endothelial nitric oxide synthase: Complementary roles of dual acylation and caveolin interactions. *Biochemistry* **37**, 193–200
21. Matsubara, M., Titani, K., and Taniguchi, H. (1996) Interaction of calmodulin-binding domain peptides of nitric oxide synthase with membrane phospholipids: Regulation by protein phosphorylation and Ca^{2+}-calmodulin. *Biochemistry* **35**, 14651–14658
22. Bredt, D.S., Ferris, C.D., and Snyder, S.H. (1992) Nitric oxide synthase regulatory sites. Phosphorylation by cyclic AMP-dependent protein kinase, protein kinase C, and calcium/calmodulin protein kinase: Identification of flavin and calmodulin binding sites. *J. Biol. Chem.* **267**, 10976–10981
23. Brune, B. and Lapetina, E.G. (1991) Phosphorylation of nitric oxide synthase by protein kinase A. *Biochem. Biophys. Res. Commun.* **181**, 921–926
24. Hayashi, Y., Nishio, M., Naito, Y., Yokokura, H., Nimura, Y., Hidaka, H., and Watanabe, Y. (1999) Regulation of Neuronal Nitric Oxide Synthase by Calmodulin Kinases. *J. Biol. Chem.* **274**, 20597–20602
25. Pan, J., Burgher, K.L., Szczepanik, A.M., and Ringheim, G.E. (1996) Tyrosine phosphorylation of inducible nitric oxide synthase: Implications potential post-translational regulation. *Biocem. J.* **314**, 889–894
26. Garcia-Cardena, G., Fan, R., Stern, D.F., Liu, J., and Sessa, W.C. (1996) Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with caveolin-1. *J. Biol. Chem.* **271**, 27237–27240
27. Michel, T., Li, G.K., and Busconi, L. (1993) Phosphorylation and subcellular translocation of endothelial nitric oxide synthase. *Proc. Natl Acad. Sci. USA* **90**, 6252–6256
28. Fulton, D., Gratton, J.P., McCabe, T.J., Fontana, J., Fujio, Y., Walsh, K., Franke, T., Papapetropoulos, A., and Sessa, W.C. (1999) Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* **399**, 597–601
29. Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R., and Zeiher, A.M. (1999) Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* **399**, 601–605
30. Michell, B.J., Griffiths, J.E., Mitchelhill, K.I., Rodriguez-Crespo, I., Tiganis, T., Bozinovski, S., Ortiz de Montellano, P.R., Kemp, B.E., and Pearson, R.B. (1999) The Akt kinase signals directly to endothelial nitric oxide synthase. *Curr. Biol.* **9**, 845–848
31. Gallis, B., Corthals, G.L., Goodlett, D.R., Ueba, H., Kim, F., Presnell, S.R., Figeys, D., Harrison, D.G., Berk, B.C., Abersold, R., and Corson, M.A. (1999) Identification of Flow-dependent endothelial nitric-oxide synthase phosphorylation sites by mass spectrometry and regulation of phosphorylation and nitric oxide production by the phosphatidylinositol 3-kinase inhibitor LY294002. *J. Biol. Chem.* **274**, 30101–30108
32. Butt, E., Bernhardt, M., Smolenski, A., Kotsonis, P., Frohlich, L.G., Sickmann, A., Meyer, H.E., Lohmann, S.M., and Schmidt, H.H.H.W. (2000) Endothelial Nitric Oxide Synthase is activated and becomes calcium independent upon phosphorylation by cyclic nucleotide-dependent protein kinases. *J. Biol. Chem.* **275**, 5179–5187
33. Chen, Z.P., Mitchelhill, K.I., Michell, B.J., Stapleton, D., Rodriguez-Crespo, I., Witters, L.A., Power, D.A., Ortiz de Montellano, P.R., and Kemp, B.E. (1999) AMP-activated protein kinase phosphorylation of endothelial NO synthase. *FEBS Lett.* **443**, 285–289
34. Bernier, S.G., Haldar, S., and Michel, T. (2000) Bradykinin-regulated interaction of the Mitogen-activated Protein Kinase Pathway with the Endothelial Nitric oxide Synthase. *J. Biol. Chem.* **275**, 30707–30715
35. Manenti, S., Sorokine, O., Van Dorsselaer, A., and Taniguchi, H. (1992) Affinity purification and characterization of myristoylated alanine- rich protein kinase C substrate (MARCKS) from bovine brain. Comparison of the cytoplasmic and the membrane-bound forms. *J. Biol. Chem.* **267**, 22310–22315
36. Matsubara, M., Hayashi, N., Titani, K., and Taniguchi, H. (1997) Circular dichroism and ^{1}H NMR studies on the structures of peptides derived from the calmodulin-binding domains of inducible and endothelial nitric-oxide synthase in solution and in complex with calmodulin. Nascent alpha-helical structures are stabilized by calmodulin both in the presence and absence of Ca^{2+}. *J. Biol. Chem.* **272**, 23050–23056
37. Martasek, P., Liu, Q., Liu, J., Roman, L.J., Gross, S.S., Sessa, W.C., and Masters, B.S.S. (1996) Characterization of bovine endothelial nitric oxide synthase in *E. coli*. *Biochem. Biophys. Res. Commun.* **219**, 359–365
38. Michell, B.J., Chen, Z., Tiganis, T., Stapleton, D., Katsis, F., Power, D.A., Sim, A.T., and Kemp, B.E. (2001) Co-ordinate control of endothelial NO synthase phosphorylation by PKC and PKA. *J. Biol. Chem.* **276**, 17625–17628
39. Fleming, I., Bauersachs, J., Fisslthaler, B., and Busse, R. (1998) Ca^{2+}-independent activation of the endothelial nitric oxide synthase in response to tyrosine phosphatase inhibitors and fluid shear stress. *Circ. Res.* **82**, 686–695
40. Tsukahara, H., Gordienko, D.V., and Goligorsky, M.S. (1993) Continuous monitoring of nitric oxide release from human umbilical vein endothelial cells. *Biochem. Biophys. Res. Commun.* **193**, 722–729
41. Davda, R.K., Chandler, L.J., and Guzman, N.J. (1994) Protein kinase C modulates receptor-independent activation of endothelial nitric oxide synthase. *Eur. J. Pharmacol.* **266**, 237–244
42. Hirata, K., Kuroda, R., Sakoda, T., Katayama, M., Inoue, N., Suematsu, M., Kawashima, S., and Yokoyama, M. (1995) Inhibition of endothelial nitric oxide synthase activity by protein kinase C. *Hypertension* **25**, 180–185
43. Craven, P.A., Studer, R.K., and DeRubertis, F.R. (1994) Impaired nitric oxide-dependent cyclic guanosine monophosphate generation on glomeruli from diabetic rats. *J. Clin. Invest.* **93**, 311–320
44. Corson, M.A., James, N.L., Latta, S.E., Nerem, R.M., Berk, B.C., and Harrison, D.G. (1996) Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. *Circ. Res.* **79**, 984–91
45. Taniguchi, H. and Manenti, S. (1993) Interaction of myristoylated alanine-rich protein kinase C substrate (MARCKS) with membrane phospholipids. *J. Biol. Chem.* **268**, 9960–9963
46. Hayashi, N., Matsubara, M., Titani, K., and Taniguchi, H. (1997) Circular dichroism and ^{1}H nuclear magnetic resonance studies on the solution and membrane structures of GAP-43 calmodulin-binding domain. *J. Biol. Chem.* **272**, 7639–7645
47. Matsuoka, Y., Li, X., and Bennett, V. (1998) Adducin is an in vivo substrate for protein kinase C: phosphorylation in the MARCKS-related domain inhibits activity in promoting spectrin-actin complexes and occurs in many cells, including dendritic spines of neurons. *J. Cell Biol.* **142**, 485–497
48. Dell'Acqua, M.L., Faux, M.C., Thorburn, J., Thorburn, A., and Scott, J.D. (1998) Membrane-targeting sequences on AKAP79 bind phosphatidylinositol-4,5-bisphosphate. *EMBO J.* **17**, 2246–2260
49. Okada, D. (1996) Differential effects of protein kinase C on neuronal nitric oxide synthase activity in rat cerebellar slices and *in vitro*. *J Chem. Neuroanat.* **10**, 213–220
50. Harris, M.B., Ju, H., Venema, V.J., Liang, H., Zou, R., Michell, B.J., Chen, Z.P., Kemp, B.E., and Venema, R.C. (2001) Reciprocal phosphorylation and regulation of the endothelial nitric oxide synthase in response to bradykinin stimulation. *J. Biol. Chem.* **276**, 16587–16591

51. Smart, E.J., Ying, Y.S., and Anderson, R.G. (1995) Hormonal regulation of caveolae internalization. *J. Cell Biol.* **131**, 929–938
52. Mineo, C., Ying, Y.S., Chapline, C., Jaken, S., and Anderson, R.G. (1998) Targeting of protein kinase Calpha to caveolae. *J. Cell Biol.* **141**, 601–610
53. Oka, N., Yamamoto, M., Schwencke, C., Kawabe, J., Ebina, T., Ohno, S., Couet, J., Lisanti, M.P., and Ishikawa, Y. (1997) Caveolin interaction with protein kinase C. Isoenzyme-dependent regulation of kinase activity by the caveolin scaffolding domain peptide. *J. Biol. Chem.* **272**, 33416–33421