Interaction of Calmodulin-Binding Domain Peptides of Nitric Oxide Synthase with Membrane Phospholipids: Regulation by Protein Phosphorylation and Ca^{2+}-Calmodulin†

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ABSTRACT: Endothelial nitric oxide synthase (eNOS) is unique among the NO synthase isozymes in being modified with myristoyl group, which appears to be necessary for its membrane association. However, the presence of myristoylated eNOS in cytosolic fraction after the stimulation-dependent translocation of the enzyme from membrane to cytosol suggests that other regions may be involved in the eNOS−membrane interaction and its regulation. In this study, we have synthesized a 20-amino acid peptide corresponding to the putative calmodulin-binding domain of human eNOS and studied the interaction of the peptide with calmodulin and with various membrane phospholipids. The peptide formed a stoichiometric complex with calmodulin. Upon addition of various acidic phospholipids, the peptide showed a drastic conformational change from random coil to α-helix, as was evidenced by circular dichroism spectroscopy. These results suggest that the same domain of eNOS binds both calmodulin and membrane phospholipids. Furthermore, we found that the synthetic peptide was phosphorylated *in vitro* by protein kinase C. Phosphorylation of the peptide decreased its interaction with membrane phospholipids. Thus, our results raise the possibility that the calmodulin-binding domain is directly involved in the membrane association of eNOS and that phosphorylation of the domain and Ca^{2+}-calmodulin may regulate the interaction. Synthetic peptides corresponding to the calmodulin-binding domains of macrophage and neuronal isozymes showed similar abilities to bind phospholipids, suggesting that the calmodulin-binding domains of NO synthase serve as the phospholipid-binding domains as well.

The enzyme nitric oxide synthase (NOS)[1] in diverse mammalian tissues catalyzes the formation of one of the major intracellular messengers, nitric oxide (NO), from L-arginine (Nathan, 1992; Marletta, 1993; Nathan & Xie, 1994a). Until now three isoforms of NOS were known, *i.e.*, type I (neuronal, nNOS) (Schmidt et al., 1989; Bredt et al., 1991), type II (inducible, iNOS) (Stuehr et al., 1991; Xie et al., 1992), and type III (endothelial, eNOS) (Pollock et al., 1991). These isoforms are the products of distinct genes and show the distinct tissue-specific patterns of expression (Nathan & Xie, 1994b). The fundamental structure of the three isoforms consists of three well-conserved domains; a cytochrome P-450-like heme protein domain and a cytochrome P-450 reductase-like flavoprotein domain are connected by a putative calmodulin-binding domain (Bredt et al., 1991; Klatt et al., 1992; MacMillan et al., 1992).

The endothelial isoform of NOS is targeted to the membrane fraction in endothelial cells (Pollock et al., 1991), in contrast to the other isoforms found in neural tissues and in macrophages, which are principally located in cell cytosol (Nathan, 1992). Furthermore, the endothelial isoform shows translocation from the membrane to the soluble fractions upon various stimulation of the endothelial cells (Michel et al., 1993), suggesting that its subcellular localization and the translocation play an important role in its function. Phosphorylation of the protein seems to be directly involved in the stimulation-dependent translocation (Michel et al., 1993). This characteristic of eNOS is partly due to the presence of N-terminal myristoylation, which is lacking in nNOS and iNOS (Busconi & Michel, 1993; Liu & Sessa, 1994). Mutation of the myristoylation consensus sequence of eNOS inhibits the incorporation of myristic acid into eNOS and converts the enzyme from a membrane-bound to soluble protein, implying that myristoylation is necessary for its membrane association (Busconi & Michel, 1993; Sessa et al., 1993). Due to its intermediate hydrophobicity, a myristoyl group is considered ideal for reversible membrane anchoring (Gordon et al., 1991). However, it is unlikely that myristoylation is sufficient for membrane localization; the change in the unitary Gibbs free energy associated with the translocation of a myristoyl group (around 8 kcal/mol) is too small to anchor a large hydrophilic protein such as eNOS to membranes (Peitzsch & McLaughlin, 1993). Additional factors are clearly needed for the reversible interaction of the enzyme with the membranes.

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[1] Abbreviations: NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; MARCKS, myristoylated alanine-rich C kinase substrate; PKC, protein kinase C; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; CD, circular dichroism; TFE, trifluoroethanol.

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We have previously shown that a major *in vivo* substrate of protein kinase C (PKC), myristoylated alanine-rich C kinase substrate (MARCKS), shows phosphorylation-dependent interaction with acidic phospholipid membranes and that the calmodulin binding domain of the protein is directly involved in the interaction (Taniguchi & Manenti, 1993). Phosphorylation of the domain by PKC decreases its affinity to phospholipids, resulting in translocation of the MARCKS protein from membrane to cytosol (Taniguchi & Manenti, 1993; Kim et al., 1994a,b). Similarly, two brain-specific PKC substrates, GAP-43 and neurogranin, have been reported to show phosphorylation-dependent membrane binding through the calmodulin-binding domain (Houbre et al., 1991). Thus, calmodulin-binding domains of basic amphiphilic nature may be a common motif for reversible membrane binding regulated by protein phosphorylation. From deletion mutant protein studies, involvement of the putative calmodulin-binding domain of eNOS in its interaction with membranes has been suggested (Venema et al., 1995). Although a synthetic peptide corresponding to the domain has recently been shown to bind to calmodulin (Venema et al., 1996), the direct interaction of the domain with membrane phospholipids is yet to be demonstrated.

In the present study, we have synthesized a peptide corresponding to the putative calmodulin-binding domain of eNOS, and studied the interaction of the peptide with calmodulin and with phospholipids. The results obtained clearly showed that the two components bind to the same peptide. Furthermore, we found that the peptide was stoichiometrically phosphorylated by PKC and that the phosphorylated peptide lost its ability to bind lipids. These results established the importance of the calmodulin-binding domain in the eNOS−membrane interaction and suggested that the direct phosphorylation of the domain as well as Ca^{2+}-calmodulin is involved in the regulation of the interaction. Studies with the corresponding peptides of the other two NOS isozymes suggested further that the calmodulin-binding domain commonly serves as a phospholipid-binding motif.

EXPERIMENTAL PROCEDURES

Materials. PKC was purified from the cytoplasmic fractions of bovine brain as described (Manenti et al., 1992). Phosphatidylcholine (PC) from egg yolk, phosphatidic acid made from egg PC, phosphatidylglycerol made from egg PC, phosphatidylinositol (PI) from bovine liver, and phosphatidylserine (PS) from bovine brain were purchased from Avanti Polar Lipids. ATP and 1,2-dioleoyl-*rac*-glycerol were obtained from Sigma. Calmodulin purified from bovine brain was obtained from Wako Pure Chemical Industries, Ltd., Japan. [^{32}P]γ-ATP was purchased from Amersham. Other chemicals used were of the highest grade commercially available.

Peptide Synthesis. The 20-residue peptide corresponding to the calmodulin-binding domain of human eNOS peptide, RKKTFKEVANAVKISASLMG (corresponding to residues 492−511), and the 23-residue peptide corresponding to that of human iNOS, KRREIPLKVLVKAVLFACMLMRK (residues 509−531), were synthesized with standard *t*-Boc chemistry using an Applied Biosystems peptide synthesizer 430A. The 20-residue peptide corresponding to the calmodulin-binding domain of human nNOS, RRAIGFKKLAEAVKFSAKLM (residues 730−749), synthesized using standard Fmoc chemistry, was obtained from Research Genetics. The peptides were purified by reversed-phase high-performance liquid chromatography (HPLC) using a C-18 column (Waters μBondasphere 5 μ C18-300 Å, 1.9 × 15 cm). They were judged to be of greater than 95% purity by analytical HPLC and electrospray mass spectrometry. Peptide concentrations were determined by quantitative amino acid analysis.

Phospholipid Vesicle Preparation. Phospholipids in chloroform solution were dried to a thin film under a stream of argon gas and kept under vacuum for 30 min to remove any residual solvent. The lipid films were dispersed in 50 mM phosphate buffer (pH 7.2) by vigorous shaking for 5 min and sonication with a probe-type sonicator (Branson Sonifier 250) for 30 min on ice under nitrogen using a duty cycle of 30−50%. After brief centrifugation, the supernatants were used as lipid vesicles.

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra were measured in a JASCO J-720 CD spectropolarimeter. Spectra were recorded over a 190−260 nm range using a 1 mm quartz cuvette. Temperature was controlled at 25 °C by using a water bath. Peptide concentration was about 25 μM, unless otherwise indicated. The results are expressed as the mean residue molar ellipticity, $[\theta]$, which is defined as $[\theta] = 100\theta_{obs}/lc$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration of the residue in moles per liter, and l is the path length in centimeters.

Phosphorylation of eNOS Peptide by PKC. Phosphorylation of eNOS peptide by PKC was carried out in 25 mM Tris-HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, 100 μM $CaCl_2$, 100 μM PS, 10 μM dioleoylglycerol, and 1 mM ATP at 35 °C for 90 min. The reaction was stopped by adding 0.1% final concentration of trifluoroacetic acid (TFA). The extent of the phosphorylation was analyzed with electrospray mass spectrometry as described previously (Taniguchi et al., 1994a,b). The time course of the phosphorylation reaction was followed by incorporation of radioactivity from [^{32}P]γ-ATP as described (Manenti et al., 1992). The phosphorylated eNOS peptide was purified by reversed-phase HPLC using a Vydac C-18 column (218TP52, 0.46 × 25 cm).

Gel Mobility Shift Assay. Binding of the peptide to calmodulin was assayed using nondenaturing urea−polyacrylamide gel electrophoresis as described previously (Erickson-Viitanen & DeGrado, 1987).

Sedimentation Assay. The direct binding measurement of NOS peptides with membrane phospholipids was assessed by cosedimentation analysis. Each peptide (10−14 μM) was mixed with indicated amounts of PS liposomes or PC liposomes in 10 mM Tris-HCl (pH 7.5) buffer, incubated at 25 °C for 20 min, and centrifuged at 200 000*g* for 90 min at 25 °C. Peptides that remained in supernatants were detected by SDS−polyacrylamide gel electrophoresis (16.5% gel) using 100 mM Tris containing 100 mM tricine and 0.1% SDS as electrode buffer.

Effects of Calmodulin on Peptide−Membrane Interaction. eNOS peptide (14 μM) was mixed with PS liposomes (1.25 mg/mL) in 10 mM Tris-HCl (pH 7.5) buffer. After the mixture had been incubated at 30 °C for 30 min, one-third was removed. To the rest were added calmodulin and $CaCl_2$ to make final concentrations of 30 μM and 0.1 mM, respectively. After a second incubation at 30 °C for 30 min, the reaction mixture was divided, and EGTA was added to

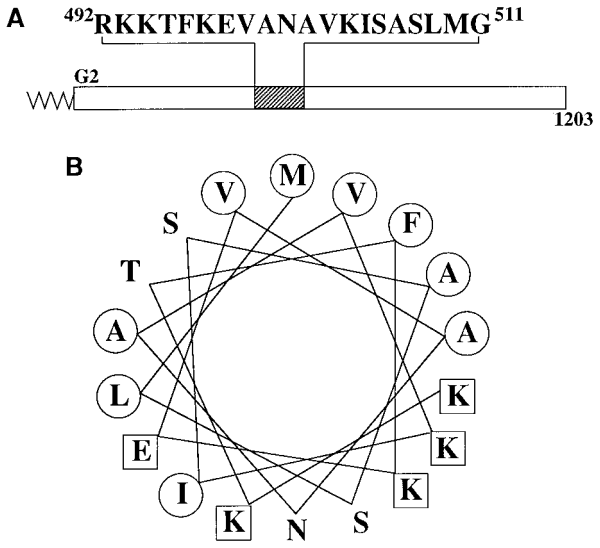

FIGURE 1: Schematic representation of human eNOS primary structure (A) a helix wheel representation of the synthetic calmodulin-binding domain (B). (A) The hatched box represents the sequence (492−511) synthesized in this study. Glycine 2 is Nα-myristoylated after removal of initial methionine. (B) Amino acids (493−510) in helix wheel representation are surrounded by circles (hydrophobic residues) or by squares (hydrophilic residues) to emphasize their segregation on the two sides of the helical structure.

make a final concentration of 1 mM to one sample. The three samples thus obtained were centrifuged at 200 000*g* for 90 min in a Beckman table top ultracentrifuge, and the peptide remained in supernatants was determined with SDS−polyacrylamide gel electrophoresis as above.

Capillary High-Performance Liquid Chromatography/ Electrospray Mass Spectrometry. A capillary high-performance liquid chromatography column (0.3 × 50 mm) packed with Perfusion Chromatography material (PerSeptive Biosystems, R2/H) was connected on-line to a Sciex API-III electrospray mass spectrometer as described previously (Taniguchi et al., 1994a,b). Phosphorylation mixtures were directly injected into the column, and the peptides that were desalted and concentrated with the column were directly analyzed by mass spectrometry.

RESULTS

Characterization of Calmodulin Binding Peptide of eNOS. The amino acid sequence of the peptide synthesized is shown in Figure 1A. The sequence corresponds to the putative calmodulin-binding domain that is located in the middle of the molecule between the N-terminal cytochrome P450-like heme protein domain and the C-terminal flavoprotein domain which is highly homologous to NADPH-cytochrome P450 reductase. As clearly seen in the helical wheel projection of the peptide, hydrophobic and basic hydrophilic amino acids segregate on the opposite sides when the peptide assumes an α-helical structure (Figure 1B). This so-called basic amphiphilic α-helix is considered to be a typical calmodulin-binding motif (O'Neil & DeGrado, 1990).

To establish that the domain is really the calmodulin binding domain of eNOS, interaction with calmodulin was examined using urea−polyacrylamide gel electrophoresis assay. In the presence of an equimolar amount of the peptide, calmodulin migrated faster in the gel, suggesting that the peptide binds to calmodulin (Figure 2). When $CaCl_2$ was omitted, no band shift was observed, indicating that the binding of eNOS peptide to calmodulin is calcium-dependent.[2] At a 0.5:1 ratio of peptide to calmodulin, roughly half of the calmodulin formed a calmodulin−peptide complex, while the rest remained in the free form. The band of the peptide calmodulin complex does not change even in the presence of an excess amount of the peptide, suggesting that the binding is stoichiometric.

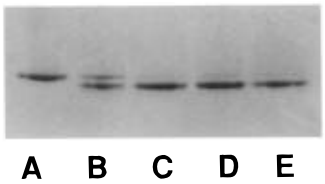

FIGURE 2: Binding of the putative calmodulin-binding domain to calmodulin. Varying amounts of the synthetic peptide were mixed with calmodulin in the presence of 0.1 mM $CaCl_2$ and subjected to nondenaturing urea−polyacrylamide gel electrophoresis. Calmodulin concentration was 12.5 μM. The peptide to calmodulin ratios were 0:1 (lane A); 0.5:1 (lane B); 1:1 (lane C); 2:1 (lane D); and 4:1 (lane E).

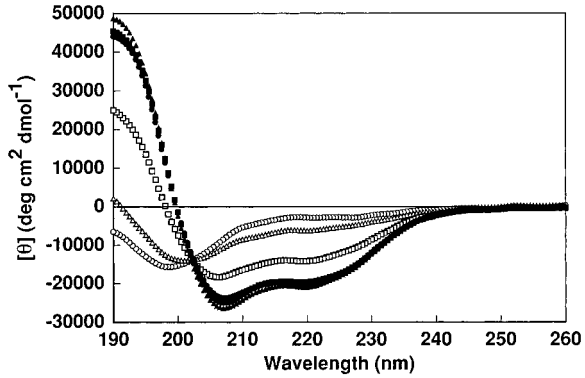

FIGURE 3: Effects of TFE on the secondary structure of eNOS peptide. CD spectra of eNOS peptide were obtained in the presence of 5 mM phosphate buffer (pH 7.2) containing varying concentrations of TFE (v/v). The eNOS peptide concentration was kept constant at 25 μM. (○) 0% TFE; (△) 10% TFE; (□) 20% TFE; (●) 30% TFE; (▲) 40% TFE; (■) 50% TFE.

Next, we studied effects of trifluoroethanol (TFE) on the structure of the peptide. The reagent mimics hydrophobic environments and induces α-helical conformation, if peptides have a tendency to form the structure (Nelson & Kallenbach, 1989; Lehrman et al., 1990). As shown in Figure 3, the CD spectrum of the peptide in aqueous buffer showed a single trough at around 198 nm, suggesting that the peptide does not have a distinct structure. With increasing concentrations of TFE, however, two negative peaks at 208 and 222 nm with a large positive peak at shorter wavelengths appeared. These are typical characteristics of α-helix, and the content of α-helix was calculated to be 57% in 50% TFE (Forood et al., 1993). This clearly demonstrates that the calmodulin-binding domain of eNOS has strong tendency to form α-helix if it is put under certain environments.

Interaction of eNOS Peptide with Membrane Phospholipids. To examine whether the eNOS peptide can interact with membrane phospholipids, effects of phospholipids on the conformation of eNOS peptide were studied using CD spectroscopy. The peptide alone in 50 mM phosphate buffer showed a CD spectrum typical for random coil, although

[2] M. Matsubara, N. Hayashi, K. Titani, and H. Taniguchi, manuscript in preparation.

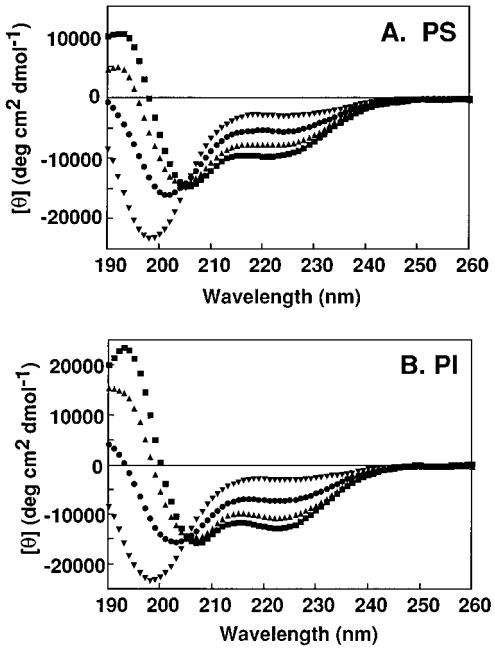

FIGURE 4: Effects of acidic phospholipids on CD spectra of eNOS peptide. CD spectra of eNOS peptide were obtained in the presence of varying concentrations of PS (A) or PI (B). The eNOS peptide concentration was kept constant at 25 μM. (A) (▼) Buffer alone; (●) 0.05 mg/mL PS; (▲) 0.1 mg/mL PS; (■) 0.15 mg/mL PS. (B) (▼) Buffer alone; (●) 0.05 mg/mL PI; (▲) 0.1 mg/mL PI; (■) 0.15 mg/mL PI.

the trough is more pronounced than that observed under lower ionic strength. When PS, an acidic phospholipid, was added, the spectrum changed drastically, and spectra typical for α-helix were again observed (Figure 4A). Although the overall shape of the spectra obtained at higher lipid concentrations was very similar to that of the spectrum obtained in 50% TFE, the content of α-helix remained at around 29%. The presence of isodichroic point means a two-state helix random coil equilibrium that is shifted toward a regular secondary structure by interacting with the phospholipid. Similar results were obtained with PI, another typical acidic phospholipid, found in membrane cells (Figure 4B). These results clearly indicated that the calmodulin-binding domain of eNOS binds to acidic phospholipid membrane and assumes α-helical structure in the membrane-bound state. Similar observations have been reported previously with a calmodulin-binding domain peptide of GAP-43 (Hayashi et al., 1995) and with bioactive peptides such as melittin and mastoparan that have a similar basic amphiphilic α-helical nature (Schwarz & Beschiaschvili, 1989; Schwarz & Blochmann, 1993).

To study the specificity of the interaction between eNOS peptide and phospholipids, various phospholipids at a fixed concentration were mixed with the peptide, and the CD spectra were measured (Figure 5). When PC, a neutral phospholipid, was added, no significant change was observed, suggesting that the neutral phospholipid does not interact with the peptide. On the other hand, all of the acidic phospholipids tested, *i.e.*, phosphatidylglycerol, phosphatidic acid, PI, and PS, affected the CD spectrum of the peptide, although the extent of the spectral changes differed depending on the lipid used. Among various acidic phospholipids, phosphatidic acid had the highest affinity to eNOS peptide, while PS had the lowest and the other two showed intermediate affinities. Although the affinities toward various acidic

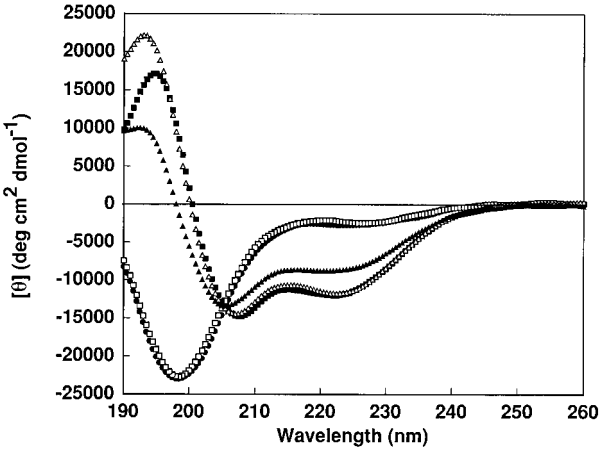

FIGURE 5: Specificity of eNOS peptide−phospholipid interaction. CD spectra of eNOS peptide (25 μM) in 50 mM phosphate buffer (pH 7.2) were measured in the presence of a fixed concentration of various phospholipids (0.15 mg/mL). (●) Buffer alone; (□) PC; (■) phosphatidylglycerol; (△) PI; (▲) PS.

phospholipids differed significantly, the final extents of the change and the spectral shape observed at saturating lipid concentrations were almost similar (data not shown). These results suggest that the interaction with between the eNOS peptide and membrane phospholipids involves not only electrostatic interaction but also the specific structures.

Phosphorylation of eNOS Peptide by PKC. In the case of MARCKS, the calmodulin-binding domain of the protein is, at the same time, the phosphorylation domain by PKC, and contains four serine residues phosphorylated by PKC. Phosphorylation of the domain peptide, and that of the whole protein by PKC significantly reduce their binding to membrane phospholipids (Taniguchi & Manenti, 1993; Kim et al., 1994a,b; Graff et al., 1989). Since the calmodulin-binding domain of eNOS contains one threonine which is within a consensus motif for PKC phosphorylation sites (Kennelly & Krebs, 1991), and since the fact that the protein shows stimulation-dependent translocation suggests the direct involvement of protein phosphorylation in the process, it is of interest to examine whether the peptide can be phosphorylated by PKC and, if this is the case, whether the phosphorylation can affect the interaction of the peptide with phospholipids.

To answer the question, the eNOS peptide was incubated with purified PKC in the presence of ATP, and the reaction mixture was analyzed using the liquid chromatography/mass spectrometry technique. In this method, the peptide was desalted and concentrated in an on-line capillary reversed-phase column, and the mass of the peptide was determined by electrospray mass spectrometry (Taniguchi et al., 1994a,b). The mass of the peptide incubated with PKC and ATP was larger than that of the original peptide by 80 Da (Figure 6B). This is exactly the mass change associated with the incorporation of one phosphoryl group to the peptide. Since no significant peak corresponding to the non-phosphorylated peptide was observed (Figure 6A), PKC stoichiometrically phosphorylated the calmodulin-binding domain peptide of eNOS. When the phosphorylation reaction was followed by radioactive assay using [^{32}P]γ-ATP, the maximal level of the phosphorylation was reached within 10 min of the incubation time under the conditions employed (Figure 6, inset), suggesting that the eNOS peptide is a good substrate of PKC. We conclude that Thr^{4} in the peptide (Thr^{445} in

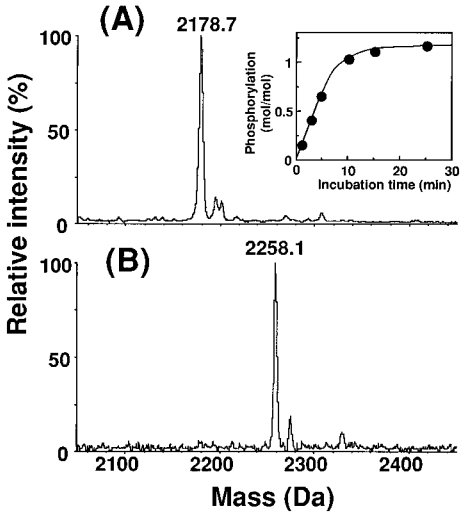

FIGURE 6: Deconvoluted electrospray mass spectra of eNOS peptide incubated with PKC. eNOS peptide was incubated with PKC at 35 °C for 90 min with (B) and without (A) ATP. The reaction mixtures were subjected to the LC/MS analysis as described under Experimental Procedures. The time course of the reaction was followed using [^{32}P]γ-ATP (inset).

the protein) is phosphorylated by PKC; two other phosphorylatable residues, Ser^{15} and Ser^{17}, are not in the consensus motif for PKC phosphorylation sites.

Effects of PKC Phosphorylation on Peptide−Membrane Interaction. We next investigated the influence of the PKC-dependent phosphorylation of the peptide on the peptide−phospholipid interaction. Phosphorylation peptide was purified over a reversed-phase HPLC column, and CD spectra of the phosphopeptide were measured in the presence or absence of PS. In the absence of phospholipids, the phosphopeptide showed a spectrum similar to that of non-phosphorylated peptide, suggesting that the peptide still assumes predominantly random structures, although a slight difference was noted. However, addition of PS to the phosphopeptide caused only a marginal change in the CD spectra. The appearance of a small negative peak at around 224 nm and the decrease in the intensity of the trough at around 198 nm with increasing concentrations of the phospholipid suggest that the phosphopeptide still binds to the lipid with much reduced affinity (Figure 7, inset). This was confirmed by direct binding measurements using sedimentation assay. Phosphorylated and non-phosphorylated peptides were incubated with PS liposomes, and the amounts of the remaining peptide in the supernatant after sedimentation of the lipid membranes were determined. As shown in Figure 8, the phosphorylated peptide did not bind to PS liposomes, while the non-phosphorylated peptide bound to the membranes almost quantitatively under the conditions employed. The phosphorylation of the peptide, therefore, almost abolished its binding to phospholipids.

Interaction of Calmodulin-Binding Domains of nNOS and iNOS with Membrane Phospholipids. Since the calmodulin-binding domains of nNOS and iNOS possess similar basic amphiphilic α-helical nature, it is of interest to examine whether the calmodulin binding domains of these isozymes also bind to membrane phospholipids. Binding of synthetic peptides corresponding to the calmodulin-binding domains to membrane phospholipids was directly studied by sedimentation assay. When each peptide was mixed with PS liposomes, a similar binding to the liposomes was observed

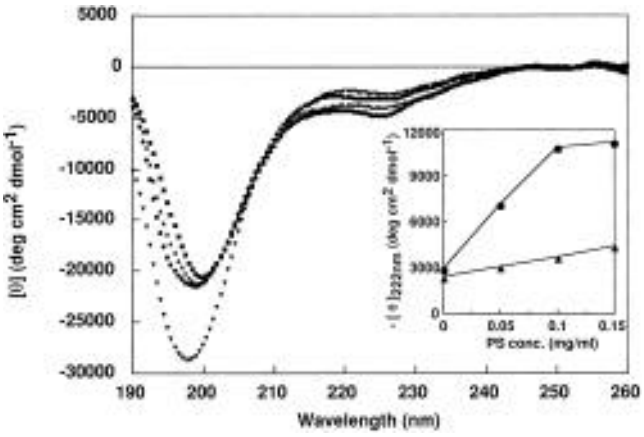

FIGURE 7: Effects of PKC phosphorylation on the eNOS peptide−phospholipid interaction. The eNOS peptide phosphorylated by PKC was purified over a reversed-phase column, and CD spectra were measured in the presence of varying concentrations of PS as described under Experimental Procedures. The eNOS peptide concentration was 12 μM. (▼) Buffer alone; (●) 0.05 mg/mL PS; (▲) 0.1 mg/mL PS; (■) 0.15 mg/mL PS. The inset shows the titration curve of the CD spectra change at 222 nm obtained with non-phosphorylated (●) and phosphorylated (▲) peptide.

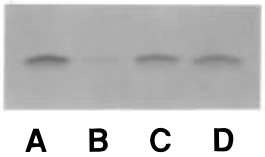

FIGURE 8: Effects of phosphorylation of cosedimentation of eNOS peptide with phospholipid membranes. Binding of non-phosphorylated and phosphorylated eNOS peptides to PS liposomes (0.05 mg/mL) was analyzed by sedimentation assay as described under Experimental Procedures. Peptides that remained in supernatant after centrifugation were determined by SDS polyacrylamide gel electrophoresis. Lane A, non-phosphorylated eNOS peptide alone; lane B, non-phosphorylated peptide plus PS liposomes; lane C, phosphorylated eNOS peptide alone; lane D, phosphorylated eNOS peptide plus PS liposomes.

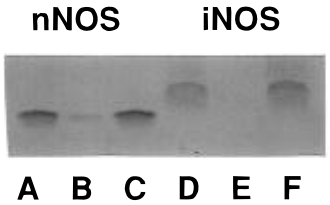

FIGURE 9: Binding of nNOS and iNOS peptides to membrane phospholipids. nNOS peptide or iNOS peptide was mixed either with PS liposomes (0.1 mg/mL) or with PC liposomes (0.1 mg/mL). After centrifugation, peptides that remained in supernatant were analyzed as in Figure 8. Lane A, nNOS peptide alone; lane B, nNOS peptide plus PS liposomes; lane C, nNOS peptide plus PC liposomes; lane D, iNOS peptide alone; lane E, iNOS peptide plus PS liposomes; lane F, iNOS peptide plus PC liposomes.

with both peptides (Figure 9, lanes B and E). On the contrary, no significant binding was observed with either peptide when PC liposomes were used (lanes C and F). Thus, the calmodulin-binding domains of nNOS and iNOS, like their counterpart in eNOS, bind to acidic phospholipids selectively. These results suggested that the calmodulin-binding domains of the three NOS isozymes serve as membrane-binding domains as well.

Effects of Calmodulin on Peptide−Membrane Interaction. Since the same peptide binds both calmodulin and acidic phospholipids, it is of interest to examine the effect of calmodulin on the peptide−membrane interaction. The

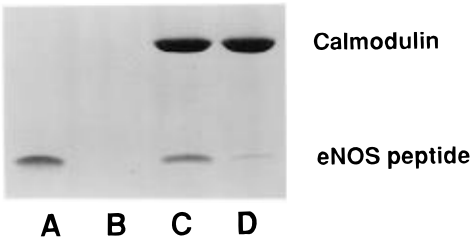

FIGURE 10: Effects of calmodulin on the eNOS peptide−membrane interaction. Effects of calmodulin on the peptide−membrane interaction were studied as described under Experimental Procedures, and the supernatants were analyzed after centrifugation on SDS−polyacrylamide gel electrophoresis. Lane A, eNOS alone; lane B, eNOS peptide plus PS liposomes; lane C, eNOS peptide plus PS liposomes plus Ca^{2+}-calmodulin (in the presence of 0.1 mM $CaCl_2$); lane D, eNOS peptide plus PS liposomes plus apocalmodulin (in the presence of 1 mM EGTA).

eNOS peptide was first mixed with PS liposomes. After the mixture was incubated at 30 °C for 30 min, one-third was removed. To the rest were added 30 μM calmodulin and 0.1 mM $CaCl_2$. After a second incubation at 30 °C for 30 min, the reaction mixture was divided into two, and 1 mM EGTA was added to one sample. The three samples thus obtained were centrifuged, and the peptide that remained in the supernatants was determined with gel electrophoresis. As shown in Figure 10, the addition of calmodulin and Ca^{2+} to the peptide−membrane complex released the peptide into supernatant (lane C). However, the addition of EGTA, a Ca^{2+} chelator, restored the binding of the peptide to the phospholipid membranes (lane D). Therefore, calmodulin can affect the eNOS peptide−membrane interaction depending on Ca^{2+} concentration. Similar effects of calmodulin on the peptide−membrane interaction were also observed with the nNOS peptide. The effects of calmodulin were dependent on Ca^{2+} concentration, as was observed with the eNOS peptide (data not shown). In contrast, the addition of calmodulin to iNOS peptide−PS mixtures only partially reversed the binding both in the presence and absence of Ca^{2+} (data not shown), suggesting that the iNOS peptide has a higher affinity to the phospholipid membranes.

DISCUSSION

So-called basic amphiphilic α-helix has been initially identified as a binding motif for calmodulin (O'Neil & DeGrado, 1990; Blumenthal et al., 1985). There are no clear-cut conserved amino acids among calmodulin-binding domains of various proteins, except that basic hydrophilic and hydrophobic amino acids appear alternately at certain intervals. When they assume α-helical structures, the two groups of amino acids segregate on opposite sides of the helices. Formation of such helical structures, in fact, has been demonstrated in the three-dimensional structures of several substrate peptide−calmodulin complexes (Ikura et al., 1992; Meador et al., 1992). The calmodulin-binding domain of eNOS has been putatively assigned solely from the sequence analysis based on these observations (Lamas et al., 1992). The present study together with recently published studies with deletion mutant proteins and peptides (Venema et al., 1995, 1996) clearly established that the region (residues 492−511 in the human sequence) contains the calmodulin-binding domain of eNOS. Like other calmodulin-binding peptides, the eNOS peptide studied in the present report showed a strong tendency to form an α-helical structure which could be induced or stabilized by TFE, which is known as an α-helix-promoting solvent (Nelson & Kallenbach, 1989; Lehrman et al., 1990). Formation of α-helix was also observed when the eNOS peptide was complexed with calmodulin,[2] suggesting that the secondary structure is crucial in the substrate−calmodulin interaction.

Furthermore, the same peptide showed an ability to bind membrane phospholipids. In addition to calcium-dependent membrane-binding motifs found in various component proteins of signal transduction pathways, two types of the membrane-binding motif, that are calcium independent, have been so far described (Resh, 1994). One is a simple polybasic cluster, a typical example being found in $pp60^{src}$ (Walker et al., 1993). The other is the calmodulin-binding domain of basic amphiphilic nature found in MARCKS, a major *in vivo* PKC substrate (Taniguchi & Manenti, 1993). Not only ionic but also hydrophobic interactions seem to be involved in the interaction of the latter type domain with phospholipid membranes. A second example of this type is found in GAP-43, another *in vivo* PKC substrate (Houbre et al., 1991; Hayashi et al., 1995). It is of interest to note that all three of the proteins are acylated with either myristoylation or palmitoylation (Aderem et al., 1989; Skene & Virag, 1989). MARCKS has been reported to show a stimulation-dependent translocation between the membrane and soluble fractions similar to that found with eNOS. We have already demonstrated that phosphorylation of the calmodulin-membrane-binding domain of MARCKS abolishes the lipid-binding ability of the domain, which results in the translocation of the protein (Taniguchi & Manenti, 1993). As described in the present report, PKC stoichiometrically phosphorylated the eNOS peptide *in vitro*, and the phosphorylation abolished the binding of the peptide to acidic phospholipids. It should be noted that the phosphorylation of the synthetic peptide by PKC *in vitro* does not necessarily imply that the phosphorylation of the intact eNOS protein by PKC also occurs in cells. However, our present study indicated a high affinity of the calmodulin-binding domain peptide derived from eNOS to PKC. This suggests that the phosphorylation may well occur *in vivo*, since the domain is not embedded inside of the protein but exposed to the outer surface and is accessible by a protein, *i.e.*, calmodulin. It is, therefore, intriguing to assume that the same mechanism as found in the translocation of MARCKS is operational in the stimulation-dependent translocation of eNOS, which is dependent on protein phosphorylation of the enzyme (Michel et al., 1993). Although the protein kinase and the phosphorylation sites involved in the eNOS translocation *in vivo* are yet to be determined, the present results suggest that the domain of basic amphiphilic α-helical nature, which connects the heme protein and the flavoprotein domains of eNOS, works as a membrane targeting motif which is regulated by both protein phosphorylation and calmodulin binding.

Although involvement of the N-terminal myristoylation in the reversible membrane targeting of eNOS has been well established (Busconi & Michel, 1993; Liu & Sessa, 1994; Sessa et al., 1993), it is clear from thermodynamical considerations that the fatty acid modification alone is not enough to stable anchor a large hydrophilic protein such as eNOS to membranes (McLaughlin & Aderem, 1995). One region containing polybasic residues (RRKRK) has been previously implicated in the eNOS−membrane interaction (Busconi & Michel, 1994). The region is located within an

insert found in the FMN binding domain of the protein. A deletion mutant of the region, however, still binds to phospholipid membranes, suggesting that the region does not play a central role in the membrane anchoring (Busconi & Michel, 1994). In contrast, a mutant lacking the calmodulin-binding domain has been recently shown to completely lose the membrane-binding ability (Venema et al., 1995). Since one cannot rule out the possibility that the loss of the binding is due to secondary effects of deletion on three-dimensional structure of the protein (Venema et al., 1995, 1996), we have conducted direct binding studies on the synthetic peptides as described in the present study. Taken together, the results obtained clearly established that the region (residues 492−511 in the human sequence) contains the calmodulin-binding domain of eNOS and that the domain serves as a membrane-binding domain as well. Other reports have recently demonstrated that eNOS is also palmitoylated at two cysteine residues, Cys^{15} and Cys^{26}, although the stoichiometry of the modification is still unknown (Robinson et al., 1995; Robinson & Michel, 1995; Liu et al., 1995). In contrast to protein myristoylation, a palmitoyl moiety is more hydrophobic, and palmitoylation−depalmitoylation cycles are believed to be necessary for regulation of the reversible membrane interaction. However, there are contradicting reports on the regulation of palmitoylation/depalmitoylation of eNOS (Robinson et al., 1995; Liu et al., 1995), and whether the modification is involved in the stimulation-dependent translocation of eNOS remains still to be established.

Needless to say, the involvement of various protein domains and modifications are not necessarily mutually exclusive; a complex mechanism may be involved in the process. For example, both the recombinant eNOS protein and the calmodulin-binding peptide showed an absolute requirement for acidic phospholipids, but the specificities among the acidic phospholipids showed a slight difference. This may indicate the involvement of regions other than the calmodulin domain in the eNOS−membrane interaction. Another point is protein myristoylation. This type of modification has been believed to occur co-translationally and be static, but we have recently demonstrated that the process is reversible and that the enzymatic myristoylation/demyristoylation process may regulate the subcellular localization of MARCKS (Manenti et al., 1994). A similar mechanism may well be involved in the eNOS−membrane interactions. The binding of various components, *i.e.*, calmodulin, acidic phospholipids, and probably a protein kinase such as PKC to a single site of eNOS also suggests that interactions between them and the regulation of the subcellular localization as well as the enzymatic activity of eNOS are, in fact, very complex. Since the regulation of the enzymatic activities of eNOS and iNOS by acidic phospholipids have been well documented (Ohashi et al., 1993; Venema et al., 1995; Hirata et al., 1995; Calderon et al., 1994; Aramaki et al., 1996), it seems reasonable to assume that the direct binding of phospholipids to the calmodulin-binding domain of these NOS isozymes affects their enzymatic activities. The results presented in this report, therefore, demonstrate that the calmodulin-binding domain of basic amphiphilic α-helical nature in NOS isozymes plays an important role as one of the cross-talk points between various signal transduction pathways.

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