Endothelial Nitric-oxide Synthase (Type III) Is Activated and Becomes Calcium Independent upon Phosphorylation by Cyclic Nucleotide-dependent Protein Kinases*

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The family of homodimeric nitric-oxide synthases (NOS) catalyze the formation of nitric oxide (NO) and L-citrulline from the precursor amino acid, L-arginine. To date, three genetically distinct NOS isoforms have been identified, originally isolated from cerebellum (nNOS, NOS-I), macrophages (iNOS, NOS-II), and endothelial cells (eNOS, NOS-III). All NOSs share an overall 50% amino acid sequence homology and have similar cofactor requirements. Each NOS monomer contains an N-terminal oxygenase domain with a binding site for the arginine substrate, tetrahydrobiopterin, Ca²⁺/CaM, and zinc, as well as a C-terminal reductase domain with an autoinhibitory region, and one binding site each for FAD, FMN, and NADPH as electron donor (1–4).

Only NOS-I and NOS-III, the constitutively expressed forms prominent in neurons, and skeletal muscle or endothelial cells, respectively, are activated by agonist-induced elevation of the intracellular free Ca²⁺ concentration with subsequent binding of Ca²⁺/CaM to NOS (5). In contrast, NOS-II is regulated predominantly at the transcriptional level by endotoxins and cytokines in macrophages, hepatocytes, and vascular smooth muscle cells (for review, see Ref. 6), and binds CaM with such high affinity that it is already maximally activated at the Ca²⁺ level of a resting cell (7). Additional regulatory mechanisms which have been proposed to regulate NOS include autoinhibition, protein-protein interactions, subcellular localization, acylation, changes in substrate supply, phosphorylation (for review, see Ref. 8), and enzyme monomerization (9, 10). Interestingly, shear stress and isotonic contraction have been shown to activate endothelial NOS-III synthase in a Ca²⁺/CaM-independent manner that is sensitive to tyrosine kinase inhibitors (11, 12). This Ca²⁺-independent activation is not understood molecularly. For example, a tyrosine kinase-dependent step has been suggested, however, shear stress-dependent tyrosine phosphorylation of NOS-III could not be shown (13). Alternatively, tyrosine kinase-dependent phosphorylation of yet unknown NOS-III-binding proteins was suggested to play a role in Ca²⁺-independent endothelial NO release (14).

The abbreviations used are: NOS, nitric-oxide synthase; eNOS or NOS-III, endothelial NOS; iNOS or NOS-II, inducible NOS; nNOS or NOS-I, neuronal NOS; CaM, calmodulin; cGK, cGMP-dependent protein kinase; cAK, cAMP-dependent protein kinase; GSH, glutathione; HUVEC, human umbilical vein endothelial cell; MALDI-TOF-MS, matrix assisted-laser desorption/ionization-time of flight-mass spectrometry; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; CHAPSO, 3-(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propane-sulfonic acid.

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completely understood. Purified NOS-I was shown to be phosphorylated by cAMP- and cGMP-dependent protein kinase (cAK, cGK), protein kinase C, and Ca²⁺/CaM kinase II, with each kinase phosphorylating a different Ser. In all cases, Ser phosphorylation resulted in an inhibition of NOS-I catalytic activity, suggesting a negative feedback regulation of neuronal NO formation (16, 17). In rat heart, NOS-III is phosphorylated on Ser by AMP-activated protein kinase both in vitro and during ischemia (18). In cultured endothelial cells, NOS-III is phosphorylated on Ser in response to bradykinin and then translocated to the cytosol (19). Several studies have shown that NOS-III is targeted to plasmalemmal caveolae, and it has been suggested that the caveolin-NOS-III complex undergoes cycles of dissociation (activates NOS-III) and re-association (inactivates NOS-III) which are modulated by increases and decreases in Ca²⁺, respectively (20, 21). However, treatment of bovine aortic endothelial cells with hydrogen peroxide increased tyrosine phosphorylation of NOS-III and inhibited its activity, but did not disturb its association with caveolin (22).

In the present study, we demonstrate that phosphorylation of NOS-III by cAMP- and cGMP-dependent protein kinases not only stimulates Ca²⁺/CaM-dependent partial activation of NOS-III both in vitro and in vivo that does not appear to involve enzyme translocation to the cytosol.

**EXPERIMENTAL PROCEDURES**

*Materials*—l-[^3]H|Arginine hydrochloride and [γ-[^32]P]ATP were purchased from Amersham Pharmacia Biotech (Braunschweig, Germany), [γ-[^32]P]orthophosphate (HCl free) from NEN Life Science Products Inc., NADPH from AppliChem (Darmstadt, Germany), reduced glutathione (GSH) from Roche Molecular Biochemicals (Germany), endopeptidase Glu-C from Roche Diagnostic (Mannheim, Germany), trypsin (sequencing grade) from Promega (Heidelberg, Germany), (6R, 5R, 3S)-3-Tryptophanhydrobenzil from Applied Biosystems, Ltd. (Darmstadt, Germany), FAD, FMN, l-arginine hydrochloride, phosphoamino acid standards, and CaM from Sigma (Deisenhofen, Germany), Sp-5,6-DCl-cBMPs from Biolot (Bremen, Germany), nihinhydro and cellulose thin layer chromatography plates (20 × 20 cm) from Merck (Darmstadt, Germany), Dulbecco’s modified Eagle’s medium, M199 medium, and fetal calf serum from Life Technologies (Eggenstein, Germany), Protein A-Sepharose from Amersham Pharmacia Biotech (Buckinghamshire, UK), and NOS-III antibody from Transduction Laboratories (Hamburg, Germany). Ilprocistin was a kind gift of Schering Pharma (Berlin, Germany), cGK Iα and the catalytic (C) subunit of cAK type II were purified from bovine lung and bovine heart, respectively, as described earlier (23). cGK Iβ and cGK II were expressed in and purified from the baculovirus-Sif9 cell system (24). All other chemicals, reagents, and solvents of the highest purity available were purchased from either Merck (Darmstadt, Germany) or Sigma (Deisenhofen, Germany). Water was deionized to 18 MΩ (Milli-Q, Millipore, Eschborn, Germany). Unless otherwise indicated, all chemicals were dissolved in argon deoxygenated water.

*Preparation of Recombinant Human (h)NOS-I, II, and III, and Native Porcine NOS-I*—Recombinant human NOS-I, II, or III were purified from Sp-5,6-DCl-cBMP-Sepharose for NOS-II, or CaM-Sepharose for NOS-I and III (9); native porcine cerebellum NOS-I was purified as described (25). Specific NOS activities were (nmol/min/mg): 401 (hNOS-I), 125 (pNOS-I), 10 (partially purified hNOS-II), and 105 (hNOS-III). All NOS were stored at −80 °C in 50-μl aliquots containing 10% glycerol until the day of use. Protein concentrations were determined spectrophotometrically according to the method of Bradford (26). Protein concentration was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA), Protein purity was determined by densitometry of Coomassie-stained SDS-PAGE gels using a flatbed scanner and NIH Image software (National Institutes of Health, Bethesda, MD). NOS-III immunoreactive bands were examined by Western blot analysis using a NOS-III-specific antibody and enhanced chemiluminescence detection.

*In Vitro Phosphorylation of NOS-I, II, and III*—NOS-I, II, and III were incubated at 30 °C for the times indicated with 10 mM HEPES, pH 7.4, 5 mM MgCl₂, 1 mM EDTA, 0.2 mM dithiothreitol, and either 5 mM cGMP or 0.5 mM cGMP. The reaction was started by the addition of 100 μM ATP. In control experiments, heat-inactivated (10 min at 95 °C) cGK and C subunit were used.

*Determination of NOS Activity*—Catalytic activity of NOS-III was assayed under steady state conditions by the Ca²⁺/CaM-dependent conversion of l-[¹⁴C]arginine to l-[¹⁴C]citrulline (25) at 37 °C during a standard incubation period of 15 min. If not otherwise stated, reaction mixtures contained NOS (various amounts used are given in figure legends), 50 mM CaM, 1 mM CaC₂, 250 μM CHAPS, 7 mM reduced glutathione, 10 μM FAD, 5 μM FMN, 5 μM tetrahydrobipterin, 50 μM 5,6,7,8-tetrahydrobiopterin from Dr. Schirks Laboratories (Jona, Switzerland), 2 μM AMP, 50 μM NADPH, in 50 mM triethanolamine buffer (pH 7.2), and a total volume of 100 μl. For assaying NOS-III activity in human umbilical vein endothelial cells (HUVEC), arginine was reduced to 2 μM and tetrahydrobipterin was increased to 10 μM. The l-citrulline formed was separated by cation exchange chromatography and measured by liquid scintillation counting. NOS activity was expressed either as a percent of the maximum rate of l-citrulline formation (V₅₀max) in the presence of Ca²⁺/CaM, or as picomole or nanomole mg⁻¹ min⁻¹.

*Phosphoamino Acid Analysis*—For phosphoamino acid analysis, 32P-phosphorylated NOS-III was digested in gel with trypsin (2:1, w/w) and then subjected to acid hydrolysis with 6 N HCl at 110 °C for 3 h in a sealed tube. The samples were dried, dissolved in water, and analyzed for phosphoamino acids by one-dimensional electrophoresis (Bruker, Amersham Pharmacia Biotech) on cellulose plates in 1.8% formic acid, 7.3% acetic acid (pH 1.9), at 1400 V, 15 °C for 2 h. Phosphoserine and phosphothreonine were used as internal standards and visualized by staining with ninhydrin. 32P-Labeled amino acids were visualized by autoradiography.

*Mass Spectrometric Analysis of NOS Phosphopeptides*—Maximally 32P-phosphorylated NOS-III (20 pmol) was digested and the tryptic fragments were separated by high pressure liquid chromatography (HPLC) using a 0.3 × 150-mm PL-SAX (8 μm, 1000 Å) anion exchange column (LC Packings, Amsterdam Pharmacia Biotech, Netherlands). Peptides were eluted at a flow rate of 4 μl/min with a linear gradient from 95% solvent A (20 mM ammonium acetate, pH 7.0) to 9% solvent B (0.5 M KH₂PO₄, 25% acetonitrile, pH 4.0) over 55 min. The eluate was monitored by UV absorbance at 214 nm and fractions of 4 μl were collected. Fractions containing radioactivity were further separated using a 300 μm × 250 mm (5 μm, 1000 Å) reversed phase C₁₈ column (LC Packings, Amsterdam Pharmacia Biotech). Peptides were eluted at a flow rate of 16 μl/min with a complex gradient which started at 5% solvent B (0.08% trifluoroacetic acid, 84% acetonitrile) in solvent A (0.1% trifluoroacetic acid) for 30 min, was increased to 50% solvent B at 120 min and 99% solvent B at 136 min, and ended with 99% solvent B at 146 min. The eluate was monitored by UV absorbance at 214 nm. Fractions containing 32P were analyzed by matrix-assisted laser-desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) on Brucker Reflex III, Bruker Daltonik, Bremen, Germany) and Edman degradation (Procise cLC 494 system, Applied Biosystems), commonly used for analysis of an HPLC column. As MALDI matrix, a solution of 4-hydroxy-α-cinnamic acid (Sigma, Deisenhofen, Germany) in 0.1% trifluoroacetic acid/acetonitrile was used. Samples were mixed on target with the matrix solution.

*Size Exclusion Chromatography*—Purified human recombinant NOS-III (20 μg) was incubated as described above for the NOS activity assay after in vitro phosphorylation, except that in these experiments the concentration of l-arginine, tetrahydrobipterin, and CaM were increased to 2 mM, 50 μM, and 2 μM, respectively. The reaction was stopped by the addition of 20 μl of ice-cold EGTA (30 mM) and the samples were immediately snap frozen in liquid nitrogen. After thawing, the samples were centrifuged (10,000 × g, 4 °C for 10 min), and then a 100-μl aliquot (equivalent to 17 μg of NOS protein) was analyzed by fast protein liquid chromatography using a Superose 6 HR 10/30 gel filtration column (Amersham Pharmacia Biotech, Freiburg, Germany) that was equilibrated with 20 mM triethanolamine buffer (pH 7.5) containing 150 mM NaCl and 5% (v/v) ethylene glycol. Proteins were eluted at a flow rate of 0.25 ml/min and monitored by their absorbance at 280 nm.

*Determination of NOS-associated Flavins*—Phospho- and dephospho-NOS-III were incubated at 37 °C with 4 mM urea in 50 mM HEPES (pH 7.5). The low molecular mass fraction, containing flavins that dissociated from NOS, was removed by centrifugation (10,000 × g, 4 °C, 20 min) using filters with a 10-kDa molecular mass cut-off (Ultrafree MC, Millipore). Samples were analyzed for NOS-associated flavins by reverse phase HPLC with fluorometric detection (λₑₑₑ = 480 nm, λₘₘₘ = 530 nm) on LiChroCart 250/4, LiChrospher 100 RP18 (5 μm) columns.
NOS-III Is Partially Ca Independent after Phosphorylation

**Fig. 1. Multiple sequence alignment of the CaM-Binding domains of different NO synthases.** Shown is an alignment performed using ClustalW analysis and subsequent correction by hand. **Black boxes** indicate putative phosphorylation sites for cAMP- and cGMP-dependent protein kinases. Conserved residues are **shadowed**, except that within the CaM inhibitory sequence, conserved residues are marked with an asterisk (*). The CaM-binding site is defined according to Mayer et al. (2); the CaM autoinhibitory sequence and three FMN-binding domains are based on the analysis of Salerno et al. (31).

**RESULTS**

**Consensus Phosphorylation Sites**—The key sequence for substrate recognition by cAK and cGK contains basic amino acids N-terminal to the phospho-acceptor serine or threonine, with a more stringent requirement for multiple basic residues in the case of cGK recognition. Most consensus phosphorylation sequences for cAK and cGK are described by the motif R(K/E)XX(S/T) and R(K/E)XXX(S/T), respectively (28). Using this information, we identified several putative cAMP- and cGMP-dependent protein kinase phosphorylation sites in human NOS forms I (Thr<sup>196</sup>, Ser<sup>631</sup>, Ser<sup>1002</sup> and Thr<sup>1432</sup>) and III (Thr<sup>495</sup> and Ser<sup>633</sup>). For NOS-II, no protein kinase recognition sequence motif was recognized. Fig. 1 shows a multiple sequence alignment of selected NOS sequences. A comparison of human NOS-III with mouse and bovine NOS-III revealed that the two possible phosphorylation sites are conserved within the CaM-binding site (Thr<sup>495</sup>) and the CaM autoinhibitory sequence (Ser<sup>633</sup>).

**Effect of Cyclic Nucleotide-dependent Phosphorylation on Human NOS Activity**—The putative phosphorylation sites of the NO synthases were further characterized by incubating purified recombinant NOS-I, -II, and -III with cGK I<sub>a</sub>, I<sub>b</sub>, or catalytic subunit of cAK in the presence of [γ<sup>32</sup>P]ATP. An autoradiogram of a representative SDS-PAGE is shown in Fig. 2B. Only marginal incorporation of phosphate into NOS-I was observed after 5 min treatment with any of the four kinases. In contrast, VASP, a well known (control) substrate for cAK and cGK (29), was highly phosphorylated by all four kinases, similar in degree to NOS-III phosphorylation by cGK II and C

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NOS-III Is Partially Ca Independent after Phosphorylation

By direct Edman sequencing of the radioactive peptides (not shown). The phosphothreonine which had been detected by one-dimensional electrophoresis was not recovered. Tryptic digestion of NOS-III resulted in most likely too small a fragment (KpFK) not retarded by the anion exchange HPLC. In addition, the presence of an intense matrix background, extending up to 1000 Da, would have made the detection of this small peptide very difficult. To circumvent these problems, phosphorylated NOS-III (up to 200 pmol) was digested with endoproteinase Glu-C to obtain a theoretical 35-mer peptide containing Thr195. However, the digest was incomplete (less than 10%), and no radioactive peptides were recovered.

**Effect of Phosphorylation on NOS-III Quaternary Structure**—Since all NOS forms are only active as homodimers, we tested the effects of cGMP and cAMP-dependent protein kinase phosphorylation on NOS-III quaternary structure by size exclusion chromatography. Phosphorylated NOS-III was found to elute as two protein peaks, a main dimer peak at 13.8 ml of elution buffer and a second smaller monomer peak at 14.9 ml (Fig. 4). Both peaks contained NOS-III immunoreactive protein as analyzed by Western blot (not shown). Neither cAMP- nor cGMP-dependent protein kinase phosphorylation altered the ratio of dimeric to monomeric enzyme (Fig. 4, A and B), suggesting that changes in NOS-III catalytic activity resulting from phosphorylation are due to changes in NOS-III quaternary structure or increased dimer stability.

**Effect of Phosphorylation on NOS-III Flavin Content**—A potential effect of phosphorylation on flavin binding was investigated since two cGK and cAK consensus phosphorylation sites (Ser631 and the putative Thr195) in the autoinhibitory and CaM binding sequences are immediately flanked by FMN-binding domains (Fig. 1, D). However, phosphorylation did not change the effect of increasing FMN concentration (0.1–10 μM) on NOS-III activity (Fig. 5A). In a second experiment, the amount of flavin bound to the phospho- and dephospho-form of NOS-III was examined by HPLC. No differences in the amount of NOS-III-associated FMN and FAD was observed after phosphorylation by C subunit (Fig. 5B) or cGK II (data not shown).

**Effect of Phosphorylation on Apparent Calmodulin Affinity of NOS-III**—To determine the effect of phosphorylation on CaM binding, phosphorylated NOS-III was incubated with various CaM concentrations up to 1 μM. As shown in Fig. 6A, phosphorylation did not change the half-maximal concentration of CaM (15 nM) that activated NOS-III, but considerably increased Vmax. Surprisingly, phosphorylated NOS-III was found to be active even under assay conditions in which Ca2+/CaM was omitted, although this NOS isoform is defined as being strictly dependent on CaM and physiological increases in free Ca2+. To further investigate this effect, NOS-III activity was examined either in the absence or presence of 100 nM CaM, in either the absence (0.5 mM EDTA) or presence of 1 mM Ca2+. When NOS-III was assayed in the absence or presence of Ca2+/CaM, both cGK II and C subunit activated NOS-III and caused a 10–20% fraction (Fig. 6, B and C, three left black columns) of the maximal Ca2+/CaM-stimulated NOS-III activity (Fig. 6, B and C, open columns), or 5–10% of the maximal activity attained by Ca2+/CaM in the presence of active kinases (Fig. 6, B and C, black column at far right) to become independent not only of Ca2+ but also of CaM. No activity was observed for nonphosphorylated NOS-III (denatured cGK II and C used) under the same Ca2+/CaM-free assay conditions (Fig. 6, B and C). As shown in Fig. 6, A–C, NOS-III activity in the presence of calmodulin (10–1000 nM) was also considerably enhanced by C subunit of cAK or cGK II.

**In Vivo Studies of NOS-III Activation in HUVEC**—In order to investigate NOS phosphorylation and activity in intact cells,
HUVEC were incubated either with 100 μM Iloprost, which activates adenylyl cyclase via a G-protein-coupled receptor to increase intracellular cAMP levels, or with 200 μM Sp-5,6-DCI-cBiMPS (not shown), a selective activator of cAK. NOS-III activity in the presence of Ca\(^{2+}\)/CaM in the homogenate of cells that had been treated with Iloprost was significantly increased (138%, compare open columns in Fig. 7A). Moreover, a significant fraction (29%) of total NOS-III activity was Ca\(^{2+}\) independent (black column in Fig. 7A) in Iloprost-treated samples. These results correlated with those obtained upon co-incuba-

**Fig. 3.** Phosphoamino acid analysis of human NOS-III. A, the NOS-III amino acid residues phosphorylated by cAK and cGK II were detected by one-dimensional electrophoresis as described under “Experimental Procedures.” The migration positions of phosphoserine and phosphothreonine standards determined by ninhydrin staining are indicated at the left. The autoradiography is representative of three experiments. B, MALDI-MS spectra of radioactive HPLC fractions from a tryptic digest of the NOS-III in vitro phosphorylated by cGK II. (Equivalent data obtained with C subunit-phosphorylated NOS-III was obtained but not shown.) The ions with a mass to charge ratio (m/z) of 1886.7 Da and 1444.7 Da were selected for MALDI-PSD experiments (see C). Peaks labeled with arrows (↓) represent typical fragments of phosphoserine and phosphothreonine resulting from loss of H\(_2\)PO\(_4\) and HPO\(_3\)\(_2\). C, PSD (post-source decay) fragment ion spectra of the designated phosphopeptides KEpSSNTDSAGALGTLR and IRTpSFSLQER. Nearly complete b-ion (N-terminal fragmentation) and y-ion (C-terminal fragmentation) series are visible and unequivocally identify phosphorylation of Ser\(^{633}\) (C, left panel) and Ser\(^{1177}\) (C, right panel). The typical generation of dehydroalanine from phosphoserine through loss of phosphate during analysis is observed by a –98 Da shift of b- and y-ions.
Iloprost treated cells, Fig. 7

The membrane association of NOS-III remained unchanged (89% in untreated, 86% in Iloprost treated cells) of HUVEC. However, the membrane association of NOS-III, we performed Western blot analysis of the 100,000 g supernatant and an associated flavin (FMN, FAD) were analyzed by FPLC as described under “Experimental Procedures.” The representatives shown are representative of two independent experiments which yielded identical results.

We investigated whether phosphorylation by cAK affected the subcellular distribution of NOS-III, we performed Western blot analysis of the 100,000 g supernatant and associated flavin (FMN, FAD) were analyzed by FPLC as described under “Experimental Procedures.” The representations shown are representative of two independent experiments which yielded identical results.

FIG. 4. Effects of cAMP- and cGMP-dependent protein kinase II phosphorylation on NOS-III quaternary structure. Purified recombinant NOS-III (3 μM) was incubated for 5 min at 30 °C with either active (dashed line) or denatured, inactive (solid line) C subunit (A) or cGK II (B) (each 0.15 μM) in a total volume of 50 μl as described under “Experimental Procedures” and then analyzed by FPLC. Phosphorylation had no demonstrable effect on monomer (M/dimer (D)) equilibrium. The chromatogram shown is representative of two independent experiments which yielded identical results.

**DISCUSSION**

The present study establishes that endothelial NOS-III is phosphorylated in vitro on Ser1283, Ser1777, and most likely Thr495, by cAMP- and cGMP-dependent protein kinase II. Furthermore, this phosphorylation can activate NOS-III in the absence of Ca2+/CaM which is unusual for NOS-III since this isoform is classified as a constitutively expressed, strictly Ca2+/CaM-dependent enzyme. Recent experiments have, however, suggested that Ca2+-independent NO release can occur in response to fluid shear stress or isometric contraction and a parallel rapid Ser/Thr phosphorylation of NOS-III was suggested. In addition, general tyrosine kinase activation upon shear stress was observed. However, it remained unclear whether NOS-III activity indeed became Ca2+/CaM-independent or whether intracellular increases in free Ca2+ occurred as highly localized Ca2+ spikes in compartments rich in NOS-III (e.g. caveolae) which were not detected by whole cell Ca2+ analysis. Moreover, the protein kinases involved in these effects were not characterized. Our present results show for the first time that cAMP- and cGMP-dependent protein kinase II-evoked Ser/Thr phosphorylation of purified NOS-III causes a partial Ca2+/CaM-independent activation of the enzyme.

Besides evoking Ca2+/CaM-independent activation, cAK and cGK also enhanced the v_max of NOS-III activity in the presence of Ca2+/CaM in vitro. Furthermore, stimulation of cAK in intact HUVEC also increased both Ca2+/CaM-dependent and -independent activation of NOS-III. This could account for an earlier observation by Graier et al. (30) that forskolin-induced elevation of endothelial cell cAMP levels increased NO release, an effect that was abolished by inhibitors of cAK.

With respect to the possible molecular mechanism underlying phosphorylation-dependent activation of NOS-III, we considered previous evidence presented by Salerno et al. (31) for an autoinhibitory control element, a 45-amino acid insert located near the CaM-binding region of only constitutive NOS forms (NOS-I and III, see Fig. 1), not NOS-II. The autoinhibitory element is postulated to stabilize an inhibited NOS conform-
RRKRK immediately before Ser633, one of the cAK/cGK II phosphorylation sites (Fig. 1). Phosphorylation of Ser633 by cAK/cGK II would increase the negative charge in the autoinhibitory domain, and could thereby facilitate displacement of this domain from the site with which it interacts, thus partially activating NOS in the absence of Ca\textsuperscript{2+}/CaM, as well as facilitating Ca\textsuperscript{2+}/CaM binding and enhancing maximal NOS activity.

The autoinhibitory domain insert is present only in NOS-I and NOS-III and, interestingly, Ser633 is conserved only in human, mouse, and bovine NOS-III, but not in NOS-I. Human NOS-I contains four possible cAK/cGK phosphorylation sites, however, our experiments detected only weak phosphorylation and no significant change in catalytic activity of hNOS-I after 5 min incubation with either C subunit or cGK. At the same time, NOS-III was highly (maximal 0.82 mol of phosphate/mol of NOS-III) phosphorylated. Previously, phosphorylation of rat neuronal NOS-I (rNOS-I) by cGK I\textsubscript{a} or C subunit of cAK was shown to result in a 25–40% decrease in rNOS-I activity (17) and translocation of rNOS-I from the membrane to the cytoplasm (16). However, incorporation of only 0.1 mol of phosphate/mol of rNOS-I was observed after treatment with cGK I\textsubscript{a} for 5 min, and half-maximal phosphorylation was evident only after 30 min. The lack of a cAK/cGK effect on phosphorylation and activity of hNOS-I in our experiments may be explained by the presence of a consensus sequence for cAK/cGK phosphorylation in the autoinhibitory domain of rat NOS-I (S\textsuperscript{RKSSGD}) that is absent in human NOS-I (SQKSSGD) (see Fig. 1). It remains to be shown whether such low levels and slow rates of NOS-I phosphorylation ever become physiologically relevant, e.g. for negative feedback inhibition of neuronal NO synthesis. NOS-III, in contrast, is phosphorylated with much faster kinetics.
Salerno et al. (31) also described a basal activity of purified NOS-I or NOS-III (5% of maximal) in the presence of EGTA and absence of CaM which they speculate may arise from a low steady-state concentration of the disinhibited NOS conformer. However, our recombinant NOS-III preparations from ST9 cells did not display Ca\(^{2+}\)/CaM-independent activity, this appeared only after cAK/cGK II-dependent phosphorylation. Chen et al. (18) recently reported that isolated rat heart NOS-III displayed already detectable phosphorylation of Thr\(^{495}\) and Ser\(^{1177}\) (the cAK/cGK phosphorylation sites) and that phosphorylation of Ser\(^{1177}\) by an AMP-activated protein kinase would further activate the enzyme in concert with Ca\(^{2+}\)/CaM.

While our article was under review, several publications appeared demonstrating the phosphorylation and activation of NOS-III by Akt (protein kinase B) (32–35). All reports demonstrated that phosphorylation of Ser\(^{1177}\) enhanced NOS-III enzyme activity and NOS sensitivity to low Ca\(^{2+}\) concentrations. However, none of the reports demonstrated Ca\(^{2+}\)-independent activation of purified NOS-III like that we have shown for cAMP- and cGMP-dependent protein kinase. Interestingly, protein kinase B and cAK not only have overlapping substrate phosphorylation site consensus sequences, as shown here for NOS-III Ser\(^{1177}\) and elsewhere for cAMP response element-binding protein Ser\(^{133}\) (36), but also a recent report has shown cAK activation of protein kinase B via a phosphatidylinositol 3-kinase-independent pathway (37). One of the reports of protein kinase B phosphorylation of NOS-III on Ser\(^{1177}\) (35) also stated (data was not shown) that \(\beta\)-(para-chlorophenylthio)-cGMP, a selective activator of cGK, a selective activator of cGK II, and a co-localization of NOS-III and cGK II needs further investigation. Llamentely, they extend the mechanisms for regulating NO synthesis to include direct induction of a Ca\(^{2+}\)-independent state of NOS-III by phosphorylation. Additionally, phosphorylation-activated Ca\(^{2+}\)/CaM-independent or -dependent NOS-III activity may constitute, in the case of cGK II, an example of internal positive feedback enhancement of NO production, or, in the case of cAK, an example of signaling cross-talk between the cAMP and NO/cGMP pathways.

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Note Added in Proof—During the review of this article, reports of deletions (53, 54) of the autoinhibitory insert described by Salerno et al. (31) demonstrated that the insert had a Ca\(^{2+}\)/CaM-dependent inhibition of eNOS and nNOS and also lowers intrinsic activity of the reductase domain. Deletions of the autoinhibitory insert produced similar effects as did our phosphorylation of eNOS by cAK and cGK, i.e. relief of autoinhibition resulting in both Ca\(^{2+}\)-independent cGK II activity and increased maximal Ca\(^{2+}\), NOS sensitivity to low Ca\(^{2+}\).

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