

Caveolin versus Calmodulin

COUNTERBALANCING ALLOSTERIC MODULATORS OF ENDOTHELIAL NITRIC OXIDE SYNTHASE*

(Received for publication, July 1, 1997, and in revised form, August 7, 1997)

Jeffrey B. Michel[‡], Olivier Feron[§], Kazuhiro Sase, Prakash Prabhakar, and Thomas Michel[¶]

From the Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

Nitric oxide is synthesized in diverse mammalian tissues by a family of calmodulin-dependent nitric oxide synthases. The endothelial isoform of nitric oxide synthase (eNOS) is targeted to the specialized signal-transducing membrane domains termed plasmalemmal caveolae. Caveolin, the principal structural protein in caveolae, interacts with eNOS and leads to enzyme inhibition in a reversible process modulated by Ca^{2+} -calmodulin (Michel, J. B., Feron, O., Sacks, D., and Michel, T. (1997) *J. Biol. Chem.* 272, 15583–15586). Caveolin also interacts with other structurally distinct signaling proteins via a specific region identified within the caveolin sequence (amino acids 82–101) that appears to subserve the role of a “scaffolding domain.” We now report that the co-immunoprecipitation of eNOS with caveolin is completely and specifically blocked by an oligopeptide corresponding to the caveolin scaffolding domain. Peptides corresponding to this domain markedly inhibit nitric oxide synthase activity in endothelial membranes and interact directly with the enzyme to inhibit activity of purified recombinant eNOS expressed in *Escherichia coli*. The inhibition of purified eNOS by the caveolin scaffolding domain peptide is competitive and completely reversed by Ca^{2+} -calmodulin. These studies establish that caveolin, via its scaffolding domain, directly forms an inhibitory complex with eNOS and suggest that caveolin inhibits eNOS by abrogating the enzyme's activation by calmodulin.

family of three related proteins and modulate diverse biological processes ranging from neurotransmission to vascular homeostasis to immunological surveillance (1, 2). These homodimeric proteins share similar overall catalytic schemes to produce NO by the NADPH-, heme-, and O_2 -dependent oxidation of L-arginine in a complex reaction involving numerous redox cofactors, and are absolutely dependent on the ability of the enzymes to be allosterically activated by Ca^{2+} -calmodulin. The roles of calmodulin in NOS catalysis have been extensively studied, and the binding of calmodulin appears to facilitate interdomain electron transfer for all three enzyme isoforms. However, the NOS isoforms differ in important aspects of their Ca^{2+} -dependence for enzyme activation by calmodulin. For the endothelial and neuronal nitric oxide synthase isoforms (termed eNOS and nNOS, respectively), transient changes in intracellular Ca^{2+} promote calmodulin binding and enzyme activation; eNOS and nNOS are characteristically activated as a short-term response to receptor-dependent calcium transients. By contrast, the inflammation-related NOS (iNOS) binds calmodulin avidly and appears to be fully active even at low ambient intracellular calcium levels in immunostimulated cells.

The different NOS isoforms may also be distinguished by their subcellular distribution. eNOS is unique among the NOS isoforms in being targeted to the signal-transducing membrane microdomains termed plasmalemmal caveolae (3). Plasmalemmal caveolae are cholesterol- and glycosphingolipid-enriched domains that appear to function as sites for the sequestration of diverse membrane-targeted signaling proteins (4). Several signaling proteins, including eNOS, have been shown to interact with caveolin, an oligomeric integral membrane protein that appears to serve as the structural “scaffold” within caveolae. The work of Lisanti and colleagues (5–7, 24) has led to the discovery of a direct interaction between caveolin and such structurally diverse signaling proteins as c-Src, Ha-Ras, and G protein α_s . These interactions appear to be mediated through a scaffolding domain comprising a 20 amino acid region (residues 82–101) within the caveolin molecule (5). Synthetic oligopeptides corresponding to this caveolin scaffolding domain directly interact with c-Src, Ha-Ras, and $\text{G}\alpha_s$ and have been shown to inhibit enzymatic activities associated with these signaling proteins (5–7). The ability of these peptides to recapitulate the inhibitory action of caveolin suggests that the caveolin scaffolding domain mediates both association with and inhibition by caveolin for diverse proteins. In the current studies, we have extended this paradigm to the analysis of the interactions between caveolin and eNOS and explored the central role of calmodulin in the modulation of this interaction.

Recently, we reported the results of transient transfection experiments in COS-7 cells in which we showed that the co-expression of eNOS and caveolin leads to a marked inhibition of eNOS enzyme activity (8). The enzyme activities of iNOS and nNOS, which are not targeted to caveolae, are not affected by

The mammalian nitric oxide (NO)¹ synthases comprise a

* This work was supported in part by awards (to T. M.) from the National Institutes of Health, the American Heart Association, and the Burroughs Wellcome Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[‡] Bugher-American Heart Association Fellow in Cardiovascular Molecular Biology.

[§] Supported by a fellowship from the Belgian American Educational Foundation and by a grant from the Patrimoine Facultaire de l'Université Catholique de Louvain (Belgium).

[¶] Established Investigator of the American Heart Association and a Burroughs Wellcome Scholar in Experimental Therapeutics. To whom correspondence should be addressed: Brigham and Women's Hospital, Harvard Medical School, Thorn Building 1110A, 75 Francis St., Boston, MA 02115. Tel.: 617-732-7376; Fax: 617-732-5132; E-mail: Michel@calvin.bwh.harvard.edu.

¹ The abbreviations used are: NO, nitric oxide; NOS, NO synthase; eNOS, endothelial NOS isoform; nNOS, neuronal NOS isoform; iNOS, inflammation-related NOS isoform; BAEC, bovine aortic endothelial cells; GST, glutathione S-transferase; Cav-1, oligopeptide corresponding to the caveolin scaffolding domain, residues 82–101 of caveolin; Cav-X, control peptide for Cav-1; CaM-B, oligopeptide corresponding to the calmodulin-binding domain of bovine eNOS, residues 493–512; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

co-transfection with caveolin. Additionally, we found that the interaction between eNOS and caveolin is entirely abrogated by Ca^{2+} -calmodulin, and we also documented that the inhibitory effect of caveolin co-expression on eNOS activity is entirely reversed by the addition of exogenous calmodulin. These data have led us to hypothesize that caveolin and calmodulin regulate eNOS through reciprocal interactions with the enzyme. However, several important questions remain following analyses of these co-transfection experiments. Does caveolin interact directly with eNOS, or are additional docking or adapter proteins required for their association? Is acylation of eNOS required for its interactions with and inhibition by caveolin? Are membrane lipids required? Which domain(s) of caveolin are required for eNOS interaction? Can interactions between purified eNOS and caveolin be reconstituted *in vitro*? How do caveolin and calmodulin interact in the reciprocal regulation of eNOS activity? In the current studies, we have utilized purified components of the eNOS-caveolin-calmodulin signaling system to pursue answers to these questions. We demonstrate that the caveolin scaffolding domain can potently and specifically mediate inhibition of eNOS activity and that direct interactions involving this domain are required for eNOS-caveolin heteromer formation. In addition, we document that the caveolin scaffolding domain functions as a competitive inhibitor of the allosteric activation of eNOS by Ca^{2+} -calmodulin.

MATERIALS AND METHODS

Peptide Synthesis—As shown in schematic form in Fig. 1, peptides were synthesized corresponding to the amino acids 82–101 scaffolding domain of caveolin-1 (DGIWKASFTTFTVTYKYWFYR, termed Cav-1 herein), to a rearranged version of the caveolin-1 peptide (WGIDKAF-TTSTVITYKWFYR, termed Cav-X), and to the calmodulin-binding domain of bovine eNOS (9), which corresponds to amino acids residues 493–512 (TRKKTFFKEVANAVKISASLM, termed CaM-B). These peptides were synthesized by the Biopolymers Facility of the Howard Hughes Medical Institute at Harvard Medical School and purified by high pressure liquid chromatography to a purity of >98% as confirmed by mass spectrometric analysis.

Preparation of Recombinant GST-eNOS—The bovine eNOS cDNA (10) was cloned into the *EcoRI* site of pGEX3X (Pharmacia Biotech Inc.) to create a glutathione *S*-transferase (GST)-eNOS fusion construct under transcriptional regulation by the inducible bacterial *lac* promoter. This GST-eNOS construct was used to transfect *Escherichia coli* (XL-2 cells, Stratagene); synthesis of the fusion protein was induced for 2 h with 0.1 mM isopropyl-1-thio- β -D-galactopyranoside in a freshly diluted overnight bacterial culture. Bacteria were pelleted and resuspended in a lysis buffer (phosphate-buffered saline, pH 7.4, 10 $\mu\text{g}/\text{ml}$ lysozyme, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 1 μM FMN, 1 μM FAD, 1 μM L-arginine, and 10 μM tetrahydrobiopterin), sonicated for 10 s on ice (output level-10; Branson Sonifier), and centrifuged (5 min, 14,000 $\times g$) to pellet unbroken cells. The supernatant was then incubated with glutathione-Sepharose beads (Pharmacia) for 30 min at 4 °C according to the manufacturer's instructions; the beads were then washed three times in lysis buffer. GST-eNOS fusion protein was eluted with lysis buffer containing 10 mM glutathione. The GST-eNOS fusion protein, a soluble protein, was further characterized in enzyme activity assays and by polyacrylamide gel electrophoresis, as discussed below. NO synthase activity was determined by measuring conversion of L-[^3H]arginine to L-[^3H]citrulline, as described previously (11). Protein concentrations were determined using the Bradford method (12).

Endothelial and Sf9 Cell Culture—Bovine aortic endothelial cells (BAEC) were cultured and harvested (between passage 4 and 10) as described previously (11). Expression of recombinant baculovirus expressing eNOS in insect Sf-9 cells followed our previously reported protocols (13, 14).

Immunoprecipitation, SDS-PAGE, and Immunoblotting—Immunoprecipitation of eNOS and caveolin followed our previously reported protocols (8, 11) as summarized below. BAEC were harvested, lysed and sonicated, then solubilized with a Ca^{2+} -free CHAPS buffer containing 50 mM Tris-HCl (pH 7.4), 16 mM CHAPS, 1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, 10 μM tetrahydrobiopterin, 4 μM FAD, 4 μM FMN, 1 μM L-arginine, and protease inhibitors. Solubilized lysates were incubated for 1 h at 4 °C either with polyclonal α -caveolin antibody (Transduction Laboratories) at a final concentration of 4 $\mu\text{g}/\text{ml}$ or with a

previously characterized rabbit eNOS polyclonal antiserum (15, 16) at a final dilution of 1:100. Protein G-Sepharose was then added for 1 h, and the immunoprecipitated complexes were washed four times, eluted by boiling in Laemmli sample buffer, separated on 12% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Mouse monoclonal α -eNOS and α -caveolin-1 antibodies (Transduction Laboratories) were then used to detect eNOS and caveolin-1 as described previously (8, 11). Quantitation of proteins detected by immunoblotting was performed using laser densitometry of x-ray films.

Gel Mobility Shift Assays for Calmodulin Binding—Purified calmodulin was incubated with peptides as noted below and then subjected to non-reducing polyacrylamide gel electrophoresis (using 18% polyacrylamide separating gel) in a modified Laemmli buffer system (17). Urea (4 M) was present in both the stacking and separating gels. To determine the calcium dependence of calmodulin binding, either CaCl_2 (0.1 mM) or EGTA (2 mM) as noted below were added to the electrophoresis running buffer and the gel itself. Calmodulin (1.5 μM) was incubated with various oligopeptides (2.5 μM) for 1 h at room temperature in 4 M urea, 0.1 M Tris-HCl, pH 7.2, in the presence of either EGTA (2 mM) or CaCl_2 (0.1 mM) as indicated and followed by SDS-PAGE analysis.

RESULTS

Effects of the Caveolin Scaffolding Domain Peptide on Interaction between eNOS and Caveolin—We have previously shown that eNOS and caveolin can be immunoprecipitated in a stable heteromeric complex (11) and that addition of Ca^{2+} -calmodulin disrupts the association between eNOS and caveolin (8). To explore whether sequences corresponding to the caveolin scaffolding domain could be implicated in the calmodulin-regulated interactions between eNOS and caveolin, we designed synthetic oligopeptides based on the scaffolding domain of caveolin (5) and the calmodulin-binding domain of eNOS (9). As shown in Fig. 1A, the caveolin scaffolding domain peptide, corresponding to residues 82–101 of the human caveolin sequence, is termed "Cav-1." A control peptide that we have termed "Cav-X" has an identical amino acid composition, but amino acids were exchanged pairwise at 8 highly conserved positions within this 20 amino acid sequence; thus, the conserved aspartic acid residue at position 1 is exchanged for the conserved tryptophan residue at position 4 and *vice versa*. Likewise, residue Ser-7 was exchanged for Trp-11; similarly traded were Lys-15 and Tyr-16, and Tyr-19 and Arg-20. This "minimally scrambled" control peptide, Cav-X, thus has 60% identity in amino acid sequence and 100% identity in amino acid composition compared with the Cav-1 peptide. This conservatively designed Cav-X control peptide helps to provide confidence in the specificity of effects seen with the Cav-1 scaffolding domain peptide. We designed experiments exploiting the Cav-1 peptide to explore the possibility that the caveolin scaffolding domain participates in the formation of eNOS-caveolin complexes. We found that the Cav-1 peptide, but not the control Cav-X peptide, completely blocked the co-immunoprecipitation by caveolin antibody of the eNOS-caveolin complex (Fig. 1B); these peptides did not alter the recovery of caveolin. We also found that a peptide corresponding to the eNOS calmodulin binding site (9), which we have termed CaM-B (Fig. 1), had no effect on eNOS-caveolin co-immunoprecipitation.

In a parallel series of experiments, antibodies directed against caveolin were used to precipitate caveolin-eNOS immunocomplexes, which were then isolated on protein G-Sepharose. The addition of the Cav-1 peptide (but not the control Cav-X peptide) promotes the dissociation of eNOS from caveolin (Fig. 1C). The eNOS released by Cav-1 from the caveolin-protein G-Sepharose immunocomplex can be completely recovered from the supernatant; there is no change in the recovery of caveolin (Fig. 1C, lower panels).

Effects of the Caveolin Scaffolding Domain Peptide on eNOS Enzyme Activity in Endothelial Cell and Sf9 Cell Membranes—We have previously shown that the co-transfection of eNOS and caveolin-1 cDNA into COS-7 cells leads to a marked attenuation of eNOS enzyme activity and that this inhibition is

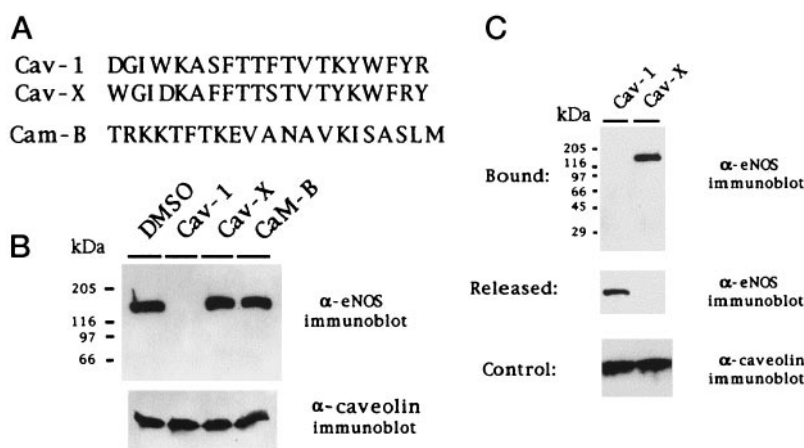


FIG. 1. Oligopeptides and the blockade of eNOS-caveolin interaction. *A*, shown are the amino acid sequences of synthetic oligopeptides used in these studies: the caveolin scaffolding domain peptide (*Cav-1*), its control peptide (*Cav-X*), and the calmodulin binding site peptide (*CaM-B*). The peptide *Cav-1* is based on amino acid residues 82–101 of caveolin-1, also termed the caveolin scaffolding domain (5). *Cav-X* represents a control peptide for *Cav-1* and is designed to maintain an identical amino acid sequence composition as the *Cav-1* peptide. Pairwise exchange of amino acid at 8 specific residues yields a control peptide that is 60% identical in sequence to *Cav-1*. The peptide labeled *CaM-B* is based on the sequence in eNOS implicated in calmodulin binding (9) and corresponds to residues 492–512 of the bovine eNOS sequence (10). *B*, this panel shows the effects of peptides on eNOS-caveolin co-immunoprecipitation from endothelial cells. For this SDS-PAGE/immunoblot analysis, peptides as shown were added to solubilized endothelial cell lysates that were then immunoprecipitated with the polyclonal caveolin antibody as described in the text. Peptides were present at a final concentration of $10 \mu\text{M}$ in 0.25% Me_2SO . The results of eNOS antibody immunoblotting are shown in the upper blot. The lower panel shows the result of reprobing this same immunoblot with the caveolin antibody. *C*, this experiment explored the release of eNOS from caveolin immune complexes as promoted by caveolin scaffolding domain peptides. Solubilized endothelial cell lysates were incubated with anti-caveolin antibody, and immune complexes were isolated using protein G-Sepharose as described in the text and then incubated with $10 \mu\text{M}$ *Cav-1* or *Cav-X* peptide for 30 min at 22°C . The Sepharose beads were then pelleted, and the proteins were released into the supernatant, and those remaining bound in the protein G-Sepharose pellet were analyzed by SDS-PAGE and immunoblotting. The upper panel shows the results of an eNOS antibody immunoblot analysis of the material remaining bound to the protein G-Sepharose after the peptide incubation, and the eNOS immunoblot shown in the middle panel analyzes the proteins released into the supernatant. The immunoblot shown in the lower panel was probed with antibody directed against caveolin and is shown in the lower panel.

relieved by adding purified calmodulin to the enzyme assay. As seen in Fig. 2A, the *Cav-1* peptide potently inhibits eNOS enzyme activity in washed BAEC membranes (assayed in the absence of added calmodulin), whereas *Cav-X* peptide had only modest inhibitory effects even at higher peptide concentrations. To test the hypothesis that calmodulin would antagonize *Cav-1* mediated NOS inhibition, separate experiments explored the effects of purified calmodulin on the inhibition of eNOS promoted by the *Cav-1* peptide (Fig. 2B). The addition of exogenous calmodulin resulted in a small increase in the eNOS activity in control BAEC membranes; however, in the presence of *Cav-1* peptide, increasing concentrations of calmodulin completely and dose-dependently reversed eNOS inhibition induced by the peptide. The IC_{50} for *Cav-1* in inhibition of eNOS activity is $1.0 \mu\text{M}$ in the absence of exogenous calmodulin; addition of increasing amounts of calmodulin increases this IC_{50} value to the point that no *Cav-1*-promoted inhibition of eNOS is seen at the highest concentrations of calmodulin.

In an effort to determine whether endothelial-specific cofactors or proteins are required for *Cav-1* inhibition of eNOS activity, we infected insect Sf-9 cells with recombinant baculovirus encoding eNOS (13). In experiments identical in design to those described for BAEC, eNOS activity was determined in washed cell membrane fractions from recombinant baculovirus-infected insect Sf9 cells at various concentrations of *Cav-1* and calmodulin. As shown in Fig. 2C, the *Cav-1* peptide markedly inhibits eNOS enzyme activity in Sf9 cell membranes. In parallel experiments, *Cav-X* had no effect on NO synthase activity (data not shown). In contrast to BAEC, NOS activity in recombinant baculovirus-infected Sf9 cells is markedly activated by the addition of exogenous calmodulin, possibly reflecting differences in the efficacy of insect calmodulin in mammalian eNOS activation (among other possibilities). The effect of *Cav-1* on inhibition of eNOS activity is even more striking in these Sf9 cell membranes, approaching 90% enzyme inhibition

at the highest *Cav-1* concentrations. The addition of calmodulin attenuated the NOS inhibitory effect of the *Cav-1* peptide, and at the highest concentrations of exogenous calmodulin, the effect of *Cav-1* is entirely abrogated in eNOS-infected Sf9 cell membranes (Fig. 2C).

Bacterial Expression of Recombinant eNOS and Inhibition of the Purified Enzyme by the Caveolin Scaffolding Domain Peptide—The interactions between eNOS and caveolin were next explored using purified bacterially expressed recombinant eNOS. Study of the interactions of caveolin with purified eNOS would help to explore the role, if any, of additional factors in this protein-protein association. The effects of membrane lipids may also be excluded by the analysis of eNOS in this *E. coli* expression system; since the acylations of eNOS that are essential for its targeting to plasmalemma do not take place in this prokaryotic system, the bacterial expression of eNOS yields a soluble rather than particulate enzyme (19). To facilitate purification of the protein using glutathione-Sepharose affinity chromatography, we used the bacterial expression vector pGEX-3X (Pharmacia) to construct a recombinant plasmid encoding a fusion protein between eNOS and GST. The fusion protein was designed such that the GST is located at the eNOS N terminus; this site was chosen because previously characterized eNOS fusion proteins involving the enzyme's N terminus appear to retain full activity (20). Indeed, the specific activity of the purified bacterially expressed GST-eNOS fusion protein averaged $50 \text{ nmol/min/mg protein}$ (see Fig. 3), comparing favorably to previous reports for the specific activity of purified eNOS (19). Using glutathione-Sepharose chromatography, the GST-eNOS fusion protein was purified to apparent homogeneity in a single step from the soluble fraction of *E. coli* transfected with this recombinant construct. SDS-PAGE of the purified protein yielded a single band in the Coomassie Blue-stained gel; this band co-migrates with the single band identified by immunoblotting with eNOS antibody (Fig. 3).

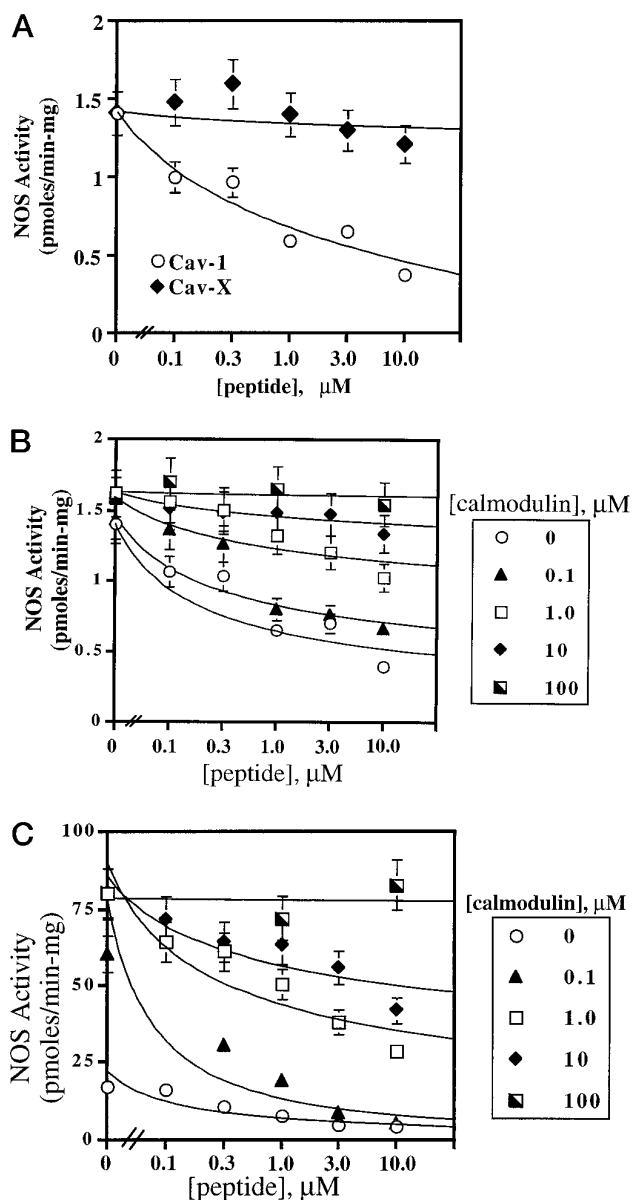


FIG. 2. Inhibition of eNOS activity in biological membranes by the caveolin scaffolding domain peptide. NOS activity was determined using the [3 H]arginine \rightarrow [3 H]citrulline assay in biological membranes in the presence of varying concentrations of the Cav-1 or Cav-X peptide. **A**, NOS activity measured in endothelial cell membranes (no exogenous calmodulin was added to these assays, which were performed three times in duplicate with equivalent result). **B**, increasing amounts of purified calmodulin were added to NOS activity assays performed in endothelial cell membranes in the presence of varying concentrations of the Cav-1 peptide as shown. This experiment, performed in duplicate, was repeated three times with similar results. **C**, recombinant baculovirus-expressing eNOS was used to infect insect Sf9 cells and washed membranes prepared from these cells used in NOS activity assays identical in design to the experiment shown in **B**. The experiment shown is representative of three similar experiments, each performed in duplicate.

In the absence of added calmodulin, the purified GST-eNOS fusion protein has no detectable NOS activity. A series of NOS activity assays were performed in the presence of 0.1 μ M calmodulin, along with varying concentrations of the Cav-1 or Cav-X peptides. The Cav-X peptide had minimal effects even at higher peptide concentrations. By contrast, the Cav-1 peptide completely inhibited enzyme activity of the purified GST-eNOS with an IC_{50} of 0.3 μ M (Fig. 3). To determine the mechanism whereby caveolin inhibits calmodulin-activated eNOS enzyme

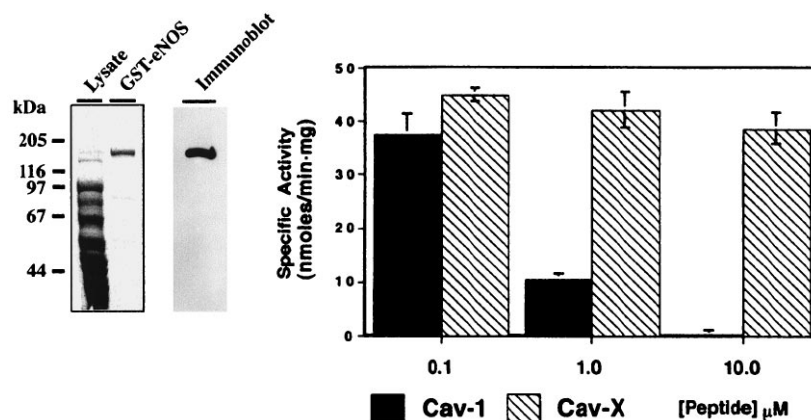
activity, we conducted a series of NOS enzyme assays in the presence of varying concentrations of calmodulin and Cav-1 peptide (Fig. 4A). The analysis of double reciprocal plots of NOS activity versus calmodulin concentration studied at different Cav-1 peptide concentrations (Fig. 4B) revealed a common y intercept, consistent with competitive inhibition of calmodulin's activation of eNOS by the Cav-1 peptide. Extrapolation of x axis intercepts permits the estimation of the K_a for calmodulin at different concentrations of Cav-1 peptide. In the absence of Cav-1 peptide, the calmodulin K_a is approximately 20 nM, increasing to 3 μ M in the presence of 10 μ M Cav-1 peptide. As a result, in the presence of 10 μ M Cav-1 peptide, NOS activity is fully inhibited even in the presence of 100 nM calmodulin (Fig. 4B).

Gel Mobility Shift Assays to Explore Interactions of the Caveolin Scaffolding Domains with Calmodulin—The key role of Ca^{2+} -calmodulin in modulating eNOS-caveolin interactions led us to explore whether caveolin might also interact with calmodulin directly, although our previous studies had found no evidence for co-immunoprecipitation of caveolin with calmodulin in endothelial cell membranes (8). The specificity of the enzyme inhibitory effect of the caveolin scaffolding domain was explored in studies of calmodulin-dependent kinase activity in endothelial cell membranes in which we found that the caveolin scaffolding domain peptide inhibits eNOS activity but *not* calmodulin-dependent kinase activity in endothelial cell lysates (data not shown). We next used the caveolin scaffolding domain peptides in gel mobility shift assays (Fig. 5), and compared the effects of these peptides with a eNOS-derived peptide, termed CaM-B, corresponding to the enzyme's calmodulin binding site. This experimental approach is based on a modified, partially denaturing non-reducing Laemmli SDS-PAGE system (17) and has been previously exploited to investigate interactions between purified calmodulin and putative calmodulin-binding peptides (18). We assessed the ability of Cav-1 and CaM-B peptides to "shift" calmodulin migration to positions of lower mobility in this modified electrophoresis system. As has been previously reported (9), the CaM-B peptide, corresponding to the eNOS calmodulin binding site, altered the migration of purified calmodulin in a Ca^{2+} -dependent fashion. By contrast, addition of the Cav-1 peptide at concentrations that fully inhibit eNOS enzyme activity could not be shown to alter calmodulin mobility in either the presence or absence of Ca^{2+} . Taken together, these results suggest, but do not prove, that the caveolin scaffolding domain is unlikely to directly interact with calmodulin.

DISCUSSION

The interaction of eNOS with caveolin appears to serve as a key determinant of enzyme activity, and this interaction may be dynamically regulated by the Ca^{2+} -calmodulin-dependent activation of NOS in endothelial cells (8). The present study utilizes the purified components of this signaling system to provide further insights into the mechanisms whereby caveolin inhibits eNOS. A synthetic oligopeptide corresponding to the caveolin scaffolding domain (Cav-1) contains sequences both necessary and sufficient to inhibit enzyme activity of purified eNOS, and the enzyme inhibition promoted by this peptide is completely reversed by Ca^{2+} -calmodulin. These results with purified components further substantiate our prior observations in biological membranes: when eNOS activity is assayed in membranes prepared from COS-7 cells co-transfected with caveolin and eNOS, caveolin co-expression inhibits eNOS activity, and the inhibitory effects of caveolin are entirely abrogated by calmodulin (8). In the present studies, it thus appears that the caveolin scaffolding domain peptide can serve as a

FIG. 3. Inhibition of purified recombinant eNOS by caveolin scaffolding domain peptide. Purification of GST-eNOS from *E. coli* (see text) was monitored by Coomassie Blue staining of SDS-PAGE of cleared bacterial lysates (*first lane*), and after elution of the recombinant fusion protein following chromatography on glutathione-Sepharose (*second lane*); immunoblots of the eluted material were probed with the eNOS antibody (*middle panel*). eNOS activity was quantitated in the purified preparation in the presence of varying concentrations of Cav-1 and Cav-X peptides (*right panel*). Similar results were obtained in three separate experiments, each performed in duplicate.



surrogate for caveolin itself, at least insofar as the regulatory interactions of caveolin and calmodulin with eNOS are concerned.

The targeting of eNOS to plasmalemmal caveolae is dependent on the myristoylation and palmitoylation of the enzyme (reviewed in Ref. 2). Since the eNOS expressed in *E. coli* is a soluble protein, being neither myristoylated nor palmitoylated, the potent inhibition of bacterially expressed eNOS by the Cav-1 peptide implies that eNOS acylation is not required for productive and appropriately regulated interactions of the enzyme with caveolin. Indeed, these data complement our recent studies of eNOS acylation mutants² and indicate that eNOS-caveolin interactions, distinctive from eNOS targeting to caveolae, may still take place even in the absence of eNOS acylation. Clearly, the direct interactions of eNOS, caveolin, and calmodulin can be reconstructed in a soluble system involving purified components, and within the limitations of this experimental system, it appears that additional protein or lipid components are not required.

Several lines of investigation provide data suggesting that caveolin and calmodulin do not interact directly; a peptide derived from the calmodulin binding site on eNOS can be shown to bind to calmodulin, but peptides derived from the caveolin scaffolding domain fail to do so (Fig. 5). Furthermore, the caveolin scaffolding domain does not have a typical calmodulin-binding sequence motif (22, 23). Our previous data failed to show evidence for co-immunoprecipitation between caveolin and calmodulin (8), and other calmodulin-dependent enzyme activities including calmodulin-dependent protein kinase do not appear to be inhibited by the caveolin scaffolding domain peptide. These negative data do not preclude the possibility that under different experimental conditions the caveolin scaffolding domain (or other sequences within the caveolin molecule) might bind directly to calmodulin. However, the caveolin scaffolding domain does not appear to interact with calmodulin under the experimental conditions utilized in our studies.

The binding site for caveolin has not yet been delineated for eNOS, but a sequence motif found in several other caveolin-associated proteins also appears to be present in eNOS (24). A recent report suggests that sequences both within the N- and C-terminal halves of caveolin appear to interact with eNOS in addition to the caveolin scaffolding domain (21). However, our data indicate that the caveolin scaffolding domain is both necessary and sufficient for caveolin to interact with eNOS, and thereby leads to enzyme inhibition. The formation of the heteromeric complex between eNOS and caveolin is completely

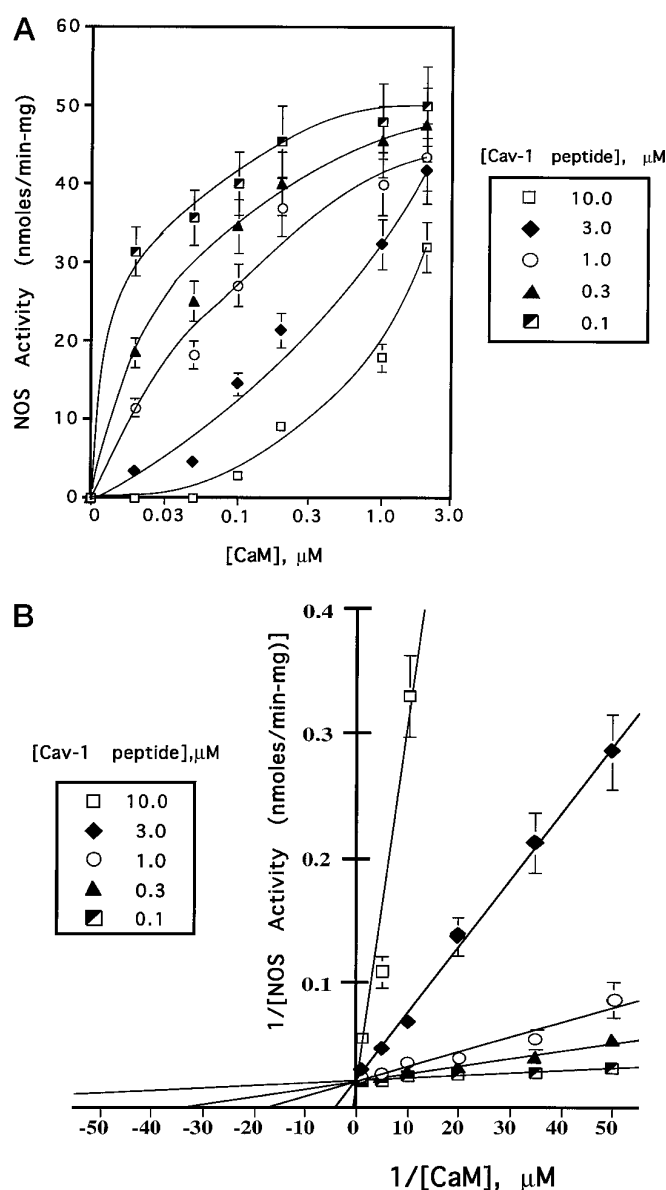


FIG. 4. Effect of calmodulin on inhibition of purified bacterially expressed eNOS by the caveolin scaffolding domain peptide. At each of several different concentrations of the Cav-1 peptide, the NO synthase activity of purified GST-eNOS was assayed at varying concentrations of calmodulin, as noted on the figure. A, plot of [calmodulin] versus NOS activity at varying concentrations of Cav-1 peptide; B, double reciprocal plot of data shown in panel A. This experiment is representative of five similar experiments, each performed in duplicate.

² O. Feron, J. B. Michel, K. Sase, and T. Michel, submitted for publication.

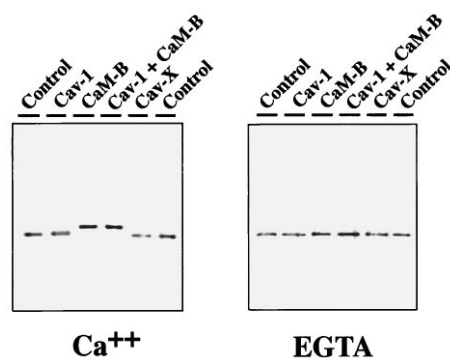


FIG. 5. **Electrophoretic mobility gel shift assay of calmodulin.** Shown are the results of peptide incubations of the electrophoretic mobility of purified calmodulin analyzed by non-reducing partially denaturing urea-SDS-PAGE performed in the presence of excess Ca^{2+} (left panel) or with excess EGTA (right panel). Cav-1, Cav-X, or CaM-B peptides were incubated with the calmodulin prior to electrophoresis in the presence of Ca^{2+} or EGTA as described in the text.

blocked by the caveolin scaffolding domain peptide, Cav-1; the calmodulin-binding domain peptide, CaM-B, which is derived from the eNOS sequence, has no effect on heteromer formation (Fig. 1). In addition, when Cav-1 and CaM-B peptides are mixed in calmodulin gel mobility shift experiments, the addition of the Cav-1 peptide failed to alter calmodulin association with CaM-B (Fig. 5). Although not definitive, these results suggest that eNOS domains distinct from (or in addition to) the eNOS calmodulin binding site may mediate the association of eNOS with the caveolin scaffolding domain.

It is unclear how caveolin inhibits such diverse enzymes, variously attenuating the GTPase activity of Ha-Ras and G protein α -subunits, the tyrosine kinase activity of Src, as well as the NO synthase activity of eNOS. Our enzyme kinetic analyses (Fig. 4) reveal that caveolin appears to serve as a competitive inhibitor of calmodulin-dependent eNOS activation. However, the precise nature of this inhibition may be more complex than can be elucidated by these limited kinetic analyses, and the eNOS-caveolin interaction may be modulated further by interactions of these proteins with other lipid and protein constituents present in biological membranes. Aside from eNOS, the other caveolin-modulated signaling proteins

are not known to be calmodulin-dependent; elements of higher order structure, as yet unidentified, may serve as the basis for caveolin inhibition of NOS activity. The ability of caveolin to dramatically alter the K_a for calmodulin likely represents an important mechanism influencing eNOS activation, as the calmodulin-modulated interactions of eNOS with caveolin may be subject to both hormonal regulation and the influences of hemodynamic shear stress associated with changes in intracellular Ca^{2+} . The interactions of eNOS with calmodulin *versus* caveolin provide a novel example of the reciprocal regulation of enzyme activity by competing allosteric protein-protein interactions.

REFERENCES

- Nathan, C., and Xie, Q.-W. (1994) *J. Biol. Chem.* **269**, 13725–13728
- Sase, K., and Michel, T. (1997) *Trends Cardiovasc. Med.* **7**, 28–37
- Shaul, P. W., Smart, E. J., Robinson, L., German, Z., Yuhanna, I. S., Ying, Y., Anderson, R. G., and Michel, T. (1996) *J. Biol. Chem.* **271**, 6518–6522
- Anderson, R. G. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 10909–10913
- Li, S., Couet, J., and Lisanti, M. P. (1996) *J. Biol. Chem.* **271**, 29182–29190
- Li, S., Okamoto, T., Chun, M., Sargiacomo, M., Casanova, J. E., Hansen, S. H., Nishimoto, I., and Lisanti, M. P. (1995) *J. Biol. Chem.* **270**, 15693–15701
- Song, K. S., Li, S., Okamoto, T., Quilliam, L. A., Sargiacomo, M., and Lisanti, M. P. (1996) *J. Biol. Chem.* **271**, 9690–9697
- Michel, J. B., Feron, O., Sacks, D., and Michel, T. (1997) *J. Biol. Chem.* **272**, 15583–15586
- Venema, R. J., Sayegh, H. S., Kent, J. D., and Harrison, D. G. (1996) *J. Biol. Chem.* **271**, 6435–6440
- Lamas, S., Marsden, P. A., Li, G. K., Tempst, P., and Michel, T. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 6348–6352
- Feron, O., Belhassen, L., Kobzick, L., Smith, T. W., Kelly, R. A., and Michel, T. (1996) *J. Biol. Chem.* **271**, 22810–22814
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Busconi, L., and Michel, T. (1995) *Mol. Pharmacol.* **47**, 655–659
- Michel, J. B., and Michel, T. (1997) *FEBS Lett.* **405**, 356–362
- Busconi, L., and Michel, T. (1993) *J. Biol. Chem.* **268**, 8410–8413
- Robinson, L. J., and Michel, T. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 11776–11780
- Erickson-Viitanen, S., and DeGrado, W. F. (1987) *Methods Enzymol.* **139**, 455–478
- Hanson, P. I., and Shulman, H. (1992) *J. Biol. Chem.* **267**, 17216–17224
- Rodriguez-Crespo, I., Gerber, N. C., and Ortiz de Montellano, P. R. (1996) *J. Biol. Chem.* **271**, 11462–11467
- Kantor, D. B., Lanzrein, M., Stary, S. J., Sandoval, G. M., Smith, W. B., Davidson, N., and Shuman, E. M. (1996) *Science* **274**, 1744–1748
- Ju, H., Zou, R., Venema, V. J., and Venema, R. C. (1997) *J. Biol. Chem.* **272**, 18522–18525
- Rhoads, A. R., and Friedberg, F. (1997) *FASEB J.* **11**, 331–340
- O'Neil, K. T., and Derado, W. F. (1990) *Trends Biochem. Sci.* **15**, 59–64
- Couet, J., Li, S., Okamoto, T., Ikezu, T., and Lisanti, M. P. (1997) *J. Biol. Chem.* **272**, 6525–6533