Interaction of Neuronal Nitric-oxide Synthase with Caveolin-3 in Skeletal Muscle

IDENTIFICATION OF A NOVEL CAVEOLIN SCAFFOLDING/INHIBITORY DOMAIN

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Neuronal nitric-oxide synthase (nNOS) has been shown previously to interact with α1-syntrophin in the dystrophin complex of skeletal muscle. In the present study, we have examined whether nNOS also interacts with caveolin-3 in skeletal muscle. nNOS and caveolin-3 are coimmunoprecipitated from rat skeletal muscle homogenates by antibodies directed against either of the two proteins. Synthetic peptides corresponding to the membrane-proximal caveolin-3 residues 65–84 and 109–130 and homologous caveolin-1 residues 82–101 and 135–156 potently inhibit the catalytic activity of purified, recombinant nNOS. Purified nNOS also binds to a glutathione S-transferase-caveolin-1 fusion protein in vitro binding assays. In vitro binding is completely abolished by preincubation of nNOS with either of the two caveolin-3 inhibitory peptides. Interactions between nNOS and caveolin-3, therefore, appear to be direct and to involve two distinct caveolin scaffolding/inhibitory domains. Other caveolin-interacting enzymes, including endothelial nitric-oxide synthase and the c-Src tyrosine kinase, are also potently inhibited by each of the four caveolin peptides. Inhibitory interactions mediated by two different caveolin domains may thus be a general feature of enzyme docking to caveolin proteins in plasma-membrane caveolae.

Nitrergic nerve fibers have been identified as an important and highly versatile signaling molecule in the nervous, immune, and cardiovascular systems. Endogenous NO in these systems is produced by one of three isoforms of nitric-oxide synthase (NOS)1 termed neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). nNOS is so termed because it was first localized in neurons (1). The NOS isoform, however, is also highly expressed in skeletal muscle (2, 3), where it appears to be involved in modulating contractile force (3). Protein-protein interactions of NOS function both to regulate enzyme activity and to target the protein to specific subcellular locations. nNOS is activated by interaction with Ca2+/calmodulin (CaM) (4) and is inhibited by interaction with a 10-kDa protein designated PIN (5). The NOS protein is targeted to neuronal postsynaptic densities by interaction with the postsynaptic density proteins PSD-95 and PSD-93 (6, 7) and to the sarcolemma of skeletal muscle cells by interaction with α2-syntrophin in the membrane cytoskeleton dystrophin complex (6, 8). Interactions with PIN, PSD-95, PSD-93, and α2-syntrophin are mediated by a 230-amino acid N-terminal extension of the enzyme that contains a PDZ domain. This domain is not found in either eNOS or iNOS (9).

eNOS activity and subcellular localization is also modulated by protein-protein interactions. eNOS is localized in plasma-membrane caveolae of endothelial cells and cardiac myocytes through association with the caveolae integral-membrane structural proteins, caveolins-1 and -3, respectively (10, 11). We have recently shown that caveolin-1 interacts directlywith eNOS and inhibits the catalytic activity of the enzyme (12). eNOS and nNOS are structurally similar, sharing >60% amino acid sequence identity outside of the nNOS N-terminal extension (13). Caveolins-1 and -3 are also >60% identical (14). Interestingly, it has been shown recently that caveolin-3 in skeletal muscle is a component of the dystrophin complex (15). Therefore, in the present study, we have examined whether skeletal muscle nNOS, in addition to interacting with α1-syntrophin, also interacts with caveolin-3.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal anti-nNOS antibody (clone 16), polyclonal anti-caveolin-1 antibody, monoclonal anti-caveolin-1 antibody (clone 2297), and polyclonal anti-caveolin-3 antibody were obtained from Transduction Laboratories (Lexington, KY). t-[14C]Arginine and [γ-32P]ATP were purchased from NEN Life Science Products. ECL detection reagents came from Amersham Corp. Synthetic peptides were obtained from Research Genetics, Inc. (Huntsville, AL) or from the Medical College of Georgia Biochemistry Core Facility and were >95% pure as assessed by high performance liquid chromatography. Recombinant human e-Crk kinase (1,000,000 units/mg), purified rat forebrain Ca2+/CaM-dependent protein kinase II (0.6 μmol of phosphate incorporated into Auto Camtide II substrate peptide/min/mg) and Ca2+/CaM-dependent protein kinase II assay kit were purchased from Upstate Biotechnology (Lake Placid, NY). Protein A/Protein G-agarose was obtained from Life Technologies Inc. AG 50W-X8 cation exchange resin, protein assay kit, and peroxidase-conjugated secondary antibodies came from Bio-Rad. Bovine CaM, NADPH, FAD, and FMN were obtained from Sigma, and tetrahydrobiopterin was obtained from Research Biochemicals International. CaM-Sepharose 4B was purchased from Pharmacia Biotech Inc.

Tissue Extraction, Immunoprecipitation, and Immunoblotting—Rat quadriceps skeletal muscle was minced into small pieces with a razor blade and homogenized in 10 volumes of ice-cold buffer containing 20 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 50 mM NaF, 10 mM Na3PO4, 1% Triton X-100, 1% phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin A, and 5 μg/ml aprotonin. Homogenates were centrifuged at 10,000 × g for 20 min to remove insoluble material. Immunoprecipitation with anti-nNOS antibody (0.5 μg) and anti-caveolin antibody (0.5 μg) was carried out as described previously (10, 11). Proteins in anti-nNOS immunoprecipitates were run on 12.5% gels, and proteins in anti-caveolin-3 immunoprecipitates were run on 7.5% gels. Proteins were transferred to nitrocellulose membranes by electroblotting, and
membranes were probed with anti-caveolin antibody or anti-nNOS antibody followed by a peroxidase-conjugated secondary antibody. Bound antibody was visualized by the ECL chemiluminescent detection system and autoradiography.

**Determination of the Effects of Synthetic Peptides on nNOS, eNOS, Ca<sup>2+</sup>/CaM-dependent Protein Kinase II, and c-Src Catalytic Activities—**Synthetic peptides corresponding to bovine caveolin-1 residues 61–81 (DDDVFKIDPVEIAEGPHSTG), 82–101 (DGIWKASFTTVKTYWFYR), 135–156 (KSFLIEIQCSRVSYYHTFC), and 157–178 (DPLFAIGKIFSNIRINTQKEI) (12) and rat caveolin-3 residues 65–84 (DGVRWSYTTFTSVKCYWR) and 109–130 (KSYLIEIQSITNRTQKEI) (12) were tested for their effects on nNOS, eNOS, Ca<sup>2+</sup>/CaM-dependent protein kinase II, and c-Src catalytic activities. Bovine eNOS and human nNOS (100 pmol each) expressed and purified to >90% homogeneity from a baculovirus system (16–18) were mixed with ice on various concentrations of peptides. NOS activity was then determined as described previously (16). c-Src activity was measured in an autophosphorylation assay. Three units of purified, human recombinant c-Src was incubated with various concentrations of caveolin peptides in 50 μl of kinase reaction buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl<sub>2</sub>, and 2 mM MnCl<sub>2</sub>). Reactions were initiated by the addition of 10 μM of [γ-<sup>32</sup>P]ATP and allowed to proceed for 15 min at 25 °C. Reactions were terminated by the addition of 50 μl of 2 × SDS sample buffer and boiling of samples for 5 min. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (10%) gels) followed by autoradiography. Ca<sup>2+</sup>/CaM-dependent protein kinase II activity was assayed using a CaM-dependent protein kinase II assay kit to phosphorylate Auto CaM II with [γ-<sup>32</sup>P]ATP, 100 ng of purified enzyme was used to phosphorylate the Auto CaM II substrate according to the manufacturer’s instructions in the presence of the same final concentration of Ca<sup>2+</sup>/CaM as used in the nNOS activity assays. To determine the stoichiometry of Ca<sup>2+</sup>/CaM-dependent protein kinase II autophosphorylation under these assay conditions, the <sup>32</sup>P-labeled kinase protein was separated from <sup>32</sup>PATP on SDS-polyacrylamide gels. The number of moles of phosphate incorporated into the enzyme in vitro was then calculated based on the amount of <sup>32</sup>P present in the excised protein band and the known specific radioactivity of the [γ-<sup>32</sup>P]ATP used, as described previously (19).

**CaM-Sepharose Chromatography—**Purified human nNOS (1 μg) was incubated with or without synthetic peptides (100 μM) in 50 mM Tris-HCl, pH 7.5 buffer for 5 min at 37 °C and then subjected to CaM-Sepharose chromatography as described previously (12, 17). Bound nNOS was eluted from the column with 2 mM EGTA and quantitated by immunoblotting with anti-nNOS antibody as described previously (12, 19).

Interaction of nNOS with a GST-Caveolin-1 Fusion Protein—**In vitro binding assays of baculovirus-expressed, purified human nNOS and a full-length bovine GST-caveolin-1 fusion protein were carried out under the same conditions described previously for eNOS binding to GST-caveolin-1 fusion proteins (12).

**RESULTS AND DISCUSSION**

Rat quadriceps muscle homogenates were immunoprecipitated with either an anti-nNOS or an anti-caveolin-3 antibody. Anti-nNOS immunoprecipitates were subjected to immunoblotting with anti-caveolin-3. Anti-caveolin-3 immunoprecipitates were subjected to immunoblotting with anti-nNOS. As shown in Fig. 1, caveolin-3 (20 kDa) was specifically coimmunoprecipitated by the anti-nNOS antibody and nNOS (160 kDa) was specifically coimmunoprecipitated by the anti-caveolin-3 antibody. A different anti-caveolin antibody that recognizes both caveolins-1 and -3 also coprecipitated nNOS. However, a caveolin-1-specific antibody (clone 2297) did not react with the caveolin protein precipitated by the anti-caveolin-3 antibody.

Caveolin-3 is structurally and functionally similar to caveolin-1, sharing 175-residue protein containing three distinct domains: a 101-residue N-terminal cytoplasmic domain, a 44-residue C-terminal cytoplasmic domain, and a 33-residue membrane-spanning domain (21–23). We have shown previously that interaction of eNOS with either cytoplasmic domain of caveolin-1 significantly inhibits eNOS catalytic activity. The eNOS inhibitory region of the caveolin-1 N-terminal cytoplasmic domain is identified as a membrane-proximal region containing residues 82–101 (12). This 82–101 region has also been shown to inhibit the catalytic activities of Gα subunits, Ha-Ras, and Src family tyrosine kinases (24–26). It has been proposed, therefore, that this 20-amino acid sequence of caveolin-1 represents the caveolin-1 scaffolding domain. Other regions of caveolin-1 have not been suspected of participating in caveolin-1 protein-protein interactions (24–26). In the present study, however, we have identified a second membrane-proximal caveolin scaffolding/inhibitory domain that exists in the C-terminal cytoplasmic tails of both caveolins-1 and -3. The C-terminal cytoplasmic tail of caveolin-1 is comprised of residues 135–178. We therefore prepared synthetic peptides corresponding to bovine caveolin-1 residues 135–156 and 157–178 (12). Peptides were tested for their abilities to inhibit recombinant bovine eNOS expressed and purified from a baculovirus system (16–18). Enzyme activity was determined by arginine-to-citrulline conversion assay in the presence of excess cofactors and in the absence or the presence of varying concentrations of the peptides. The 135–156 caveolin-1 peptide inhibited eNOS with a potency similar to that previously reported for inhibition of eNOS by the 82–101 peptide (IC<sub>50</sub> = 1.0 μM). In contrast, the 157–178 peptide had no effect on activity. The effects of caveolin-1 peptides on the activity of human recombinant nNOS expressed and purified from a baculovirus system (16, 18) were also determined. The 82–101 peptide inhibited activity of purified nNOS (Fig. 2A) with a potency (IC<sub>50</sub> = 1.2 μM) similar to that reported previously for inhibition of eNOS (12), suggesting that nNOS, like eNOS, can interact directly with caveolin proteins. The caveolin-1 peptide corresponding to residues 135–156 was also effective in inhibiting nNOS (IC<sub>50</sub> = 0.9 μM) (Fig. 2B). In contrast, peptides corresponding to caveolin-1 residues 61–81 and 157–178 had no effect on nNOS activity.

Bovine caveolin-1 residues 82–101 and 135–156 (12) are equivalent to rat caveolin-3 residues 65–84 and 109–130, respectively (14). The primary structures of caveolin-1 residues 82–101 and corresponding caveolin-3 residues 65–84 contain conservative substitutions at seven positions. To determine whether these amino acid substitutions significantly alter the inhibitory potency of the two caveolin scaffolding domains, we prepared synthetic peptides corresponding to rat caveolin-3 residues 65–84 and 109–130 and determined the effects of the peptides on nNOS activity. As shown in Fig. 2 (C and D), low micromolar concentrations of both caveolin-3 peptides inhibited nNOS, indicating that nNOS can interact in vitro with both inhibitory domains of both caveolin-3 and caveolin-1. The 109–130 caveolin-3 peptide was 8-fold more potent than the 65–84 peptide in inhibiting nNOS (IC<sub>50</sub> = 0.5 μM versus IC<sub>50</sub> = 4.0 μM), suggesting that the second caveolin-3 scaffolding do-
main identified in this study may contribute significantly to nNOS inhibition when nNOS and caveolin-3 interact in skeletal muscle cells. To determine whether inhibition of nNOS activity was due to binding of the caveolin-3 peptides to nNOS or to Ca\(^{2+}\)/CaM, we tested the effects of the peptides on the activity of another Ca\(^{2+}\)/CaM target enzyme, the Ca\(^{2+}\)/CaM-dependent protein kinase II. Concentrations of caveolin-3 peptides (10 mM) that inhibited nNOS by 100% only partially inhibited this Ca\(^{2+}\)/CaM-dependent enzyme. Inhibition of 56 ± 2 and 50 ± 3% were determined for the 65–84 and 109–130 peptides, respectively (mean ± S.E., n = 4). Interactions of the peptides with Ca\(^{2+}\)/CaM may thus contribute to the peptide inhibition observed for nNOS. However, because complete inhibition was not observed, this mechanism can only account for about half of the inhibitory effects of the peptides on nNOS. The ~50% residual CaM kinase II activity determined in these experiments cannot be explained by partial activation of the enzyme due to autophosphorylation, which yields a CaM-independent enzyme. This can be concluded from experiments in which the stoichiometry of autophosphorylation under identical assay conditions was quantitated and found to be <0.001 mol phosphate incorporated/mol of enzyme in the presence or the absence of the caveolin peptides (n = 3). Moreover, the peptides could also inhibit Ca\(^{2+}\)/CaM-dependent protein kinase II by direct interaction with the enzyme rather than with Ca\(^{2+}\)/CaM. This interpretation is supported by the results of our previous study in which we reported that Ca\(^{2+}\)/CaM does not bind to a GST-caveolin-1 fusion protein in in vitro binding assays (12).

Dose-response relationships for caveolin-3 peptide inhibition of eNOS were very similar to those shown for nNOS in Fig. 2 (C and D) (IC\(_{50}\) values of 0.8 and 5.4 mM for the 65–84 and 109–130 peptides, respectively). Similar IC\(_{50}\) values of the four peptides for nNOS and eNOS suggest that both NOS enzymes contain the structural features necessary for direct interaction with caveolin proteins. These data suggest further that eNOS interactions with caveolin-3, known to occur in cardiac myocytes (10), are likely to have similar functional consequences to those of eNOS with caveolin-1 in endothelial cells. Addition-

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**Fig. 2. Effects of caveolin peptides on nNOS activity.** Purified, baculovirus-expressed human nNOS was mixed with various concentrations of the indicated synthetic peptides. nNOS activity was then determined by monitoring the rate of conversion of L-arginine to L-citrulline. The results shown are the means ± S.E. of duplicate determinations from three separate experiments.

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**Fig. 3. In vitro binding of nNOS to a GST-caveolin-1 fusion protein.** Purified nNOS was preincubated with or without the caveolin-3 65–84 and 109–130 peptides (100 mM) and used in an in vitro binding assay with a GST-caveolin-1 fusion protein (GST-cav 1–178) or GST alone. Bound proteins were visualized by immunoblotting with anti-nNOS antibody. Equivalent results were obtained in three separate experiments.

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ally, in cells where eNOS and nNOS are coexpressed (i.e. neurons in the hippocampus) (27), it is not likely that caveolin proteins will preferentially bind one of the two NOS isoforms and not the other.

To demonstrate unequivocally the capacity of nNOS to interact directly with caveolin proteins, we performed in vitro binding assays with purified human nNOS and a full-length bovine caveolin-1-GST fusion protein (12). The GST-caveolin-1 fusion protein (GST-cav 1–178) and a GST-nonfusion protein were expressed in *Escherichia coli* and purified by affinity chromatography on glutathione-agarose. The GST-caveolin-1 fusion or GST alone prebound to beads were then used in in vitro binding assays with recombinant nNOS expressed and purified from a baculovirus system (16, 18). Purified nNOS was preincubated for 5 min at 37 °C with or without the caveolin-3 65–84 and 109–130 peptides (100 mM). As a negative control nNOS was also preincubated with the caveolin-1 61–81 peptide. Following preincubation, nNOS was incubated overnight a 4 °C with GST-caveolin-1 or GST prebound to beads. Beads were washed extensively (six consecutive washes), and bound proteins were eluted with reduced glutathione. Eluted proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with anti-nNOS antibody. As shown in Fig. 3, nNOS bound specifically to the GST-caveolin-1 fusion or GST alone prebound to beads were then used in in vitro binding assays with recombinant nNOS expressed and purified from a baculovirus system (16, 18). Purified nNOS was preincubated for 5 min at 37 °C with or without the caveolin-3 65–84 and 109–130 peptides (100 mM). As a negative control nNOS was also preincubated with the caveolin-1 61–81 peptide. Following preincubation, nNOS was incubated overnight a 4 °C with GST-caveolin-1 or GST prebound to beads. Beads were washed extensively (six consecutive washes), and bound proteins were eluted with reduced glutathione. Eluted proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with anti-nNOS antibody. As shown in Fig. 3, nNOS bound specifically to the GST-caveolin-1 fusion or GST alone prebound to beads were then used in in vitro binding assays with recombinant nNOS expressed and purified from a baculovirus system (16, 18). Purified nNOS was preincubated for 5 min at 37 °C with or without the caveolin-3 65–84 and 109–130 peptides (100 mM). As a negative control nNOS was also preincubated with the caveolin-1 61–81 peptide. Following preincubation, nNOS was incubated overnight a 4 °C with GST-caveolin-1 or GST prebound to beads. Beads were washed extensively (six consecutive washes), and bound proteins were eluted with reduced glutathione. Eluted proteins were separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with anti-nNOS antibody. As shown
in Fig. 4, both of the caveolin-3 peptides completely blocked the binding of nNOS to CaM-Sepharose, demonstrating that both caveolin scaffolding/inhibitory domains inhibit nNOS by a common mechanism. The caveolin-1 82–101 and 135–156 peptides also blocked the binding of nNOS to CaM-Sepharose. Control peptides (caveolin-1 residues 61–81 and 157–178), however, had no effect on binding.

The results of our previous study and of the present study demonstrate that the NOS enzymes interact with both N- and C-terminal cytoplasmic domains of caveolin proteins. We have therefore suggested that the caveolin-NOS interaction may be fundamentally different from that of caveolin with Gα subunits, Ha-Ras, and Src tyrosine kinases (12). These particular proteins are reported to interact with (and be inhibited by) caveolin-1 exclusively through residues 82–101 in the N-terminal cytoplasmic domain (24–26). However, in the present study, we have found that c-Src activity is actually more potently inhibited by the caveolin-1 135–156 peptide than by the caveolin-1 82–101 peptide. Auto-activation of c-Src occurs through auto-phosphorylation of tyrosine 416 and has been tentatively inhibited by the caveolin-1 82–101 peptide than by the caveolin-3 65–84 and 109–130 peptides in the low micromolar range also completely inhibited c-Src.

In summary, the results of this study demonstrate several important but previously unrecognized features of the protein-protein interactions of both nNOS and the caveolins. Notable among these is the fact that nNOS in the skeletal muscle dystrophin complex interacts not only with α1-syntrophin but also with caveolin-3. Interaction of the two proteins appears to be direct and to involve two distinct and physically separated caveolin scaffolding domains. Furthermore, interaction serves to suppress or inhibit nNOS catalytic activity. The capacity to interact with caveolin proteins is thus a general property of the Ca2+/CaM-dependent NOS enzymes and is not unique to eNOS. Two distinct caveolin scaffolding domains are also involved in caveolin inhibition of the c-Src tyrosine kinase. We propose, therefore, that caveolin proteins contain both N- and C-terminal scaffolding/inhibitory domains and suggest that these domains be referred to as scaffolding domains 1 and 2, respectively.

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