Inhibitory Interactions of the Bradykinin B2 Receptor with Endothelial Nitric-oxide Synthase*

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It has been shown previously that the endothelial nitric-oxide synthase (eNOS) interacts reversibly with the plasmalemmal caveolar structural protein, caveolin-1. The eNOS-caveolin-1 interaction inhibits eNOS catalytic activity. In the present study, we show that eNOS also participates in reversible inhibitory interactions with the G protein-coupled bradykinin B2 receptor. eNOS and the B2 receptor are coimmunoprecipitated from endothelial cell lysates by antibodies directed against either of the two proteins. A glutathione S-transferase fusion protein containing intracellular domain 4 of the receptor is bound by purified recombinant eNOS in vitro binding assays. The fusion protein selectively inhibits the activity of purified eNOS. A synthetic peptide corresponding to membrane-proximal residues 310–334 in intracellular domain 4 also potently inhibits eNOS activity (IC50 < 1 μM). Treatment of cultured endothelial cells with bradykinin or Ca2+-ionophore promotes a rapid dissociation of the eNOS-B2 receptor complex. These data demonstrate that the bradykinin B2 receptor physically associates with eNOS in a ligand- and Ca2+-dependent manner. Reversible and inhibitory membrane-docking interactions of eNOS, therefore, are not restricted to those with caveolin-1 but also occur with the bradykinin B2 receptor.

Endothelial nitric-oxide synthase (eNOS) is targeted to endothelial plasmalemmal caveolae through direct interaction with the caveolar structural protein, caveolin-1. Binding of caveolin-1 by eNOS serves to inhibit or suppress eNOS catalytic activity (1–4). Inhibition appears to be due to competition between caveolin-1 and Ca2+-calmodulin (CaM) for interaction with the enzyme (2, 4). Thus, one of the mechanisms for eNOS activation in endothelial cells appears to involve agonist-stimulated increases in intracellular Ca2+ and subsequent displacement by Ca2+-CaM of caveolin-1 from its inhibitory interaction with eNOS. For example, it has recently been shown that the eNOS-caveolin-1 complex undergoes dissociation in cultured endothelial cells in response to either the Ca2+ ionophore A23187 or the eNOS-activating muscarinic cholinergic agonist, carbachol (5).

Another endothelial eNOS-activating agonist is bradykinin (BK) (6). BK signal transduction in endothelial and other cell types is mediated by G protein-coupled cell surface receptors termed B2 receptors (7, 8). B2 receptors, like other members of the G protein-coupled receptor family, are predicted to contain seven membrane-spanning α-helices, four extracellular domains, and four intracellular domains. Recently, we have shown that the B2 receptor interacts with its downstream effector enzyme, phospholipase Cγ1 (PLCγ1) in a ligand- and tyrosine phosphorylation-dependent manner (9). In the present study, we show that the B2 receptor also interacts with another of its downstream effector enzymes, namely eNOS. This interaction is regulated in a ligand- and Ca2+-dependent manner. We have also identified the functional consequences of receptor binding on eNOS catalytic activity and have mapped the region in the receptor involved in interaction with the enzyme.

EXPERIMENTAL PROCEDURES

Materials—Monoclonal anti-B2 receptor antibody (Clone 2) and monoclonal anti-eNOS antibody (Clone 3) were obtained from Transduction Laboratories, Protein A/G Plus agarose was obtained from Santa Cruz Biotechnology. Protein molecular weight standards, a detergent-compatible protein assay kit, AG 50W-X8 cation exchange resin, and peroxidase-conjugated anti-IgG antibodies were purchased from Bio-Rad. The glutathione S-transferase (GST) fusion protein cloning vector, pGEX-4T-1, glutathione-Sepharose 4B, and CaM-Sepharose 4B were purchased from Amersham Pharmacia Biotech. L-[14C]Arginine and ECL reagents came from Amersham Pharmacia Biotech. The cDNA encoding the human B2 receptor was kindly provided by Dr. Tanya MacNeil, Merck Research Laboratories, Rahway, NJ. The cDNA encoding the rat angiotensin II AT1A receptor intracellular domain 4 (residues 306–359)–GST fusion protein has been described previously (10). Synthetic peptides were obtained from the Medical College of Georgia Biochemistry Core Facility and were >95% pure as determined by high performance liquid chromatography. Bovine CaM, BR, Ca2+-ionophore A23187, NADPH, FAD, and FMN were purchased from Sigma, and tetrahydrobiopterin was purchased from Research Biochemicals International.

Cell Culture—Bovine aortic endothelial cells were passaged from primary cultures and maintained in M199 medium supplemented with 10% fetal bovine serum, 5% iron-supplemented calf serum, 20 μg/ml l-glutamine, IX minimum Eagle’s medium amino acid and vitamin solutions, 0.6 μg/ml thymidine, 500 IU/ml penicillin, and 500 μg/ml streptomycin. Cells were used for immunoprecipitation experiments during passages two to five.

Immunoprecipitation and Immunoblotting—Bovine aortic endothelial cell cultures were lysed in ice-cold buffer containing 20 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1 mM Na2VO4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Lysates were separated from the plates and precleared by centrifugation at 10,000 × g for 25 min at 4 °C. Anti-B2 receptor antibody (5 μg/ml) or anti-eNOS antibody (5 μg/ml) were then added to the precleared lysates for 2 h at 4 °C followed by the addition of protein A/G Plus agarose (50 μl) and rocking overnight at 4 °C. The immunoprecipitates were then pelleted and washed twice in 1 ml of ice-cold wash buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Triton X-100, and 1 mM Na2VO4. Immunoprecipitated proteins were then eluted from the beads by boiling for 5 min in SDS sample buffer and reducing conditions.

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The abbreviations used are: eNOS, endothelial nitric-oxide synthase; CaM, calmodulin; PLCγ1, phospholipase Cγ1; BK, bradykinin; GST, glutathione S-transferase; B2EAC, bovine aortic endothelial cells; ID, intracellular domain; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.
separated by SDS-polyacrylamide gel electrophoresis. Proteins in gels were transferred to nitrocellulose membranes by electroblotting for 1 h at 100 mA. Anti-eNOS immunoprecipitates were then immunoblotted with anti-B2 receptor antibody (1:500 dilution) and anti-B2 receptor immunoprecipitates were immunoblotted with anti-eNOS antibody (1:500 dilution). Immunoreactive proteins were visualized using a horseradish peroxidase-conjugated goat anti-mouse antibody and an enhanced chemiluminescence (ECL) detection kit.

**Construction and Purification of GST-B2 Receptor Fusion Proteins—**
cDNA constructs encoding GST-B2 receptor fusion proteins were created by subcloning into the GST fusion protein cloning vector, pGEX-4T-1. The construct encoding the B2 receptor intracellular domain 4 (residues 310–364) was generated by polymerase chain reaction amplification of the full-length human cDNA using primers containing 5′ EcoRI and SalI sites to allow for digestion and subcloning. Construct inserts encoding the human B2 receptor intracellular domains 1 (residues 57–65), 2 (residues 128–147), and 3 (residues 222–240) were created by annealing two reverse complementary coding sequence oligonucleotides containing 5′ overhangs that created the EcoRI and SalI sticky ends required for subcloning. The cDNA sequence encoding each GST or GST-B2 receptor residues 310–334 (KRFRKKSWEVYQGVCQKGGCRSEPI), 330–347 (SVERQIHKLQDWAGSRQ), and 348–364 (RSEPIQMENSMGTLRTSI) were tested for their effects on eNOS catalytic activity. GST or GST-B2 receptor fusion proteins were expressed in Escherichia coli and purified by affinity chromatography on glutathione-agarose as described previously (9, 11).

**Expression and Purification of eNOS—** Bovine eNOS was expressed in a baculovirus/Sf9 insect cell system and purified to apparent homogeneity by affinity chromatography on 2′,5′-ADP-Sepharose as described previously (12, 13). The enzyme was purified in buffers containing 2 mM EGTA and was thus completely dependent on exogenous CaM for activity.

**In Vitro Binding of eNOS to GST-B2 Receptor Fusion Proteins and the GST-AT, Receptor Fusion Protein—** GST, GST-B2 receptor fusion proteins, or the GST-AT, receptor fusion protein (100 pmol of each, quantitated by Bio-Rad detergent-compatible protein assay), were prebound to glutathione-agarose beads, were incubated overnight (at 4 °C with shaking) with 100 pmol of purified eNOS in 1 ml of buffer containing 50 mM Tris-HCl, pH 7.4, 20% glycerol and the following protease inhibitors: 1% phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM pepstatin A, and 5 μM aprotinin. Following the overnight incubation, beads were washed six times in 1 ml of buffer containing 50 mM Hepes, pH 7.5, 1 mM NaCl, 1 mM EDTA, 0.5% CHAPS, and the protease inhibitors listed above. Proteins remaining bound to the beads after washing were eluted with 100 mM reduced glutathione, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, plus protease inhibitors. Eluted proteins were separated in SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with anti-eNOS antibody (1:1000 dilution).

**Determination of the Effects of GST-B2 Receptor Fusion Proteins and Synthetic Peptides on eNOS Catalytic Activity—** GST or GST-B2 receptor fusion proteins or synthetic peptides corresponding to the human B2 receptor residues 310–329 (KRFRKKSWEVYQGVCQKGGC), 330–347 (SVERQIHKLQDWAGSRQ), and 310–334 (KRFRKKSWEVYQGVCQKGGCRSEPI) were tested for their effects on eNOS catalytic activity. GST or GST-B2 receptor fusion proteins (300 pmol of each, quantitated by Bio-Rad detergent-compatible protein assay) were incubated for 5 min at 37 °C with purified eNOS (100 pmol) in 50 mM Tris-HCl, pH 7.5, buffer. eNOS activity was measured by a coupled enzyme assay involving activation of eNOS (14). To determine whether eNOS interacts with the B2 receptor in endothelial cells, we lysed bovine aortic endothelial cells (BAEC) in membrane-solubilizing buffer containing 1% Triton X-100 and subjected the lysates to immunoprecipitation with anti-eNOS and anti-B2 receptor antibodies. Anti-B2 receptor immunoprecipitates were then immunoblotted with anti-eNOS antibody and anti-eNOS immunoprecipitates were immunoblotted with anti-B2 receptor antibody. As shown in Fig. 1, eNOS (130 kDa) was specifically coimmunoprecipitated from endothelial cell lysates by the anti-B2 receptor antibody. In addition, the ligand-binding, glycosylated form of the B2 receptor (70 kDa) (15) was specifically coimmunoprecipitated by the anti-eNOS antibody. eNOS and the B2 receptor thus appear to form a complex in endothelial cells in a manner similar to that of eNOS and caveolin-1 in endothelial eNOS in BAEC that is complexed with the B2 receptor cannot be obtained using coimmunoprecipitation protocols because detergent-containing buffers used to solubilize membrane proteins may also disrupt certain protein-protein interactions. However, if it is assumed that none of the eNOS-B2 receptor complex is dissociated during solubilization, our results indicate that less than 5% of total cellular eNOS exists in a complex with the receptor. Feron et al. (5) have reported previously that eNOS can be almost quantitatively immunoprecipitated from endothelial cells with anti-caveolin-1 antibody. Therefore, the proportion of eNOS in BAEC that is complexed with the B2 receptor may be small compared with that complexed with caveolin-1. It is also possible that eNOS exists, to at least some extent, in a trimeric complex with both caveolin-1 and the B2 receptor.

eNOS Interacts with the Bradykinin B2 Receptor C-terminal Intracellular Domain 4 in Vitro—To examine whether eNOS and the B2 receptor interact directly, we prepared B2 receptor-GST fusion proteins for use in in vitro binding assays. GST fusion proteins containing the human B2 receptor intracellular domain 4 (ID4, residues 57–65), 2 (ID2, residues 128–147), 3 (ID3, residues 222–240), and 4 (ID4, residues 310–364) were each expressed in E. coli. In addition, a GST nonfusion protein was expressed as a control. The GST fusion proteins and GST alone were purified by affinity chromatography on glutathione-agarose. The GST-B2 receptor fusion proteins and GST alone, prebound to agarose beads, were then used in in vitro binding assays with recombinant bovine eNOS, expressed and purified from a baculovirus system (2, 11–13). Beads were incubated with eNOS at 4 °C overnight and then washed six consecutive times in buffer containing 1 mM NaCl. Proteins remaining bound to the beads after the washing were eluted with reduced glutathione, separated on SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with anti-eNOS antibody. As shown in Fig. 2, eNOS (130 kDa) bound specifically to the GST-B2R-ID4 fusion protein but not to GST alone or to the
GST-B2R-ID1, GST-B2R-ID2, or GST-B2R-ID3 fusion proteins. eNOS can thus bind directly to the B2 receptor intracellular domain 4. Furthermore, binding of eNOS to this domain is very tight because it is stable during extensive washing in buffer containing 1 M NaCl.

The Bradykinin B2 Receptor Intracellular Domain 4 Inhibits eNOS Catalytic Activity—To determine whether interaction of eNOS with the B2 receptor C-terminal intracellular domain 4 alters eNOS catalytic activity, we incubated equal quantities of purified, baculovirus-expressed eNOS in the absence and presence of equimolar quantities of purified GST alone or the GST-B2R-ID1, GST-B2R-ID2, GST-B2R-ID3, and GST-B2R-ID4 fusion proteins. eNOS catalytic activity was then determined by arginine-to-citrulline conversion assay in the presence of excess cofactors, Ca²⁺, CaM, and Mn²⁺. As shown in Fig. 3, neither GST alone nor the GST-B2R-ID1, GST-B2R-ID2, and GST-B2R-ID3 fusion proteins affected eNOS activity. The GST-B2R-ID4 fusion protein, however, significantly inhibited eNOS activity. Interaction of eNOS with the B2 receptor C-terminal intracellular domain thus appears to inhibit eNOS catalytic activity in a manner analogous to that of the eNOS-caveolin-1 interaction (1–4).

A Membrane-proximal Region of the Bradykinin B2 Receptor Intracellular Domain 4 Is Responsible for eNOS Inhibition—To map the region within the B2 receptor C-terminal intracellular domain responsible for eNOS inhibition, we prepared synthetic peptides corresponding to three contiguous segments of the human B2 receptor intracellular domain 4. Each of the peptides was tested for its ability to inhibit the activity of purified, baculovirus-expressed eNOS. eNOS activity was measured by the arginine-to-citrulline conversion assay as before. A peptide corresponding to residues 310–329 potently inhibited eNOS activity (IC₅₀ < 1 μM) (Fig. 4A). Peptides corresponding to residues 330–347 and residues 348–364, however, had little or no effect on eNOS activity even at concentrations as high as 100 μM (Fig. 4B and C). A small degree of inhibition by 100 μM of the 330–347 peptide suggested that residues C-terminal to position 329 may have a low-affinity inhibitory interaction with eNOS. Therefore, we prepared an additional peptide corresponding to the B2 receptor residues 310–334 and determined its inhibitory potency on eNOS activity. This peptide was an even more potent inhibitor of eNOS (IC₅₀ < 1 μM) than the 310–329 peptide. Thus, it appears that eNOS inhibition by the B2 receptor C-terminal intracellular domain occurs through a membrane-proximal subdomain encompassed by residues 310–334. Furthermore, a peptide comprised of this subdomain is an even more potent inhibitor of eNOS in vitro than the caveolin-1 membrane-proximal scaffolding domains 1 (residues 82–101) and 2 (residues 135–156) peptides shown previously to inhibit eNOS in independent reports from ours and two other laboratories (2–4, 11).

The Bradykinin B2 Receptor Inhibits eNOS by a Mechanism That Is Distinct from That of Caveolin-1 Inhibition—Inhibition of eNOS by caveolin-1 scaffolding domain peptides occurs...
through a mechanism involving peptide interference with enzyme binding of Ca\(^{2+}\)-CaM. Thus, we have shown previously that preincubation of eNOS with caveolin-1 peptides (10 \mu M) almost completely blocks subsequent binding of the enzyme to CaM-Sepharose (2, 11). To determine whether the B2 receptor peptide inhibits eNOS by a similar mechanism, we preincubated eNOS with and without 10 and 100 \mu M of the B2 receptor 310–334 peptide. The enzyme was then subjected to affinity chromatography on CaM-Sepharose. eNOS was allowed to bind to the column in the presence of 2 mM CaCl\(_2\) and was eluted with 2 mM EGTA. The amount of enzyme eluted (and thus bound to CaM-Sepharose) in each condition was quantitated by immunoblotting with anti-eNOS antibody. No effect of the peptide on eNOS binding to CaM-Sepharose was detected even at a peptide concentration of 100 \mu M. We have also shown previously that inhibition of eNOS by the caveolin-1 82–101 peptide is reversible by Ca\(^{2+}\)-CaM. When the concentration of Ca\(^{2+}\)-CaM (1.25 \mu M) routinely used in the arginine-to-citrulline conversion assay is increased by 10-fold (to 12.5 \mu M), the inhibitory effects of the caveolin-1 peptide is completely reversed (2). In the present study, we have performed identical experiments with the B2 receptor 310–334 peptide. No reversal of peptide inhibition by Ca\(^{2+}\)-CaM was observed. Inhibition of eNOS by the B2 receptor thus appears to occur through a different mechanism than that of caveolin-1. Furthermore, the B2 receptor interacts with a different domain in eNOS than that bound by caveolin-1 because caveolin-1 scaffolding domain peptides (residues 82–101 and 135–156, 100 \mu M) had no effect on eNOS binding to the GST-B2R-ID4 fusion protein in *in vitro* binding assays.

**eNOS Dissociates from the Bradykinin B2 Receptor in a Ligand- and Ca\(^{2+}\)-dependent Manner**—To determine whether association of eNOS with the B2 receptor is a ligand-regulated process, BAEC were treated or not treated with BK (1 \mu M for 0.5 min) prior to lysis of the cells and immunoprecipitation of lysates with anti-eNOS antibody. Immunoprecipitates were then immunoblotted with anti-B2 receptor antibody. As shown in Fig. 5A, BK treatment of endothelial cells resulted in a rapid dissociation of the eNOS-B2 receptor complex. Loss of receptor immunoprecipitation from treated cells by the anti-eNOS antibody was not due to differences in the amount of eNOS that was immunoprecipitated in each condition because, when membranes were stripped and reprobed with anti-eNOS antibody, equal amounts of the protein were detected for both treated and untreated conditions (Fig. 5B). BK-dependent eNOS-B2 receptor dissociation may occur through a Ca\(^{2+}\)-dependent process. Support for this hypothesis is provided by the results of additional experiments in which BAEC were treated or not treated with the Ca\(^{2+}\) ionophore A23187 (5 \mu M for 0.5 min) prior to lysis of the cells and immunoprecipitation and immunoblotting as above. Like BK, the Ca\(^{2+}\) ionophore also promoted a rapid dissociation of the eNOS-B2 receptor complex (Fig. 5C), which could not be attributed to different amounts of eNOS being preprecipitated in the two conditions (Fig. 5D). Thus, elevation of intracellular Ca\(^{2+}\) promotes dissociation of eNOS from the receptor. These experiments suggest that eNOS activation by BK in endothelial cells involves a ligand- and Ca\(^{2+}\)-dependent dissociation of the enzyme from its inhibitory interactions with the B2 receptor. This mechanism of activation is analogous to that described previously involving dissociation of eNOS from caveolin-1 in response to either Ca\(^{2+}\) ionophore or the eNOS-activating muscarinic cholinergic agonist, carbachol (5). Dissociation, however, is not due simply to displacement of the receptor by Ca\(^{2+}\)-CaM because, as shown in the experiments described above, the B2 receptor inhibitory peptide does not compete with Ca\(^{2+}\)-CaM for interaction with eNOS.

**eNOS Also Binds to the Angiotensin II AT\(_1\), C-terminal Intracellular Domain 4**—It is conceivable that binding and inhibition of eNOS is a general property of G protein-coupled receptors and thus occurs with receptors for other eNOS-activating agonists such as those for angiotensin II, thrombin, histamine, adenosine nucleotides, and endothelin-1 (6). Because it is not likely that eNOS would bind to more than one receptor at a time, each receptor would sequester a small pool of the total cellular eNOS. Vascular endothelial cells, like vascular smooth muscle cells, possess functional angiotensin II receptors known as AT\(_1\) receptors (16, 17) which are coupled to eNOS activation (18). We, therefore, examined whether eNOS could bind *in vitro* to the AT\(_1\) receptor C-terminal intracellular domain 4. GST alone, GST-B2R-ID4, and a GST-fusion protein containing intracellular domain 4 (residues 306–359) of the rat AT\(_1\) receptor (GST-AT\(_1\)-ID4) (10) were expressed in *E. coli* and purified by affinity binding to glutathione-agarose beads. The proteins, prebound to beads, were then washed in buffer containing 1 M NaCl and bound proteins were eluted with reduced glutathione. Eluted proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-eNOS antibody. The results shown are representative of three separate experiments.
recombinant bovine eNOS. As shown in Fig. 6, the GST-AT_1-ID4 fusion protein bound eNOS in these assays equally as well as did the GST-B2R-ID4 fusion protein.

In summary, the results of the present study demonstrate several novel features of eNOS regulation in endothelial cells. First, eNOS interactions with membrane-docking proteins are not restricted to interactions with the integral membrane protein, caveolin-1 but also occur with the transmembrane bradykinin B2 receptor. Second, eNOS interactions with the receptor are direct and inhibitory and are mediated by a membrane-proximal subdomain of the B2 receptor intracellular domain 4. Third, eNOS association with the receptor is regulated in a ligand- and Ca^{2+}-dependent manner, suggesting that agonist-activation of eNOS in endothelial cells may be mediated in part by agonist-stimulated dissociation of eNOS from its inhibitory interactions with the agonist receptor. Fourth, eNOS also interacts in vitro with the AT_1 receptor, suggesting that binding of eNOS may occur with receptors for other eNOS-activating agonists. Many examples exist of cell surface receptors that activate downstream effector enzymes through mechanisms involving receptor-effector association. Ligand-induced growth factor receptor autophosphorylation, for example, promotes the interaction of these receptors with various Src homology 2-containing enzymes including PLCγ1 (19). We have shown recently that activation of the AT_1 receptor in vascular smooth muscle cells as well as the B2 receptor in vascular endothelial cells also involves a transient association of PLCγ1 with the receptors in a ligand- and tyrosine phosphorylation-dependent manner (9, 10). Evidence for signaling by cell surface receptors through a mechanism of activation involving receptor-effector dissociation (rather than association) has, not to our knowledge, been previously described. Receptor-effector dissociation, however, may represent an intriguing new paradigm for post-receptor activation of downstream effector enzymes.

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