Endothelial nitric oxide synthase interactions with G-protein-coupled receptors

Mario B. MARRERO^{*†}, Virginia J. VENEMA^{*}, Hong JU^{*}, Han HE^{*}, Haiying LIANG^{*}, Ruth B. CALDWELL^{*‡§}, and Richard C. VENEMA^{*†||¹}

^{*}Vascular Biology Center, Medical College of Georgia, Augusta, GA 30912-2500, U.S.A., [†]Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA 30912, U.S.A., [‡]Department of Cellular Biology and Anatomy, Medical College of Georgia, Augusta, GA 30912, U.S.A., [§]Department of Ophthalmology, Medical College of Georgia, Augusta, GA 30912, U.S.A., and ^{||}Department of Pediatrics, Medical College of Georgia, Augusta, GA 30912, U.S.A.

The endothelial nitric oxide synthase (eNOS) is activated in response to stimulation of endothelial cells by a number of vasoactive substances including, bradykinin (BK), angiotensin II (Ang II), endothelin-1 (ET-1) and ATP. In the present study we have used *in vitro* activity assays of purified eNOS and *in vitro* binding assays with glutathione S-transferase fusion proteins to show that the capacity to bind and inhibit eNOS is a common feature of membrane-proximal regions of intracellular domain 4 of the BK B2, the Ang II AT1 and the ET-1 ETB receptors, but not of the ATP P2Y2 receptor. Phosphorylation of serine or tyrosine residues in the eNOS-interacting region of the B2 receptor results in a loss of eNOS inhibition due to a decrease in the binding affinity of the receptor domain for the eNOS enzyme.

Furthermore, the B2 receptor is transiently phosphorylated on tyrosine residues in cultured endothelial cells in response to BK stimulation. Phosphorylation occurs during the time in which eNOS transiently dissociates from the receptor accompanied by a transient increase in nitric oxide production. Taken together, these data support the hypotheses that eNOS is regulated in endothelial cells by reversible and inhibitory interactions with G-protein-coupled receptors and that these interactions can be modulated by receptor phosphorylation.

Key words: angiotensin II, ATP, bradykinin, endothelin-1, tyrosine phosphorylation.

INTRODUCTION

The endothelial nitric oxide synthase (eNOS) is activated in endothelial cells in response to a variety of stimuli, including bradykinin (BK), acetylcholine, thrombin, histamine, substance P, ATP, endothelin-1 (ET-1), angiotensin II (Ang II), vascular endothelial growth factor (VEGF) and fluid shear stress. Agonist stimulation of endothelial-cell receptors results in activation of one or more isoforms of phospholipase C, with consequent increases in the formation of inositol 1,4,5-trisphosphate [Ins(1,4,5) P_3]. Transient elevations of Ins(1,4,5) P_3 lead to transient increases in the levels of intracellular free Ca^{2+} , accompanied by activation of eNOS via interaction of Ca^{2+} -calmodulin (CaM) with the eNOS Ca^{2+} -CaM-binding domain. The molecular interactions of eNOS that regulate its activity, however, are more complex than simple reversible binding of Ca^{2+} -CaM. Heat-shock protein 90 (Hsp90), for example, also appears to be a positive allosteric effector of eNOS. Hsp90 binds to, and enhances, the activity of the enzyme in response to several different stimuli, including VEGF, histamine and shear stress [1].

Negative allosteric effectors of eNOS also contribute to regulation of enzyme activity. One such negative effector is the plasmalemmal caveolae structural protein caveolin-1, which interacts with eNOS and inhibits its catalytic activity [2–5]. The eNOS–caveolin-1 complex undergoes dissociation in response to treatment of cells with the Ca^{2+} ionophore A23187 or the eNOS-activating muscarinic cholinergic agonist carbachol [6], as well as

in response to shear stress [7], suggesting that dissociation of the enzyme from caveolin-1 contributes to the eNOS activation process. We have recently identified a second negative allosteric effector of eNOS activity, namely the BK B2 receptor [8]. eNOS interaction with the receptor is direct, inhibits enzyme activity, and is mediated by a membrane-proximal subdomain of the B2 receptor C-terminal intracellular domain 4. eNOS association with the receptor appears to be regulated in a ligand- and Ca^{2+} -dependent manner, suggesting that BK stimulation of eNOS activity in endothelial cells may be due not only to association of the enzyme with Ca^{2+} -CaM and Hsp90 and dissociation from caveolin-1, but also to dissociation of eNOS from the B2 receptor. In our previous study [8] we also demonstrated that eNOS interacts *in vitro* with another G-protein-coupled receptor, the Ang II AT1 receptor. Whether this interaction inhibits eNOS activity, however, was not determined.

In the present study, in order to determine whether binding and inhibition of eNOS is a general property of G-protein-coupled receptors for eNOS-activating agonists, we have carried out a comparative analysis of eNOS interactions with four different endothelial receptors, including the BK B2 receptor, the Ang II AT1 receptor, the ET-1 ETB receptor, and the ATP P2Y2 receptor. In addition, we have examined the potential role of phosphorylation in regulation of eNOS–B2 receptor complex dissociation and have examined whether the B2 receptor is tyrosine-phosphorylated in endothelial cells in response to BK stimulation.

Abbreviations used: BAECs, bovine aortic endothelial cells; eNOS, endothelial nitric oxide synthase; BK, bradykinin; ET-1, endothelin-1; Ang II, angiotensin II; VEGF, vascular endothelial growth factor; Ins(1,4,5) P_3 , inositol (1,4,5)-trisphosphate; CaM, calmodulin; Hsp90, heat-shock protein 90; GST, glutathione S-transferase; GRK, G-protein-coupled receptor kinase; ID4, intracellular domain 4.

¹ To whom correspondence should sent, at the Vascular Biology Center address (e-mail rvenema@mail.mcg.edu).

MATERIALS AND METHODS

Materials

Monoclonal anti-(B2 receptor) antibody (clone 2), monoclonal anti-eNOS antibody (clone 3), and monoclonal and polyclonal anti-phosphotyrosine antibodies were purchased from Transduction Laboratories, Lexington, KY, U.S.A.. Protein A/G Plus agarose was obtained from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A. Protein molecular-mass standards, AG 50W-X8 cation-exchange resin and detergent-compatible protein assay kit were purchased from Bio-Rad. The glutathione *S*-transferase (GST) fusion protein cloning vector pGEX-4T-1, glutathione-Sepharose 4B, 2',5'-ADP-Sepharose, L-[¹⁴C]arginine, cGMP radioimmunoassay kit, peroxidase-conjugated anti-IgG antibodies, and ECL[®] (enhanced chemiluminescence) reagents were obtained from Amersham Pharmacia Biotech. The cDNA encoding the human ETB receptor [9] was generously provided by Dr. Masashi Yanagisawa, University of Texas Southwestern Medical Center at Dallas, Dallas, TX, U.S.A.. The cDNA encoding the mouse ATP P2Y2 receptor [10] was generously provided by Dr. David Julius, University of California at San Francisco, San Francisco, CA, U.S.A. Synthetic peptides were prepared in the Medical College of Georgia Biochemistry Core Facility and were purified to greater than 95 % purity by HPLC. Bovine CaM, BK, Ang II, NADPH, FAD, and FMN were obtained from Sigma and tetrahydrobiopterin was obtained from Research Biochemicals International, Natick, MA, U.S.A. The RFL-6 rat lung fibroblast cell line was purchased from the American Type Culture Collection.

Cell culture

Bovine aortic endothelial cells (BAECs) were passaged from primary cultures and were used in experiments during passages 2–5. Cells were maintained in M199 medium supplemented with fetal bovine serum (10 %, v/v), iron-supplemented calf serum (10 %, v/v), L-glutamine (20 µg/ml), vitamins (1 × MeM vitamin solution, Cellgro[®]; Mediatech, Herndon, VA, U.S.A.), thymidine (0.6 µg/ml), penicillin (500 i.u./ml) and streptomycin (500 µg/ml). RFL-6 rat lung fibroblasts were maintained in Ham's F12 medium supplemented with fetal bovine serum (15 %, v/v), L-glutamine (20 µg/ml), penicillin (500 i.u./ml), and streptomycin (500 µg/ml).

Immunoprecipitation and immunoblotting

BAECs in 100 mm-diameter Petri dishes were treated with BK (1 µM) for various times at 37 °C in serum-free M199 medium. Incubations were terminated by aspirating the medium and washing the cells twice with ice-cold PBS containing 1 mM sodium orthovanadate. Cells were then lysed in ice-cold buffer containing 20 mM Tris/HCl, pH 7.4, 2.5 mM EDTA, 1 % Triton X-100, 1 mM sodium orthovanadate, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin A and 5 µg/ml aprotinin. Lysates were scraped from the dishes and precleared by centrifugation at 10000 *g* for 25 min at 4 °C. Anti-eNOS antibody (5 µg/ml) or anti-phosphotyrosine antibody (5 µg/ml) was then added to the precleared lysates for 2 h at 4 °C, followed by an overnight incubation at 4 °C with protein A/G Plus agarose (50 µl). Immunoprecipitates were washed twice in 1 ml of ice-cold buffer containing 50 mM Tris/HCl, pH 8.0, 150 mM NaCl, 0.1 % Triton X-100 and 1 mM sodium orthovanadate. Immunoprecipitated proteins were eluted from the agarose beads by boiling for 5 min in SDS sample buffer. Proteins were then separated on SDS/polyacrylamide gels and electroblotted on to nitrocellulose

membranes. Immunoprecipitated proteins were then immunoblotted with anti-B2 receptor antibody (1:500 dilution).

Construction and purification of GST–receptor fusion proteins

cDNA constructs encoding intracellular domain 4 (ID4)–GST fusion proteins of the rat AT1A and human B2 receptors have been described previously [8,11]. cDNA constructs encoding ID4–GST fusion proteins of the human ETB and mouse P2Y2 receptors were created by subcloning into the GST fusion protein cloning vector pGEX-4T-1. Constructs containing ETB receptor residues 390–442 and P2Y2 receptor residues 310–373 were generated by PCR amplification of the corresponding full-length cDNAs using primers containing 5' *Eco*RI and *Sal*I restriction sites for subcloning. DNA sequences were confirmed by automated sequencing with a Perkin–Elmer ABI 377 sequencer in the Molecular Biology Core Facility of the Medical College of Georgia.

Expression and purification of recombinant eNOS

Bovine eNOS was expressed in a baculovirus system and purified to more-than-90 % homogeneity by affinity chromatography on 2',5'-ADP-Sepharose as described previously [12,13]. eNOS was purified in buffers containing 2 mM EGTA and was therefore completely dependent on exogenous CaM for activity.

In vitro binding of eNOS to GST fusion proteins

GST alone and ID4–GST fusion proteins of the B2 receptor (GST–B2-ID4), the AT1 receptor (GST–AT1-ID4), the ETB receptor (GST–ETB-ID4) and the P2Y2 receptor (GST–P2Y2-ID4) were expressed in *Escherichia coli* and purified by affinity chromatography on GSH-agarose as described by Frangioni and Neel [14]. Expressed proteins (100 pmol) were incubated overnight at 4 °C with purified eNOS (100 pmol) in 1 ml of buffer containing 50 mM Tris/HCl, pH 7.4, 20 % glycerol, 1 % PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, and 5 µg/ml aprotinin. Beads were then washed six times in 1 ml of buffer containing 50 mM Hepes, pH 7.5, 1 M NaCl, 1 mM EDTA, 0.5 % CHAPS, 1 % PMSF, 10 µg/ml leupeptin, 10 µg/ml pepstatin A and 5 µg/ml aprotinin. Proteins bound to the beads were eluted with 100 mM GSH and subjected to immunoblotting with anti-eNOS antibody (1:1000 dilution).

Determination of the effects of synthetic peptides on eNOS catalytic activity

Synthetic peptides corresponding to human B2 receptor residues 310–329 (KRFRKKSWEVYQGVCQKGGC) as either an unmodified 20-mer (unmodified peptide), a 20-mer with a phosphorylated serine residue at position 316 (PS316 peptide), a 20-mer with a phosphorylated tyrosine residue at position 320 (PY320 peptide) or a 21-mer containing the 310–329 sequence C-terminal to a phosphorylated tyrosine residue (PY309 peptide) were tested for their effects on eNOS activity, together with peptides corresponding to the rat AT1 receptor residues 306–325 (GKKFKKYFLQLLKYIPPKAK), the human ETB receptor residues 390–409 (SKRFKNCFKSCLCCWCQSFE) and the mouse P2Y2 receptor residues 310–329 (GQRLVRFARDAK-PPTEPTPS). Purified eNOS (100 pmol) was preincubated for 5 min at 37 °C in the absence or presence of various concentrations of the synthetic peptides. eNOS activity was then determined by monitoring the rate of conversion of L-[¹⁴C]-arginine (50 µM) into L-[¹⁴C]citrulline at 37 °C in the presence of exogenously added cofactors, including Ca²⁺ (2 mM), CaM

(250 pmol), NADPH (1 mM), FAD (4 μ M), FMN (4 μ M) and tetrahydrobiopterin (40 μ M) in a 200 μ l reaction mixture, as described previously [12].

Bioassay measurements of nitric oxide release

Nitric oxide release from BAECs was measured by a modification of the procedure described by Ishii et al. [15]. Culture medium was removed from BAECs and replaced by Locke's solution containing 154 mM NaCl, 5.6 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 3.6 mM NaHCO₃, 5.6 mM glucose, 10 mM Hepes, pH 7.4, 20 units/ml superoxide dismutase and 0.3 mM 3-isobutyl-1-methylxanthine. Cells were then treated with BK (1 μ M) for various times before transferring the bathing medium to confluent RFL-6 fibroblast reporter cells in six-well plates. After the transfer, the RFL-6 cells were incubated for 3 min and then lysed in ice-cold 20 mM sodium acetate, pH 4.0. cGMP concentrations in the lysates were quantified using a radioimmunoassay kit.

RESULTS AND DISCUSSION

ID4 of some, but not all, receptors coupled to eNOS activation bind the enzyme in *in vitro* binding assays

Among the various hormones that promote nitric oxide release from endothelial cells are BK, Ang II, ATP, and ET-1. eNOS activation in response to these agonists is mediated by BK B2 [16], Ang II AT1 [17], ATP P2Y2 (formerly known as P2U) [18], and ET-1 ETB [19] receptors respectively. We have shown previously [8] that purified GST fusion proteins containing either the human BK B2 receptor ID4 (residues 310–364) [20] or the rat Ang II AT1 receptor ID4 (residues 306–359) [21] bind purified recombinant eNOS with high affinity in *in vitro* binding assays. To examine further whether the capacity for high-affinity binding of eNOS is a general property of the ID4 of other G-protein-coupled receptors for eNOS-activating agonists, we prepared two additional GST fusion proteins. GST fusion proteins containing the human ET-1 ETB receptor ID4 (residues 390–442) [9] and the mouse ATP P2Y2 receptor ID4 (residues 310–373) [10] were expressed in *E. coli*, together with the ID4–GST fusion proteins of the B2 and AT1 receptors. A GST non-fusion protein was also expressed as a negative control. The GST fusion proteins and GST alone were purified by affinity binding to GSH-agarose beads and used in *in vitro* binding assays with recombinant bovine eNOS, expressed and purified from a baculovirus expression system [12]. Fusion proteins, prebound to beads, were incubated with purified eNOS at 4 °C overnight and then washed six times under high-stringency conditions with buffer containing 1 M NaCl. Bound proteins were eluted with GSH, separated by SDS/PAGE, transferred to nitrocellulose, and immunoblotted with anti-eNOS antibody. As shown in Figure 1, eNOS (130 kDa) bound selectively to the B2, AT1 and ETB receptor ID4–GST fusion proteins (GST–B2-ID4, GST–AT1-ID4, and GST–ETB-ID4), but not to GST alone or the P2Y2 receptor ID4 fusion protein (GST–P2Y2-ID4). The capacity to bind eNOS directly is thus a common feature of the ID4 of at least three different receptors for eNOS-activating agonists. However, not all receptors coupled to eNOS activation bind the enzyme, as demonstrated in these assays by the P2Y2 receptor.

Membrane-proximal subdomains of ID4 of some, but not all, receptors coupled to eNOS activation inhibit the enzyme in *in vitro* activity assays

A synthetic peptide comprised of the 20 membrane-proximal residues of the human B2 receptor ID4 (residues 310–329) is a

Figure 1 *In vitro* binding of eNOS to GST fusion proteins containing ID4 of various G-protein-coupled receptors

GST fusion proteins containing ID4 of the human B2 receptor (GST–B2R-ID4), the rat AT1 receptor (GST–AT1-ID4), the human ETB receptor (GST–ETB-ID4), and the mouse P2Y2 receptor (GST–P2Y2-ID4), plus GST alone were expressed in *E. coli* and purified by affinity binding to GSH-agarose beads. Proteins, prebound to beads, were incubated overnight at 4 °C with recombinant bovine eNOS, expressed and purified from a baculovirus system. Beads were washed six times in buffer containing 1 M NaCl, and bound proteins were eluted with GSH. Eluted proteins were separated by SDS/PAGE, transferred to nitrocellulose, and immunoblotted with an anti-eNOS antibody. The results shown are representative of three separate experiments. 'M' is the molecular mass in kDa.

Table 1 Effect of synthetic peptides on eNOS activity

Receptor	Residues	EC ₅₀ for eNOS (μ M)*
Human B2	310–329	1 $\pm$ 0.8
Rat AT1	306–325	3 $\pm$ 1.2
Human ETB	390–409	3 $\pm$ 1.8
Mouse P2Y2	310–329	ND†

* Values represent means $\pm$ S.E.M. from three experiments.

† ND, no detectable inhibition, even at a 100 μ M concentration of peptide.

potent inhibitor of purified eNOS *in vitro* [8]. To test whether inhibition of eNOS occurs with peptides corresponding to membrane-proximal regions of ID4 of the AT1, ETB and P2Y2 receptors, we prepared synthetic peptides corresponding to 20-residue ID4 juxtamembrane regions of the rat AT1 receptor (residues 306–325), the human ETB receptor (residues 390–409), and the mouse P2Y2 receptor (residues 310–329). Each peptide was tested for its ability to inhibit the catalytic activity of purified recombinant eNOS, as measured by arginine-to-citrulline conversion assay. As shown in Table 1, peptides corresponding to membrane-proximal regions of ID4 of the B2, AT1, and ETB receptors all had similar high potencies for eNOS inhibition (EC₅₀ 1–3 μ M), comparable with that of the caveolin-1 scaffolding domains 1 (residues 82–101) and 2 peptides (residues 135–156) shown previously to inhibit eNOS *in vitro* and *in situ* [3,22,23]. In contrast, a peptide corresponding to an equivalent region of the P2Y2 receptor had no effect on eNOS activity, even at the highest concentration tested (100 μ M). Thus, in terms of its lack of ability both to bind and inhibit eNOS, the P2Y2 receptor ID4 appears to differ significantly from that of the B2, AT1, and ETB receptors.

ID4 of eNOS-binding receptors do not bind Ca²⁺-calmodulin

The ID4 eNOS-inhibitory peptides derived from the B2, AT1, and ETB receptors contain low overall sequence identity, and

Figure 2 Effect of non-phosphorylated and phosphorylated B2 receptor peptides on eNOS catalytic activity

Purified recombinant bovine eNOS was incubated for 5 min at 37 °C with various concentrations of the indicated synthetic peptides. eNOS activity was then determined by the arginine-to-citrulline conversion assay. Results shown represent means $\pm$ S.E.M. from three experiments.

inspection of the three sequences does not reveal a potential consensus sequence for eNOS binding. Interestingly, however, each of the three sequences possess characteristics reminiscent of high-affinity Ca^{2+} -CaM-binding domains in other proteins. Ca^{2+} -CaM-binding domains are typically α -helices, 15–20 amino acids in length, that contain several positively charged residues on one side of the helix and several hydrophobic residues on the other [24]. Helical wheel representations of B2 receptor residues 310–329, AT1 receptor residues 306–325, and ETB receptor residues 390–409 show that these sequences are amphiphilic in nature, with several positively charged residues on one side of the helix and several hydrophobic residues on the other. We therefore considered the possibility that the peptides inhibit eNOS by binding and sequestering Ca^{2+} -CaM, thus preventing its interaction with the enzyme. *In vitro* binding assays were carried out with purified Ca^{2+} -CaM and the GST-ETB-ID4, GST-AT1-ID4 and GST-ETB-ID4 fusion proteins under the same conditions used in the binding assays with eNOS. No Ca^{2+} -CaM binding to any of the fusion proteins was detected in these assays, suggesting that the membrane-proximal residues of ID4 of the B2, AT1, and ETB receptors do not function as high-affinity Ca^{2+} -CaM binding domains. Furthermore, the P2Y2 peptide is also amphiphilic in nature and does not inhibit eNOS, indicating that amphiphilicity by itself is not sufficient for inhibition.

Phosphorylation of the B2 receptor eNOS-inhibitory peptide markedly decreases its inhibitory potency for eNOS

G-protein- and phospholipase C-coupled receptors are known to exist as phosphoproteins whose level of phosphorylation is increased dramatically subsequent to receptor stimulation. Phosphorylation occurs primarily on serine and threonine residues and has traditionally been thought to provide a mechanism for receptor desensitization. It has been suggested recently, however, that receptor signalling and desensitization are in reality two

different aspects of receptor function that proceed simultaneously [25]. The BK B2 receptor in human HF-15 fibroblasts has been shown to be phosphorylated in a ligand-dependent manner on serine and threonine residues (but not on tyrosine residues). Phosphorylation appears to be mediated by a G-protein-coupled-receptor-specific kinase (GRK) and occurs in ID4 of the receptor [26]. Phosphorylation of the B2 receptor on tyrosine residues in unstimulated WI-38 human lung fibroblasts has also been reported [27]. Whether the receptor is phosphorylated either basally or in a ligand-dependent manner in endothelial cells, however, has not been previously investigated. The human B2 receptor eNOS-inhibitory peptide (residues 310–329) contains no threonine residues, one serine residue (at position 316) and one tyrosine residue (at position 320). To test the possibility that phosphorylation of either serine-316 or tyrosine-320 might alter the interaction of the peptide with eNOS, we prepared synthetic peptides corresponding to the human B2 receptor residues 310–329 containing either a phosphoserine at position 316 or a phosphotyrosine at position 320. These peptides (termed PS316 and PY320) were tested together with the unmodified peptide and negative control B2 receptor peptides for their abilities to inhibit the catalytic activity of eNOS as measured by arginine-to-citrulline conversion assay. As shown in Figure 2(A) and as reported previously [8], the unmodified peptide potently inhibited eNOS activity. Negative control peptides corresponding to B2 receptor residues 330–347 and 348–364, however, had little or no effect on eNOS activity (results not shown). Phosphorylation of either serine-316 or tyrosine-320 in the 310–329 peptide markedly decreased the inhibitory potency of the peptide (Figures 2B and 2C), suggesting that eNOS interactions with the membrane-proximal region of ID4 of the B2 receptor may be regulated, at least in part, by serine and/or tyrosine phosphorylation of the receptor. Furthermore, loss of inhibitory potency of the phosphorylated peptide appears not to depend on the position of the phosphorylated residue within the peptide sequence because, when an additional peptide (termed PY309) was prepared containing residues 310–329 C-terminal to a phosphotyrosine residue, the inhibitory potency of the peptide was again significantly decreased (Figure 2D).

Phosphorylation of the B2 receptor eNOS-inhibitory peptide decreases its affinity for eNOS

To determine whether phosphorylation of the B2 receptor 310–329 peptide at either serine-316 or tyrosine-320 reduces its inhibitory potency by reducing its affinity for eNOS, we carried out additional *in vitro* binding assays with the GST-B2R-ID4 fusion protein and eNOS. A GST non-fusion protein was utilized as a negative control. Purified eNOS was incubated overnight at 4 °C with purified GST alone prebound to GSH-agarose beads or with the purified GST-B2R-ID4 fusion protein prebound to beads. Incubations with the GST-B2R-ID4 fusion protein were carried out in the absence of any peptide or in the presence (100 μM) of either the unmodified B2 receptor 310–329 peptide, the serine-phosphorylated PS316 peptide or the tyrosine-phosphorylated PY320 peptide. As shown in Figure 3, the unmodified peptide completely blocked the binding of eNOS to the GST-B2R-ID4 fusion protein, owing to competition of the peptide with the fusion protein for binding of eNOS. In contrast, the PS316 and PY320 peptides had no effect on binding of eNOS to the fusion protein, owing to a much lower affinity of the phosphorylated peptides for the eNOS protein. Decreased affinity is likely due to disruption by the negatively charged phosphorylated residue of hydrophobic interactions between the enzyme and the peptide. These data also support the conclusion

Figure 3 Effect of non-phosphorylated and phosphorylated B2 receptor peptides on *in vitro* binding of eNOS to a GST fusion protein containing ID4 of the B2 receptor

A GST fusion protein containing the human B2 receptor ID4 (GST-B2R-ID4) and GST alone were expressed in *E. coli* and purified by affinity binding to GSH-agarose beads. Purified proteins, prebound to beads, were incubated overnight at 4 °C with recombinant bovine eNOS in the absence or presence of the indicated peptides (100 μ M). Beads were washed six times in buffer containing 1 M NaCl, and bound proteins were eluted with GSH. Eluted proteins were separated by SDS/PAGE, transferred to nitrocellulose, and immunoblotted with an anti-eNOS antibody. The results shown are representative of three separate experiments. 'M' is the molecular mass in kDa.

Figure 4 BK stimulation of tyrosine phosphorylation of the B2 receptor in BAECs

BAECs were treated with BK (1 μ M) for the times indicated, cells were lysed, and lysates were immunoprecipitated with an anti-phosphotyrosine antibody. Immunoprecipitated proteins were immunoblotted with an anti-B2 receptor antibody. Similar results were obtained in seven separate experiments. 'M' is the molecular mass in kDa.

that inhibition of eNOS by the 310–329 peptide occurs through peptide binding to eNOS rather than to peptide binding to CaM because the purified eNOS protein used in these binding assays was purified in buffers containing 2 mM EGTA and was thus completely devoid of bound CaM.

The B2 receptor is transiently tyrosine-phosphorylated in endothelial cells in a ligand-dependent manner

As indicated above, it is now well-established that G-protein-coupled receptors are phosphorylated on serine and threonine residues in a ligand-dependent manner by both second-messenger-regulated protein kinases such as the cAMP-dependent protein kinase and protein kinase C, as well as by specific receptor kinases known as GRKs [25]. The B2 receptor, in particular, has been shown to be phosphorylated on serine and

Figure 5 BK stimulation of dissociation and re-association of the eNOS-B2 receptor complex in BAECs

BAECs were treated with BK (1 μ M) for the times indicated, cells were lysed, and eNOS was immunoprecipitated from lysates with an anti-eNOS antibody. Immunoprecipitates were immunoblotted with an anti-B2 receptor antibody. Similar results were obtained in three experiments. 'M' is the molecular mass in kDa.

threonine residues in response to BK stimulation of human HF-15 fibroblasts [26], and it is likely that similar serine and threonine phosphorylation events occur in endothelial cells. Whether the B2 receptor is tyrosine-phosphorylated in endothelial or other cells in a ligand-dependent manner has not been previously shown. Indeed, it has traditionally been thought that ligand-dependent tyrosine phosphorylation of cell-surface receptors is restricted to growth-factor receptors possessing intrinsic tyrosine kinase activity. Recently, however, we have shown that the G-protein-coupled Ang II AT1 receptor in vascular smooth-muscle cells is tyrosine-phosphorylated in response to Ang II stimulation [11]. To determine whether the B2 receptor is phosphorylated on tyrosine residues in endothelial cells in response to BK stimulation, we treated BAECs with BK (1 μ M) for various times, lysed the cells, and immunoprecipitated the receptor with anti-phosphotyrosine antibody. The immunoprecipitated receptor was then immunoblotted with anti-B2 receptor antibody. As shown in Figure 4, the B2 receptor was transiently tyrosine-phosphorylated in response to BK, with maximal phosphorylation observed after 1 min of agonist exposure, demonstrating that the B2 receptor is indeed tyrosine-phosphorylated in a ligand-dependent manner.

Transient tyrosine-phosphorylation of the B2 receptor in endothelial cells is accompanied by a transient dissociation of eNOS from the receptor and an increase in nitric oxide release

Tyrosine phosphorylation and/or serine/threonine phosphorylation of the receptor could conceivably promote a dissociation of the B2 receptor–eNOS complex. We have shown previously that a 30 s exposure of BAECs to BK results in a complete dissociation of the B2 receptor–eNOS complex and have suggested that dissociation of eNOS from the receptor may contribute to the eNOS activation process [8]. If dissociation contributes to eNOS activation, it is likely that re-association of the two proteins contributes to subsequent de-activation. To determine whether BK-stimulated eNOS dissociation from the B2 receptor is followed by a re-association, we treated BAECs with BK (1 μ M) for various times, lysed the cells, and immunoprecipitated the lysates with anti-eNOS antibody. Immunoprecipitates were then immunoblotted with anti-B2 receptor antibody in order to quantify the extent of eNOS–B2 receptor complex formation. As shown in Figure 5, BK stimulated a dissociation of eNOS from the receptor that was followed by a re-association. Furthermore, dissociation occurs during a period

Figure 6 BK stimulation of nitric oxide release from BAECs

BK were treated with BK (1 μ M) for the times indicated, and nitric oxide release was quantified by radioimmunoassay of cGMP production by RFL-6 reporter cells. The results shown represent means $\pm$ S.E.M. from three experiments.

of time (0.5–1 min) when the B2 receptor is maximally tyrosine-phosphorylated. To determine whether a temporal correlation exists between eNOS dissociation from the B2 receptor and eNOS activation, we also determined the time course of BK-stimulated nitric oxide release from BAECs. Cells were treated with BK (1 μ M) for various times and nitric oxide release was quantified by a sensitive bioassay method as described previously [15,28]. As shown in Figure 6, eNOS was activated in response to BK stimulation of BAECs and was subsequently gradually deactivated during the time period when the enzyme became re-associated with the B2 receptor (after 2 min).

Conclusions

In summary, the results of the present study demonstrate that the capacity to bind and inhibit eNOS is a common feature of membrane-proximal regions of the ID4 of the BK B2, the Ang II AT1 and the ET-1 ETB receptors. Not all receptors coupled to eNOS activation bind and inhibit the enzyme, however, as shown here for the ATP P2Y2 receptor. Phosphorylation of potentially phosphorylatable residues in the eNOS-interacting domain of the B2 receptor results in a loss of eNOS inhibition by the domain due to a significant decrease in binding affinity of the B2 receptor domain for the eNOS enzyme. Furthermore, the B2 receptor is transiently phosphorylated on tyrosine residues in response to BK stimulation of cultured endothelial cells. Phosphorylation occurs during the time during which eNOS transiently dissociates from the receptor and is accompanied by a transient increase in nitric oxide release. Taken together, these data support the hypotheses that eNOS is regulated in endothelial cells by reversible and inhibitory interactions with G-protein-coupled receptors and that these interactions can be modulated by receptor phosphorylation.

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