Cyclic AMP-independent Activation of Protein Kinase A by Vasoactive Peptides*

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Protein kinase A (PKA) is an important effector enzyme commonly activated by cAMP. The present study focuses on our finding that the vasoactive peptide endothelin-1 (ET1), whose signaling is not coupled to cAMP production, stimulates PKA in two independent cellular models. Using an in vivo assay for PKA activity, we found that ET1 stimulated PKA in HeLa cells overexpressing ET1 receptors and in aortic smooth muscle cells expressing endogenous levels of ET1 receptors. In these cell models, ET1 did not stimulate cAMP production, indicating a novel mechanism for PKA activation. The ET1-induced activation of PKA was found to be dependent on the degradation of inhibitor of κB, which was previously reported to bind and inhibit PKA. ET1 potently stimulated the nuclear factor-κB pathway, and this effect was inhibited by overexpression of the inhibitor of κB dominant negative mutant (IκBαm) and by treatment with the proteasome inhibitor MG-132. Importantly, IκBαm and MG-132 had similar inhibitory effects on ET1-induced activation of PKA without affecting Gαi-mediated activation of PKA or ET1-induced phosphorylation of mitogen-activated protein kinase. Finally, another vasoactive peptide, angiotensin II, also stimulated PKA in a cAMP-independent manner in aortic smooth muscle cells. These findings suggest that cAMP-independent activation of PKA might be a general response to vasoactive peptides.

Endothelin-1 (ET1)* is a vasoactive peptide implicated in embryonic development and in pathophysiology of cardiovascular, renal, and respiratory systems (1, 2). Two types of ET1 receptors, namely ET_A and ET_B, have been cloned and identified as typical G protein-coupled receptors (3, 4). ET_A receptors are coupled to G<sub>α11</sub>, G<sub>α12</sub>, and G<sub>α13</sub>, whereas ET_B receptors are coupled to G<sub>αq</sub>, G<sub>α11</sub>, and G<sub>α12</sub>, leading to stimulation of phospholipase C, small GTPase RhoA, and inhibition of adenyl cyclase, respectively (5–8). The coupling of ET1 receptors to G<sub>i</sub> is controversial. A modest cAMP response to ET1 was reported by some investigators (9–11), whereas no response or inhibition of cAMP levels was shown by others (5, 7, 12–15). Moreover, there was no convincing evidence that the main target of cAMP, the protein kinase A (PKA), could be activated by ET1. The PKA holoenzyme is a tetrameric complex consisting of two catalytic subunits (PKAc) bound to a homodimer of two regulatory subunits (PKAr). The established mechanism of PKA activation in response to various hormones involves stimulatory G proteins, Gs, which activate adenyl cyclase resulting in production of cAMP. Binding of cAMP to PKAr leads to a release and activation of PKAc (16, 17). Recently, a novel mechanism for PKA activation by lipopolysaccharide (LPS) has been described that is related to the nuclear factor-κB (NFκB) pathway (18). NFκB is a transcription factor that is commonly activated during immune and inflammatory responses (19, 20). Under basal conditions, NFκB exists in an inactive state bound to its natural inhibitor IκB. Activation of NFκB occurs as a result of agonist-induced phosphorylation and degradation of IκB followed by a release of free NFκB. Apparently, a certain pool of PKAc also exists in a complex with IκB (18). Under basal conditions, IκB retained PKAc in the inactive state, presumably by masking its ATP binding site. LPS-induced phosphorylation and degradation of IκB results in a release and activation of PKAc (18). However, except for bacterially derived LPS, there was no evidence that other physiological agonists are able to activate PKA by this mechanism. The present study demonstrates for the first time that ET1 stimulates PKA activity by a cAMP-independent mechanism involving degradation of IκB. Moreover, our data suggest that this is most likely a general phenomenon common for vasoactive peptides.

MATERIALS AND METHODS

Reagents—The cDNA for ET<sub>A</sub> receptor was kindly provided by Dr. Masashi Yamagisawa (University of Texas, South Western Medical Center, Dallas, TX). The cDNA for FLAG-tagged vasodilator-stimulated phosphoprotein (VASP) was a gift from Dr. Michael Uhler (University of Michigan, Ann Arbor, MI). The cDNA for the dominant negative mutant of PKA (6R1α) was a gift from Dr. Stanley McKnight (University of Washington, Seattle, WA). The cDNA for the phosphorylation-deficient S19A,S25A mutant of mouse IκBα (IκBαm) was a gift from Dr. Indra Verma (The Salk Institute, La Jolla, CA). The phosphorylation-deficient S19A,S25A mutant of mouse IκBβ (IκBβm) was generated by polymerase chain reaction, and its identity was confirmed by sequencing. The NFκB-driven luciferase reporter plasmid was described previously (21). Endothelin-1, isoproterenol, tumor necrosis factor α, and MG-132 were from Calbiochem. Angiotensin II was from Dr. Inder Verma (The Salk Institute, La Jolla, CA). The phosphorylation-deficient S19A,S25A mutant of mouse IκBβ (IκBβm) was a gift from Dr. Sergei Orlov (University of Montreal, Montreal, Canada). The RASMC were cultured for up to 10 passages in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 units/ml streptomycin, and 100 units/ml penicillin, and 10% fetal bovine serum (FBS). The primary culture of rat aortic smooth muscle cells (RASMRC) from Wistar-Kyoto rats was kindly provided by Dr. Sergei Orlov (University of Montreal, Montreal, Canada). The RASMC were cultured for up to 10 passages in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 units/ml streptomycin, and 100 units/ml penicillin as described elsewhere (22). For transient
overexpression of proteins, the HeLa cells or RASMC were transfected with desired DNA in the presence of serum, using LipofectAMINE-2000 or LipofectAMINE-Plus reagents (Life Technologies, Inc.), respectively, following the manufacturer’s protocol. The cells were serum-starved in 0.2% FBS for 24 h before the experiment.

**PKA Activity in Intact Cells**—Phosphorylation-induced electrophoretic mobility shift of the VASP is a highly sensitive functional assay for the activity of cyclic nucleotide-dependent protein kinases in intact cells (23, 24) and was used in this study. The specificity of PKA-mediated phosphorylation of VASP was confirmed by overexpression of the dominant negative mutant of PKA, δR1α, which abolished VASP phosphorylation induced by isoproterenol (see “Materials and Methods”) or by 8-bromo-cAMP (25) but not by 8-bromo-cGMP (25). The assay involved transient transfection of cells with FLAG-tagged VASP cDNA, stimulation of quiescent cells with desired agonists, cell lysis followed by immunoblotting of cell lysates with FLAG antibodies (see below), and monitoring the phosphorylation-dependent electrophoretic mobility shift of VASP, as described previously (25).

**Immunoblotting**—After stimulation of quiescent cells with desired agonists, the cells were lysed in the buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 5 mM EDTA, 1 mM NaF, 200 μM sodium orthovanadate, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride). The lysates were cleared from insoluble material by centrifugation at 20,000 × g for 10 min, subjected to polyacrylamide gel electrophoresis, transferred to nitrocellulose, and analyzed by Western blotting with 0.5 μg/ml primary antibodies followed by 0.3 μg/ml horseradish peroxidase-conjugated secondary antibodies and developed by ECL (Amersham Pharmacia Biotech).

**Cyclic AMP Assay**—Cyclic AMP accumulation was determined as described previously (26). Briefly, cells were serum-starved and labeled with 3 μCi/ml [3H]adenine for 24 h, washed twice with serum-free DMEM, and stimulated with desired agonists for various times at 37°C. Reactions were terminated by aspiration of medium followed by addition of ice-cold 5% trichloroacetic acid. Acid-soluble nucleotides were separated on ion-exchange columns and subjected to scintillation spectroscopy. The radioactivity of cAMP-containing fractions was normalized on the total (cAMP + ATP) radioactivity in each sample and finally expressed as -fold increase over control (zero time point).

**RESULTS**

**ET1-induced Activation of PKA**—Fig. 1 shows a time course of PKA activation in response to ET1 (Fig. 1A) and β2-adrenergic receptor agonist isoproterenol (ISO) (Fig. 1B) after transient transfection of HeLa cells with ETα and β2-adrenergic receptor, respectively, as measured by gel retardation of the PKA substrate VASP (see “Materials and Methods”). ET1 induced a transient phosphorylation of VASP with a maximum at 5 min. In contrast, ISO-induced phosphorylation of VASP was much stronger and persisted for at least 1 h (Fig. 1B). To confirm that phosphorylation of VASP is mediated by PKA, we employed a cAMP-unresponsive dominant negative mutant of PKA, δR1α. As shown in Fig. 1C, phosphorylation of VASP, induced by ET1 and ISO, was abolished by overexpression of δR1α. Confirming the specificity of δR1α, it had no effect on ET1-induced MAP kinase phosphorylation (Fig. 1D) or on cGMP-mediated phosphorylation of VASP (25).

**ET1-induced Activation of PKA Is Mediated by Degradation of IκB**—Because two mechanisms of PKA activation have been described, it was important first to examine whether the effect of ET1 on PKA activity was mediated by cAMP. As shown in Fig. 2, ET1 did not stimulate cAMP production but rather reduced basal levels of cAMP in ETα-transfected HeLa cells. By contrast, ISO (positive control) increased cAMP levels by more than 8-fold in β2-adrenergic receptor-transfected cells (Fig. 2). This suggests that ET1-induced activation of PKA is cAMP-independent and confirms that in our cellular model, ET1 signaling is not coupled to Gs and adenyllyl cyclase.

We next addressed the possibility of a cAMP-independent mechanism of ET1-induced PKA activity, described previously for LPS, wherein PKA activation was mediated by proteasome-dependent degradation of IκB (18). ET1 stimulated NFκB activity in HeLa cells by 35.8 ± 4.4-fold, as measured by κB-dependent expression of the luciferase gene (Fig. 3). This effect of ET1 was inhibited by the proteasome inhibitor MG-132, as well as by overexpression of the phosphorylation-deficient dominant negative mutant of IκB, IκBα (Fig. 3). These data indicate that ET1 stimulates NFκB via phosphorylation and degradation of IκB.

Peincubation of cells with increasing concentrations of MG-132 resulted in a dose-dependent inhibition of ET1-induced PKA activity, reaching maximum at 15 μM MG-132 (Fig. 4A). By contrast, up to 50 μM MG-132 had no significant effect on ET1-induced phosphorylation of MAP kinase (Fig. 4B) or the ISO-induced VASP shift (Fig. 4F). This suggests that ET1-induced activation of PKA is mediated by proteasome-dependent protein degradation. To examine whether this PKA activation is dependent on the degradation of IκB, we employed phosphorylation-deficient dominant negative mutants of IκB.
I artifact of ETA overexpression. Therefore, we next examined Smooth Muscle Cells—
It was important to confirm that cAMP-
by immunoblotting with anti-FLAG and anti-phospho-MAP kinase (P-
pressured as -fold activation over control (mean ± S.D. from one of three independent experiments with similar results, performed in triplicates).

FIG. 3. ET1-induced activation of NF-κB. HeLa cells grown on 12-well plates were transfected with 100 ng of β-driven luciferase reporter plasmid, 100 ng of pCDNA3-LacZ, 400 ng of ETA cDNA, and 400 ng of empty vector or the cDNA for IκB dominant negative mutant, IκBα, as indicated. Quiescent cells were pretreated with or without proteasome inhibitor MG-132 (50 μM) for 1 h as indicated, followed by stimulation with 100 nM ET1 for 6 h. Luciferase activity in cell lysates was then measured, normalized on β-galactosidase activity, and expressed as -fold activation over control (mean ± S.D. from one representative experiment performed in triplicate). Note the difference between A and B in stimulation time points. Shown are the representative data from at least three (A) or two (B) experiments. P-VASP, phospho-VASP.

PKA was previously shown to bind IκBα, as well as IκBβ isoforms (18). Therefore, we examined the effects of IκBα-S32A,S36A (IκBαom) and IκBβ-S19A,S23A (IκBβm) overexpression on ET1-induced PKA activity. Overexpression of increasing amounts of IκBom resulted in a dose-dependent inhibition of ET1-induced PKA activity (Fig. 4C) without affecting MAP kinase phosphorylation (Fig. 4D) or the ISO-induced VASP shift (Fig. 4F). By contrast, overexpression of IκBβm had no significant effect on ET1-induced VASP phosphorylation (Fig. 4E). Taken together, these data suggest that proteasome-dependent degradation of IκBα mediates ET1-stimulated PKA activity in HeLa cells.

Activation of PKA by ET1 and Angiotensin II in Vascular Smooth Muscle Cells—It was important to confirm that cAMP-independent activation of PKA by ET1 in HeLa cells was not an artifact of ETA overexpression. Therefore, we next examined the ability of ET1 to activate PKA in a primary culture of RASMC, which expresses endogenous levels of ETA receptors. As shown in Fig. 5A, ET1 and ISO stimulated phosphorylation of VASP in these cells with a striking similarity to their effects in the transiently transfected cellular model (compare Fig. 5A and Fig. 1). Moreover, in RASMC, PKA was also stimulated by another vasoactive peptide, angiotensin II (AII) (Fig. 5A). Importantly, ET1 and AII failed to stimulate cAMP production in RASMC, whereas ISO increased cAMP levels by more than 200-fold (Fig. 5B). This suggests that cAMP-independent activation of PKA may be a general phenomenon, common for vasoactive peptides.

DISCUSSION

The present study describes for the first time cAMP-independent activation of PKA by G protein-coupled receptor agonist endothelin-1 and provides the mechanism of this signaling event.

Cyclic AMP-independent Activation of PKA by Vasoactive Peptides—Employing two independent cellular models with overexpressed or endogenous levels of ETA receptors, we provide strong evidence for the ability of ET1 to stimulate PKA activity in a cAMP-independent manner. Moreover, this may represent a general phenomenon common for vasoactive peptides, because angiotensin II elicited similar effect on PKA in RASMC. With the exception of one study, which showed a modest, cAMP-dependent activation of PKA by ET1 in pig coronary arteries (10), the stimulation of PKA by either ET1 or AII has not been reported. In our experiments, ET1 failed to stimulate cAMP production but rather reduced the basal levels of cAMP. This is in accord with other investigators having shown that ET1 either had no effect or inhibited basal or agonist-induced cAMP production, which is consistent with the coupling of ETA receptors to Gi proteins (5, 7, 13–15). However, one might still consider the possibility of compartment-specific changes in cAMP-levels in response to ET1, which have not been detected in the present study.

ET1-induced PKA Activity Is Dependent on IκB Degradation—The cAMP-independent mechanism of PKA activation, which is mediated by LPS-induced degradation of IκB, has been described previously by Zhong et al. (18). However, except for bacterially derived LPS, no physiological ligand has been reported to activate PKA by this mechanism. The present work demonstrates for the first time that the physiologically relevant hormone ET1, which is central to cardiovascular, renal, and pulmonary physiology, also stimulates PKA in an IκB-dependent manner (Fig. 4). This suggests that this mechanism for
PKA activation is more widespread and might also be relevant to other G protein-coupled receptors.

Several important questions are still to be resolved, such as the signaling pathways, which link ET<sub>A</sub> receptors to the degradation of IkB and activation of PKA, as well as the functional significance of ET1-induced PKA activation. IkB degradation can be mediated by a variety of mechanisms, including protein kinase C (37), mitogen-activated protein kinase (29), or Akt/protein kinase B (21). ETA receptors can activate all above-mentioned molecules (30–32), suggesting several possibilities which is opposite of vasoconstrictive and proliferative effects of ET1. This suggests that activation of PKA may serve as a regulatory mechanism in the function of ET1. Future studies will address these issues.

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