Gβγ-mediated Prostacyclin Production and cAMP-dependent Protein Kinase Activation by Endothelin-1 Promotes Vascular Smooth Muscle Cell Hypertrophy through Inhibition of Glycogen Synthase Kinase-3*

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Endothelin-1 (ET1) is a vasoactive peptide that stimulates hypertrophy of vascular smooth muscle cells (VSMC) through diverse signaling pathways mediated by Gαi/G12/13 heterotrimeric G proteins. We have found that ET1 stimulates the activity of cAMP-dependent protein kinase (PKA) in VSMC as profoundly as the Gα12-linked β-adrenergic agonist, isoproterenol (ISO), but in a transient manner. PKA activation by ET1 was mediated by type-A ET1 receptors (ETA) and recruited an autocrine signaling mechanism distinct from that of ISO, involving Gα12-coupled βγ subunits of heterotrimeric G proteins, extracellular signal-regulated kinases ERK1/2, cyclooxygenase COX-1 (but not COX-2) and prostacyclin receptors. In the functional studies, inhibition of PKA or COX-1 attenuated ET1-induced VSMC hypertrophy, suggesting the positive role of PKA in this response to ET1. Furthermore, we found that ET1 stimulates a Gβγ-mediated, PKA-dependent phosphorylation and inactivation of glycogen synthase kinase-3 (GSK3), an enzyme that regulates cell growth. Together, this study describes that (i) PKA can be transiently activated by Gα12-coupled agonists such as ET1 by an autocrine mechanism involving Gβγ-calcium/ERK/COX-1/prostacyclin signaling, and (ii) this PKA activation promotes VSMC hypertrophy, at least in part, through PKA-dependent phosphorylation and inhibition of GSK3.

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EXPERIMENTAL PROCEDURES

VSMC Culture and DNA Transfection—The rat aortic smooth muscle cells RASMC were obtained by enzymatic dissection of the Wistar-Kyoto (WKY) rat aortas as described previously (15). The cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml streptomycin, 250 ng/ml amphotericin B, and 100 units/ml penicillin. The cells were serum-deprived for 24 h in DMEM containing 0.1% bovine serum albumin, and 2 mM l-glutamine. Transient DNA transfections were performed using Lipofectamine-PLUS reagent (Invitrogen) following the manufacturer’s recommendations.

DNA and Reagents—The cDNAs for Myc-tagged regulator of G protein signaling (RGS) RGS3 (16), Myc-tagged RGS domain of p115RGS (17), and FLAG-tagged vasodilator-stimulated phosphoprotein VASP (18) were previously described as indicated. Endothelin-1, isoproterenol, PKI (14–22), Ro-31-8220, U0126, SC-560, NS-398, and Wortmannin were from EMD Biosciences. Phorbol 12-myristate 13-acetate (PMA), pertussis...
toxin, BAPTA-AM and indomethacin and iloprost were from Sigma. CAY10441 was from Cayman Chemicals. Gβγ-activating peptide mSIRK and its L9A mutant were described previously (19) and were kindly provided by Dr. Alan Smrcka. Antibodies against phosho-ERK1/2, total ERK1/2, phosho-AKT, total AKT, phosho-GSK3α/β, and total GSK3α/β were from Cell Signaling Technology. Antibodies against Myc, Gαq/11, Gαs, Gα13, and cPLA2 were from Santa Cruz Biotechnology. Antibodies against FLAG were from Sigma. Antibodies against RGS3 were described previously (20).

Adenovirus Construction and Transduction of Cells—The recombinant adenovirus expressing full-length RGS3 (AdRGS3) was generated as described previously (21). Briefly the shuttle vector containing RGS3 cDNA was co-transfected with the replication-deficient adenovirus type 5 (Ad5) with deletions in the E1 and E3 genes into HEK 293 cells, to allow homologous recombination to occur. The recombinant AdRGS3 was then isolated, amplified, and purified by CsCl density gradient centrifugation. Titers of the viral stocks were determined by plaque assay using HEK 293 cells (22). The adenovirus expressing protein kinase inhibitor PKI (AdPKI) was described and used previously (23, 24). The adenovirus expressing β-adrenergic receptor kinase C terminus (Ad-βARKct) was provided by Dr. Richard Minshall (25). VSMC were transduced with desired doses of adenoviruses in DMEM containing 0.1% bovine serum albumin for 24 h.

Non-radioactive in Vitro Assay for PKA Activity (24)—The assay is based on the in vitro phosphorylation of the positively charged, fluorometric peptide substrate of PKA (kemptide), which upon phosphorylation, acquires a negative charge and can be separated from the non-phosphorylated peptide by agarose gel electrophoresis and visualized by fluorescence. Following stimulation with desired agonists, the cells (grown in 12-well plates) were lysed in 0.15 ml/well lysis buffer containing 25 mM HEPES (pH 7.5), 0.5% Nonidet P-40, protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF), and phosphatase inhibitors (1 mM NaF, 200 μM sodium orthovanadate). The lysates were cleared from insoluble material by centrifugation at 20,000 × g for 10 min, and 5 μl of cleared lysates were subjected to a kinase reaction with the fluorescence-labeled PKA substrate, kemptide (Promega), following the manufacturer’s protocol. The reaction was stopped by boiling the samples for 10 min. The phosphorylated kemptide was separated from non-phosphorylated kemptide by 0.8% agarose electrophoresis. The fluorescent images were taken by Luminescent Image Analyzer LAS-3000 (Fujifilm).

Cyclic AMP Assay—Cyclic AMP accumulation was determined as described previously (18). 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 at 37 °C. Reactions were terminated by aspiration of media 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 to the total (cAMP + ATP) radioactivity in each sample and expressed as fold increase over control (zero time point).

Western Blotting—Following stimulation of quiescent cells with desired agonists, cells were lysed in radioimmune precipitation assay buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM NaF, 200 μM sodium orthovanadate, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF). The lysates were cleared from insoluble material by centrifugation at 20,000 × g for 10 min, boiled in Laemmli buffer, subjected to polyacrylamide gel electrophoresis, and analyzed by Western blotting with desired primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies (Calbiochem), and developed by enhanced chemiluminescence reaction.

Luciferase Reporter Assays—The firefly luciferase reporter constructs for NFκB (18), SRF (24), and ELK1 (16) were previously described as indicated. Cells grown in 24-well plates were co-transfected with 50 ng/well desired firefly luciferase reporter plasmid, 10 ng/well CMV-driven Renilla luciferase plasmid (Promega), and 50 ng/well empty vector or cDNA for a desired RGS protein (Fig. 3B). Cells were serum-starved overnight following transfection, stimulated with the desired agonists for 6 h, washed with phosphate-buffered saline, lysed in protein extraction reagent. The lysates were assayed for firefly and Renilla luciferase activity using the Promega Dual luciferase assay kit. To account for differences in transfection efficiency, firefly luciferase activity of each sample was normalized to Renilla luciferase activity.

Coimmunoprecipitation of RGS Proteins with Gα Subunits (16, 20)—Cells transfected with cDNA for a desired Myc-tagged RGS protein were lysed in 25 mM HEPES (pH 7.5), 150 mM NaCl, 0.5% Nonidet P-40, 5 mM MgCl2, 1 mM dithiothreitol (DTT), 10 mM NaF, 30 μM AlCl3, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF). The lysates were cleared from insoluble material by centrifugation at 20,000 × g for 10 min and incubated with agarose-conjugated anti-Myc antibodies for 2 h at 4 °C on rotator followed by three washes with 1 ml of the same buffer. The immune complexes were boiled in Laemmli buffer for 5 min, subjected to electrophoresis, and analyzed by Western blotting with antibodies against Myc or against desired Gα subunits as described above.

Total Protein Assay—Cells were lysed in a buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM NaF, 200 μM sodium orthovanadate, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF). The lysates were cleared from insoluble material by centrifugation at 20,000 × g for 10 min. The protein concentration in cell lysates was measured by using BCA Protein Assay kit (Pierce). The desired concentrations of bovine serum albumin were used as standards.

GSK3 Activity Assay—Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl2, 0.5% Triton X-100, 1 mM sodium orthovanadate, 2.5 mM β-glycerophosphate, 1 mM EGTA, 1 mM DTT, and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF). The lysates were cleared from insoluble material by centrifugation at 20,000 × g for 10 min at 4 °C, and the protein concentrations were determined by BCA Protein Assay kit. Equal amounts (15
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μg of protein) of cell lysates were subjected to a kinase reaction containing (final concentrations) 30 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM EGTA, 1 mM DTT (1 mM), 250 μM ATP, 5 μCi of [γ-32P]ATP (GE Healthcare BioSciences), and 50 μM phosphoglycogen synthase peptide-2 substrate of GSK3 (Millipore). The non-specific (GSK3-independent) phosphorylation was dissected in each sample by a parallel kinase reaction containing a GSK3 inhibitor, LiCl (20 mM). The reactions were carried out for 30 min at 30 °C and were terminated by addition of 200 mM EDTA and 5 mM ATP (final concentrations). The samples were then transferred to a Whatman P81 filter paper and washed three times in 100 mM phosphoric acid followed by a final rinse in 95% ETOH. The filter papers were then air-dried, and the radioactivity was measured by scintillation counting.

RESULTS

Activation of PKA by ET-1 in VSMC—We have previously shown that in VSMC, ET1 stimulates phosphorylation-dependent electrophoretic mobility shift of the established PKA substrate, vasodilator-stimulated phosphoprotein (VASP) (18). To examine the activation of PKA more directly, we adapted an in vitro PKA assay that measures in vitro phosphorylation of PKA substrate (kemptide) by the extracts prepared from stimulated cells. Fig. 1A shows phosphorylation of kemptide by lysates prepared from VSMC stimulated with ET1. Confirming the specificity of the kinase assay, phosphorylation of kemptide was blocked by PKA inhibitor peptide, PKI (14–22), but not by the inhibitor of protein kinase C, of p90 and p70 ribosomal S6 kinases (26, 27), Ro-31-8220 (Fig. 1A). The efficiency of Ro-31-8220 was confirmed by its ability to inhibit ET1-induced activation of p90RSK1 when applied to intact cells (data not shown).

PKA activation by ET1 was detectable at 1 nM ET1 and reached maximum at 10 nM ET1 (Fig. 1B). The dose response of PKA activation to ET1 paralleled that of Erk1/2 phosphorylation, as assessed by electrophoretic mobility shift assay for phosphorylated ERK1/2, or by Western blotting with phosphospecific ERK1/2 antibodies (Fig. 1B). The effect of ET1 on PKA activation and Erk1/2 phosphorylation was mediated by ETₐ receptor, as the ETₐ antagonist, BQ123, but not the ETₐ antagonist, BQ788, blocked these responses to ET1 without affecting ISO-induced PKA activity (Fig. 1C). Finally, PKA activation by ET1 was transient, reaching baseline within 20–30 min of stimulation, whereas PKA activation by ISO was much more sustained and persisted for more than 1 h (Fig. 1D). Interestingly, despite a comparable (to ISO) magnitude of PKA activation, ET1 had a relatively modest (but significant) effect on cAMP level (2.7 ± 0.5-fold increase), which could be detected only in the presence of a broad phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine IBMX (Fig. 1E). This suggests that the signaling mechanisms of PKA activation by ET1 and ISO are different.

Dissection of G proteins Mediating PKA Activation by ET1 in VSMC—ETₐ receptor signaling is mediated by Gq11, Gq₁, G₁₂₁₃ and Gβγ subunits of heterotrimeric G proteins. To dissect the role of these G proteins in PKA activation by ET1 in VSMC, we initially assessed phosphorylation of ectopically-expressed, FLAG-tagged vasodilator-stimulated phosphoprotein (VASP) as a reporter for PKA activity, co-expressed with the regulators of G protein signaling (RGS) proteins. RGS proteins bind to the activated (GTP-bound) Gα subunits and inactivate...
them by promoting the hydrolysis of GTP (28). Myc-tagged RGS proteins with the following relative specificity to G proteins were used: RGS3 (specific for G\textsubscript{q/11} and G\textsubscript{i} (20, 29)), and RGS domain of p115RhoGEF (specific for G\textsubscript{12/13} (17)). Fig. 2A shows the co-immunoprecipitation of ectopic Myc-tagged RGS proteins with the corresponding endogenous G\textsubscript{i}-subunits, confirming the specificity of binding. Fig. 2B demonstrates the efficiency of these RGS proteins in dissecting G protein signaling, showing that RGS3, but not the RGS domain of p115RhoGEF, inhibits the G\textsubscript{q/11}-mediated activation of ELK1 by ET1 (16), whereas the RGS domain of p115RhoGEF, but not RGS3, inhibits the G\textsubscript{12/13}-mediated activation of serum response factor by ET1 (30). In addition, to dissect the role of G\textsubscript{\beta\gamma}, we used G\textsubscript{a} subunit of retinal transducin (G\textsubscript{\alpha}), which specifically blocks G\textsubscript{\beta\gamma}-mediated signaling in non-retinal cells by sequestering G\textsubscript{\beta\gamma} subunits (31). As shown in Fig. 2C, ET1 stimulates phosphorylation of VASP as assessed by electrophoretic mobility shift. Overexpression of RGS3 and G\textsubscript{\alpha}, but not of RGS domain of p115RhoGEF, resulted in a significant inhibition of ET1-induced phosphorylation of VASP. These results suggest that ET1-induced VASP phosphorylation is mediated by G\textsubscript{q/11} and/or G\textsubscript{i} (inhibited by RGS3) and by G\textsubscript{\beta\gamma} subunits (inhibited by G\textsubscript{\alpha}).

To confirm the data obtained from using VASP as a reporter, we next examined the effect of RGS3 expression on PKA activity with \textit{in vitro} PKA assay. To achieve an efficient expression of RGS3, which is required for \textit{in vitro} assay that measures PKA activity in the whole population of cells, we generated and purified an adenovirus carrying the RGS3 cDNA (AdRGS3). Fig. 3A shows that overexpression of RGS3 results in the inhibition of PKA activation induced by ET1 (but not by ISO). To further dissect the role of G\textsubscript{\alpha} from that of G\textsubscript{\beta\gamma}, we used pertussis toxin (PTX), which ADP-ribosylates and inactivates G\textsubscript{\alpha} subunits. As shown in Fig. 3B, PKA activation by ET1 (but not by ISO) was inhibited by PTX. Finally to assess the role of G\textsubscript{\beta\gamma} signaling, we used a cell-permeable G\textsubscript{\beta\gamma}-activating peptide, mSIRK (19). As shown in Fig. 3C, activation of G\textsubscript{\beta\gamma} by mSIRK,
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FIGURE 4. Signaling mechanism for PKA activation by ET1 in VSMC. A, effect of BAPTA-AM (50 μM, 1-h pretreatment) on PKA activation induced by 5-min stimulation of VSMC with 100 nM ET1 or 10 μM ISO as indicated. B, effect of PKC depletion (by 24-hr pretreatment with 1 μM PMA) on PKA activation (upper panel) or ERK1/2 shift (lower panel) induced by 5-min stimulation of VSMC with 100 nM ET1 or 1 μM PMA as indicated. C, effect of U0126 (10 μM, 1-h preincubation) or indomethacin (Indo, 10 μM, 1-h preincubation) on PKA activation (upper panel), or on phosphorylation (shift) of ERK1/2 (middle panel) or of cPLA2 (bottom panel) induced by 5-min stimulation of VSMC with 100 nM ET1 or 10 μM ISO as indicated. D, effect of COX-I inhibitor SC-560 (0.3 μM, 1-h preincubation) or of COX-II inhibitor NS-398 (0.3 μM, 1-h preincubation) on PKA activation (upper panel) or ERK1/2 shift (bottom panel) induced by 5-min stimulation of VSMC with 100 nM ET1 or 10 μM ISO as indicated. E, effect of prostacyclin receptor antagonist CAY10441 (1 μM, 1-h preincubation) on PKA activation (upper panel) or ERK1/2 shift (bottom panel) induced by 5-min stimulation of VSMC with 100 nM ET1, or 10 μM ISO, or 1 μM iloprost as indicated. Shown are the representative images from at least three independent experiments.

but not by the inactive mutant (L9A) peptide, was sufficient for the induction of PKA activity in VSMC. Collectively, these data suggest that the Gαi-coupled βγ proteins mediate PKA activation by ET1 in VSMC.

Signaling Mechanism for PKA Activation by ET1 in VSMC—Because Gβγ signaling is linked to Ca2+ mobilization, activation of protein kinase C (PKC) and ERK1/2 (32, 33), we next examined the role of these signaling pathways in PKA activation by ET1. As shown in Fig. 4A, administration of a cell-permeable chelator of intracellular Ca2+ (BAPTA-AM) abolished PKA activation by ET1 but not by ISO, suggesting that Ca2+ is required for the ET1 effect. In contrast, PKC (at least phorbol-sensitive isoforms) appeared dispensable for PKA activation by ET1, as depletion of PKC by a 24-h preincubation of cells with phorbol ester (PMA) did not affect PKA activation by ET1, whereas this blocked PKC-dependent ERK1/2 phosphorylation induced by acute PMA treatment (Fig. 4B). To examine the role of ERK in ET1-induced PKA activation, we used U0126—a pharmacological inhibitor of an upstream ERK effector kinase, MEK1. Fig. 4C shows that 10 μM U0126 inhibits PKA activation by ET1 but not by ISO (note that at this concentration, U0126 has little or no effect on a large number of other protein kinases (34)). One established target of ERK is the cytosolic phospholipase A2 (cPLA2) that is activated by ERK-dependent phosphorylation to produce arachidonic acid (35). Given that arachidonic acid can be metabolized by cyclooxygenases (COX) to prostaglandins and/or prostacyclins, known activators of cAMP production, we examined the possibility of this signaling pathway in mediating PKA activation by ET1. As shown in Fig. 4C, ET1 (but not ISO) stimulated the phosphorylation of cPLA2 in an ERK-dependent manner, as it was inhibited by U0126 (assessed by electrophoretic mobility shift of phosphorylated cPLA2). Furthermore, the COX inhibitor indomethacin also inhibited activation of PKA by ET1 (but not by ISO) without affecting phosphorylation of ERK or cPLA2 (Fig. 4C). Selective inhibition of COX-1 with SC-560, but not of COX-2 with NS-398, abolished PKA activation by ET1 (but not by ISO), without affecting ET1-induced ERK phosphorylation (Fig. 4D).

The failure of NS-398 to inhibit the rapid PKA activation by ET1 is not surprising given that COX-2-mediated effects commonly require the induction of COX-2 expression. Finally, the prostacyclin receptor agonist Iloprost mimicked the effect of ET1 on PKA activity; and the prostacyclin receptor antagonist CAY10441 blocked PKA activation induced by ET1 or Iloprost (but not by ISO) without affecting ET1-induced ERK phosphorylation (Fig. 4E). Taken together, our signaling studies point to the autocrine, prostacyclin-mediated mechanism of PKA activation by ET1 (but not by ISO) via Ca2+/ERK/cPLA2/SOX-1/prostacyclin pathway.

PKA Activation Promotes ET1-induced Hypertrophy of VSMC—Fig. 5A shows a dose-dependent effect of ET1 on a total protein content in VSMC, which parallels to that of ERK phosphorylation and PKA activation (Fig. 1B). To examine the role of PKA in ET1-induced VSMC hypertrophy, we overexpressed a PKA inhibitor (PKI) by adenovirus-mediated transduction of its cDNA (AdPKI). Fig. 5B shows that AdPKI inhibits ET1-induced PKA activation without affecting ERK phosphorylation. Transduction of AdPKI, but not of the control AdLacZ virus, significantly attenuated the ET1-induced total protein accumulation (Fig. 5C), suggesting that PKA promotes ET1-induced VSMC hypertrophy. If PKA activation by ET1 is mediated by COX1, then its inhibition should mimic the effect of AdPKI transduction. Indeed, indomethacin or SC-560, which block PKA activation by ET1 (Fig. 4, C and D), also attenuated ET1-induced hypertrophy of VSMC (Fig. 5D) without affecting ERK phosphorylation. Together, these data suggest that PKA partially mediates the ET1-induced hypertrophy of VSMC.
PKA-dependent Phosphorylation and Inhibition of GSK3 by ET1—Glycogen synthase kinase-3 (GSK3) is the enzyme that is widely implicated in control of cell growth (36). Under basal conditions, GSK3 phosphorylates a broad range of substrates that are critical for cell growth, and this phosphorylation leads to their inactivation and/or degradation. Inhibition of GSK3 by Wnt signaling or by protein kinase B/AKT-dependent phosphorylation results in activation of molecules targeted by GSK3 (36). Given the reports suggesting that the activity of GSK3 may be also regulated by PKA signaling (37), we examined how PKA activation by ET1 affects phosphorylation of GSK3 in VSMC. As shown in Fig. 6A, ET1 stimulated significant phosphorylation of AKT and of GSK3, which is consistent with its hypertrophic properties. PKA inhibition by AdPKI transduction did not affect ET1-induced AKT phosphorylation, whereas wortmannin (an inhibitor of an upstream effector of AKT, PI3 kinase) abolished both basal and ET1-stimulated AKT phosphorylation (Fig. 6A). This suggests that AKT activation by ET1 occurs entirely in a PI3 kinase-dependent, PKA-independent manner. In contrast, ET1-induced GSK3 phosphorylation was inhibited by AdPKI transduction. Furthermore, inhibition of AKT phosphorylation by wortmannin decreased the basal phosphorylation of GSK3, but it still allowed ET1 to induce the phosphorylation of GSK3 in an entirely PKA-dependent manner (as it was inhibited by AdPKI transduction). Finally, consistently with GSK3 phosphorylation, ET1 treatment resulted in a decrease in GSK3 activity, and this effect of ET1 was reversed by AdPKI transduction, with or without wortmannin treatment (Fig. 6A). These results suggest that ET1-induced phosphorylation and inhibition of GSK3 is mediated by PKA, whereas the basal GSK3 activity is controlled by AKT signaling.

If ET1-induced PKA activation is mediated by Gβγ signaling (Figs. 2C and 3C), then this should translate to Gβγ-mediated
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FIGURE 7. A proposed signaling mechanism for the transient PKA activation by ET1 and for the positive role of PKA in ET1-induced VSMC hypertrophy. The inhibitors and agonists used in this study are indicated by "italic" font. The established pathways (not evaluated in this study in detail) are indicated by "dashed lines". The signaling molecules representing the main focus of this study are underlined.

GSK3 phosphorylation. To test this hypothesis, we first used β-adrenergic receptor kinase C-terminal fragment (BARKct), which inhibits Gβγ-mediated signaling by sequestering Gβγ subunits, similarly to Goα (Fig. 2C). As shown in Fig. 6B, adenovirus-mediated transduction of BARKct (Ad-BARKct) resulted in inhibition of ET1-induced PKA activation (confirming again the role of Gβγ); and more importantly, this also attenuated ET1-induced GSK3 phosphorylation. Next, we examined if a direct stimulation of Gβγ by mSIRK peptide would result in a PKA-dependent GSK3 phosphorylation. As shown in Fig. 6B, mSIRK stimulated GSK3 phosphorylation, and this effect was inhibited by AdPKI transduction. Together, these data suggest that ET1 stimulates GSK3 phosphorylation in a PKA-dependent manner, through Gβγ-mediated signaling.

DISCUSSION

PKA is commonly activated via Gs-coupled receptors through a direct stimulation of adenylyl cyclase and production of cAMP. In contrast, Gi-coupled receptors are known to mediate the inhibition of adenylyl cyclases that result in a decrease in cAMP levels. The present study demonstrates that in VSMC, (i) PKA can be transiently (but profoundly) activated through Gi-coupled Gβγ signaling, and (ii) this PKA activation promotes ET1-induced hypertrophy of VSMC, at least in part, through phosphorylation and inhibition of GSK-3 (Fig. 7).

Our signaling studies suggest that PKA activation by ET1 in VSMC occurs by an autocrine mechanism involving Gi-coupled Gβγ, Ca2+, MAP kinase, cPLA2, COX-1, and prostacyclin signaling (Fig. 7). All the inhibitors and agonists that we used to dissect this autocrine pathway (Fig. 7, italic font) have no effect on PKA stimulated through a direct activation of Goα, by β-adrenergic agonist, ISO (Figs. 3 and 4). Importantl, activation of Gβγ (by mSIRK) is sufficient for PKA activation (Fig. 3C), suggesting that other Gi-Gβγ-linked stimuli may have the same effect. Indeed, our additional experiments suggest that PKA activation by extracellular ATP through P2Y purinergic receptors occurs also though Gi-coupled Gβγ signaling (data not shown). At the same time, ET1 (but not ISO) may also stimulate known regulatory mechanisms, such as Goα-mediated inhibition of adenyl cyclases and activation of Ca2+ -/calmodulin-dependent phosphodiesterases (Fig. 7, dash lines), which may explain the relatively moderate increase in bulk cAMP levels (Fig. 1E) and transient PKA response (Fig. 1D) to ET1. Nonetheless, this moderate cAMP production was sufficient for the stimulation of acute PKA activation by ET1 as profoundly as by ISO (Fig. 1D).

It is known that in various blood vessels, ET1 can stimulate endothelium-dependent production of prostanoit (including prostacyclin) that modulate contraction of VSMC in an endocrine manner (38–40). It was also shown that platelet-derived growth factor (PDGF) can stimulate transient PKA activation in VSMC through an autocrine, MAP kinase/cPLA2/COX-dependent mechanism (41). However, it has not been investigated as to how this translates into the regulation of VSMC growth. Our study suggests that the transient PKA activation by ET1 (in cooperation with ERK1/2, AKT, etc.) promotes VSMC hypertrophy (Fig. 5), at least in part through phosphorylation and inhibition of GSK3 by PKA (Fig. 6).

It was previously reported that COX activity is required for the hypertrophy of skeletal muscle (42) and of cardiac muscle (43). However, no one has previously demonstrated the role of COX in VSMC hypertrophy, and more importantly, the role of PKA in hypertrophy of any type of cell, regardless of the stimulus. It is noteworthy that this novel role of PKA in promoting VSMC growth was impossible to uncover using pharmacological PKA inhibitors, such as H-89 or KT 5720, which inhibit other protein kinases critical for cell growth (ribosomal S6 kinase, Rho kinase, phosphoinositide-dependent protein kinase, AKT, etc.) with an equal or better efficiency (34). Furthermore, this study describes one possible mechanism by which PKA may promote VSMC hypertrophy, i.e., through phosphorylation and inhibition of GSK3 (Fig. 6). Interestingly, AKT (a known regulator of GSK3) was responsible only for a basal phosphorylation of GSK3, whereas ET1-induced GSK3 phosphorylation and inhibition was mediated mainly by PKA (Fig. 6). This may suggest the spatial differences in GSK3 activity controlled by AKT or PKA, respectively. GSK3 regulates cell growth by inhibition and/or degradation of a large number of signaling molecules implicated in gene transcription, cell metabolism, and protein synthesis (36). Identification of precise signaling mechanisms by which ET1-induced, PKA-dependent inhibition of GSK-3 promotes VSMC hypertrophy is the goal of our future studies.

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