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Sphingosine 1-Phosphate and Isoform-specific Activation of Phosphoinositide 3-Kinase β

EVIDENCE FOR DIVERGENCE AND CONVERGENCE OF RECEPTOR-REGULATED ENDOTHELIAL NITRIC-OXIDE SYNTHASE SIGNALING PATHWAYS*

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Sphingosine 1-phosphate (S1P) is a platelet-derived sphingolipid that elicits diverse biological responses, including angiogenesis, via the activation of G protein-coupled EDG receptors. S1P activates the endothelial isoform of nitric-oxide synthase (eNOS), associated with eNOS phosphorylation at Ser-1179, a site phosphorylated by protein kinase Akt. We explored the proximal signaling pathways that mediate Akt activation and eNOS regulation by S1P/EDG receptors. Akt is regulated by the lipid kinase phosphoinositide 3-kinase (PI3-K). We found that bovine aortic endothelial cells (BAEC) express both α and β isoforms of PI3-K, while lacking the γ isoform. S1P treatment led to the rapid and isoform-specific activation of PI3-Kβ in BAEC. PI3-Kβ can be regulated by G protein βγ subunits (Gβγ). The overexpression of a peptide inhibitor of Gβγ attenuated S1P-induced eNOS enzyme activation, as well as S1P-induced phosphorylation of eNOS and Akt. In contrast, bradykinin, a classical eNOS agonist, neither activated any PI3-K isoform nor induced eNOS phosphorylation at Ser-1179, despite activating eNOS in BAEC. Vascular endothelial growth factor activated both PI3-Kα and PI3-Kβ via tyrosine kinase pathways and promoted eNOS phosphorylation that was unaffected by Gβγ inhibition. These findings indicate that PI3-Kβ (regulated by Gβγ) may represent a novel molecular locus for eNOS activation by EDG receptors in vascular endothelial cells. These studies also indicate that different eNOS agonists activate distinct signaling pathways that diverge proximally following receptor activation but converge distally to activate eNOS.

The endothelial isoform of nitric-oxide synthase (eNOS)[1] modulates diverse vascular functions, including blood pressure regulation, inhibition of platelet aggregation, and angiogenesis (reviewed in Ref. 1). eNOS activity is complexly modulated by a wide array of physiological and pathophysiological stimuli, including hormones such as bradykinin (2), growth factors such as vascular endothelial growth factor (VEGF) (3), and mechanical stimuli (4). However, the differences and similarities of the proximal signaling pathways elicited by these diverse eNOS activators remain less well understood.

We recently discovered that sphingosine 1-phosphate (S1P), a novel sphingolipid mediator derived from platelets, robustly activates eNOS (5). The roles of S1P in eNOS regulation have since been explored in several experimental systems (6–8). S1P exerts diverse biological responses including cell migration, survival, proliferation, and differentiation (reviewed in Ref. 9); eNOS activation elicited by S1P may represent an important molecular locus of sphingolipid-mediated responses. Importantly, the magnitude of eNOS activation by S1P in cultured endothelial cells is equivalent to that elicited by the classical eNOS agonist bradykinin (6), suggesting the quantitative importance of eNOS regulation by S1P. eNOS activation by S1P is mediated by G protein-coupled receptors termed EDG receptors (for review, see Ref. 10). EDG stimulation by S1P mediates eNOS phosphorylation at Ser-1179 (6), a putative site for the protein kinase Akt (11, 12). S1P activation of EDG led to marked enzyme activation of kinase Akt in vascular endothelial cells (6, 8). However, we found that bradykinin-mediated eNOS activation appears to proceed independently of eNOS Ser-1179 phosphorylation or activation of Akt (6, 13), suggesting the presence of important differences between the signaling events elicited by these two G protein-coupled receptor pathways.

The protein kinase Akt is modulated by its upstream regulator phosphoinositide 3-kinase (PI3-K; for review, see Refs. 14 and 15). PI3-K is a lipid kinase that mediates signal transduction pathways connecting cell surface receptors, including receptor tyrosine kinases as well as G protein-coupled receptors, to their downstream effectors, including protein kinase Akt, ultimately leading to diverse cellular responses including morphogenesis, survival, and metabolic regulation (14, 15). There are at least four independent isoforms of PI3-K as follows: PI3-Kα, -β, -γ, and -δ. Stimulation of G protein-coupled receptors in some cellular systems may lead to the activation of PI3-Kβ or PI3-Kγ (15–21), whereas the PI3-Kα or PI3-Kδ iso-

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This work is dedicated to the memory of Professor Eva J. Neer, whose seminal work on the signaling roles of G protein βγ subunits established a new paradigm in signal transduction and whose scientific insights continue to inspire.

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[1] The abbreviations used are: eNOS, endothelial isoform of nitric-oxide synthase; VEGF, vascular endothelial growth factor; S1P, sphingosine 1-phosphate; dihydro-S1P, sphinganine 1-phosphate; PI3-K, phosphoinositide 3-kinase; PI(3)P, phosphatidylinositol 3-monophosphate; PI/PS, phosphatidylinositol/phosphatidylserine; FBS, fetal bovine serum; BAPTA, 1,2-bis-*O*-aminophenoxyethane-*N, N,N′,N′*-tetraacetic acid tetra(acetoxymethyl) ester; βARK, β-adrenergic receptor kinase; HA, hemagglutinin; ERK, extracellular stimuli-regulated kinase; BAEC, bovine aortic endothelial cells; PAGE, polyacrylamide gel electrophoresis; HA, hemagglutinin.

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forms have not been found previously to be modulated by G protein-coupled receptors. The identity of the PI3-K isoform(s) expressed in vascular endothelial cells, the regulation of PI3-K by the S1P/EDG receptor pathway, and the proximal signaling pathways that connect S1P/EDG with PI3-K, all remain to be elucidated. In the present report, we studied the regulation of the PI3-K-dependent signaling pathways that connect EDG stimulation by S1P to eNOS phosphorylation and enzyme activation.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum (FBS) was from HyClone (Logan, CT). FuGENE6 transfection reagent was from Roche Molecular Biochemicals. All other cell culture reagents and media were from Life Technologies, Inc. S1P and dihydro-S1P (sphinganine 1-phosphate) were from Biomol (Plymouth Meeting, PA). Purified G protein βγ subunit (derived from bovine brain) and 1,2-bis-*O*-aminophenoxyethane-*N,N,N′,N′*-tetraacetic acid tetra(acetoxymethyl) ester (BAPTA) were from Calbiochem. Polyclonal anti-PI3-Kα, anti-PI3-Kβ, anti-PI3-Kγ, anti-p85, anti-β-adrenergic receptor kinase-1 (βARK-1), and anti-hemagglutinin (HA) epitope antibodies were from Santa Cruz Biotechnology. Anti-HA monoclonal antibody (12CA5) was from Roche Molecular Biochemicals. Agarose-conjugated monoclonal anti-phosphotyrosine antibody (4G10) and polyclonal anti-Myc epitope antibody were from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-phospho-eNOS antibody (phosphoserine 1179 in bovine eNOS sequence), anti-phospho-Akt antibody (Ser-473), anti-Akt antibody, and anti-phospho-ERK1/2 antibody (Thr-202/Tyr-204) were from Cell Signaling Technologies (Beverly, MA). Anti-eNOS monoclonal antibody was from Transduction Laboratories (Lexington, KY). SuperSignal substrate for chemiluminescence detection and secondary antibodies conjugated with horseradish peroxidase were from Pierce. L-[^{3}H]Arginine was from Amersham Pharmacia Biotech. [γ-^{32}P]ATP was from ICN (Costa Mesa, CA). Protein determinations were made with the Bio-Rad Protein Assay Kit. All other materials, including anti-FLAG monoclonal antibody, were from Sigma.

Plasmids—cDNA encoding full-length human EDG-1 receptor epitope tagged with FLAG peptide (FLAG/EDG-1 (22), provided by Timothy Hla (University of Connecticut)) was subcloned into pcDNA3 (Invitrogen) (5). Full-length wild type bovine eNOS cDNA, epitope-tagged with HA peptide (eNOS/HA), subcloned into pBK-CMV, was described previously (23). Full-length wild type murine Akt1 cDNA, epitope-tagged with Myc peptide (Akt/Myc), subcloned into pUSEamp, was from Upstate Biotechnology, Inc. cDNA encoding C-terminal fragment peptide of bovine βARK-1 (βARKct, described in Ref. 24), subcloned into pRK5, was provided by Robert J. Lefkowitz (Duke University). cDNA encoding full-length pig PI3-Kγ, subcloned into pcDNA3 (described in Ref. 16), was provided by Phillip T. Hawkins (Babraham Institute, Cambridge, UK).

Cell Culture and Transfection—BAEC were obtained from Cell Systems (Kirkland, WA) and maintained in culture as described (25). In some cultures, BAEC in a 100-mm dish were co-transfected with plasmid cDNAs encoding eNOS/HA (1 μg) and/or βARKct (3 μg) using FuGENE6 following the supplier's protocol and were analyzed 48 h following transfection.

COS-7 cells were maintained in culture as described previously (26). The day before transfection, the cells were split at a ratio of 1:8 in DMEM containing 10% FBS. Cells in a 60-mm dish were co-transfected with cDNAs encoding FLAG/EDG-1, eNOS/HA, Akt/Myc, and/or βARKct (0.5 μg each) using FuGENE6. In some experiments, cells in a 100-mm dish were transfected with plasmid cDNA encoding PI3-Kγ (6 μg). COS-7 cells were used for experiments 48 h after transfection. For both BAEC and COS-7 cells, culture medium was changed to serum-free medium and incubation proceeded overnight prior to all experiments to exclude the effects of S1P contained in FBS (6, 27). Drug treatments were performed exactly as described previously (6, 13).

Lipid Kinase Assay—The enzyme activity of PI3-K in BAEC was determined *in vitro* essentially as described previously (28–30). Briefly, cells in a 100-mm dish were washed with ice-cold phosphate-buffered saline and harvested in 1 ml of lysis buffer comprising Tris (20 mM, pH 7.5), Nonidet P-40 (1% v/v), Na_3VO_4 (1 mM), NaF (50 mM), NaCl (137 mM), $MgCl_2$ (1 mM) and $CaCl_2$ (1 mM), supplemented with a mixture of protease inhibitors (as described in Ref. 31). Harvested cells were incubated at 4 °C for 20 min with rocking and then centrifuged at 14,000 × *g* for 5 min. The protein concentration in the resulting supernatant was determined, and the samples were adjusted to an equal amount of cellular protein (750 μg) in a volume of 500 μl with lysis buffer. The cell lysate was then pre-cleared with 1.5 μg of the corresponding non-immune IgG plus protein A (or G)-agarose beads for 30 min at 4 °C. After a brief centrifugation, the pre-cleared lysate was immunoprecipitated at 4 °C for 1 h with 1.5 μg of antibody, followed by the addition of protein A (or G)-agarose beads. After being incubated further for 1 h, immunoprecipitates were extensively washed as described (28).

Equal amounts of phosphatidylinositol and phosphatidylserine (Sigma) were dissolved in chloroform together and dried under nitrogen. The sample was then resuspended in HEPES buffer (10 mM, pH 7.4), sonicated for 15 min and stored at −20 °C under nitrogen (PI/PS vesicle (28)) to yield a phospholipid concentration of 2 mg/ml. The agarose beads containing immunoprecipitates (prepared as above) were mixed with an enzyme reaction solution (40 μl) containing HEPES (20 mM, pH 7.4), 20 μg of PI/PS vesicle, $MgCl_2$ (5 mM), NaCl (40 mM), and EGTA (0.4 mM). G protein βγ subunit was diluted into a buffer containing Tris-HCl (20 mM, pH 7.4), EGTA (1 mM), NaCl (100 mM), dithiothreitol (1 mM), and sodium deoxycholate (0.9% w/v). An aliquot (5 μl) of diluted Gβγ (or its vehicle) was added to the reaction mixture. The enzyme reaction was initiated by the addition of 5 μl of 20 μM ATP supplemented with 10 μCi of [γ-^{32}P]ATP (800 Ci/mmol). After incubation at 30 °C for 10 min with vigorous shaking, enzyme reaction was terminated by the addition of ice-cold 1 M HCl (100 μl) and chloroform/methanol (200 μl, 1:1 in v/v). After mixing and brief centrifugation in a microcentrifuge, the upper (aqueous) phase was discarded. The resulting lower (organic) phase (35 μl/lane) was applied to a Whatman LK6D TLC plate that had been pretreated with 1% potassium oxalate as described (29). The plate was then developed with a solvent system comprising chloroform/acetone/methanol/acetic acid/water (40:15:13:12:7, v/v). After being air-dried, the TLC plate was subjected to autoradiography. The R_F value of lipid spots corresponding to phosphatidylinositol 3-monophosphate (PI(3)P, the principal product of PI3-K enzyme reaction) was ~0.8 in this TLC system (30). Densitometric analyses of autoradiograms were performed using a ChemiImager 4000 (Alpha Innotech). The degree of PI3-K activation was expressed as the fold increase of PI(3)P formation over basal.

Immunoprecipitation and Western Blot Analyses—The preparation of cell lysates and immunoprecipitation of HA epitope-tagged eNOS protein were performed as described previously (13). Protein expression and the degree of protein phosphorylation were assayed by Western blot analysis as described (6).

Quantitation of Intracellular NO Generation—eNOS enzyme activity was quantified as the formation of L-[^{3}H]citrulline from L-[^{3}H]arginine, as described previously (5, 6, 32). Briefly, cells labeled with L-[^{3}H]arginine (10 μCi/ml) were stimulated by various concentrations of S1P (or vehicle) at 37 °C for 10 min. The cells were then scraped into 2 ml of solution containing 20 mM sodium acetate, 1 mM L-citrulline, 2 mM EDTA, and 2 mM EGTA, pH 5.5, followed by sonication. The sample was applied to Dowex 50WX8–400 column to separate L-[^{3}H]citrulline. The flow-through fraction was analyzed by liquid scintillation counting; L-[^{3}H]citrulline formation in the cells was expressed as femtomoles of L-[^{3}H]citrulline produced per mg of cellular protein/min. Statistical differences were analyzed by analysis of variance followed by Scheffe's *F* test using STATVIEW II (Abacus Concepts). A p value less than 0.05 was considered statistically significant.

RESULTS

In exploring the proximal signaling pathways that couple S1P-mediated activation of EDG receptors to the regulation of eNOS in BAEC, we first studied the expression of PI3-K isoforms in these cells. To enhance the sensitivity of detecting PI3-K isoforms, we used isoform-specific antibodies to immunoprecipitate PI3-Kα, PI3-Kβ, or PI3-Kγ, and then we performed lipid kinase activity assay using the immunoprecipitated enzymes (20, 21). Note that the antibodies used in the present study have been shown to recognize specifically each PI3-K isoform by immunoprecipitation when used in lipid kinase activity assays but that the levels of PI3-K isoform expression are typically below the limit of detection by standard protein immunoblot techniques (20). We used phosphatidylinositol as substrate for the PI3-K assay in the presence of [γ-^{32}P]ATP. Following enzyme reaction, lipid products were separated by TLC and detected with autoradiography. When BAEC lysates were immunoprecipitated with antibodies spe-

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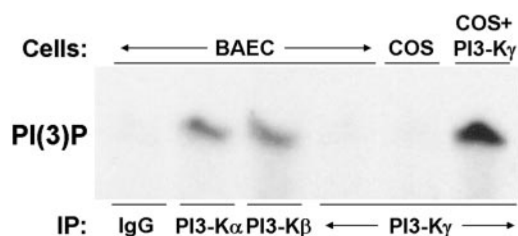

FIG. 1. **Expression of PI3-K isoforms in BAEC.** Shown are the results of *in vitro* PI3-K activity assay performed in cell lysates derived from BAEC or transfected COS-7 cells. Cell lysates were prepared from BAEC or COS-7 cells transfected with pcDNA3 (shown as *COS*) or with plasmid cDNA encoding PI3-Kγ (shown as *COS+PI3-Kγ*). Cell lysates were immunoprecipitated with antibodies that are specific to each PI3-K isoform as indicated or non-immune rabbit immunoglobulin (shown as *IgG*). Immunoprecipitates were then subjected to PI3-K assay using phosphatidylinositol as substrate as described in detail under "Experimental Procedures." After enzyme reaction, the resulting radiolabeled lipid products were separated by TLC and subjected to autoradiography. The lipid spots corresponding to phosphatidylinositol 3-monophosphate (PI(3)P), the PI3-K product, are indicated. Shown are results representative of experiments repeated independently three times with equivalent results.

cific for PI3-K isoforms PI3-Kα or PI3-Kβ, we detected significant formation of ^{32}P-labeled PI(3)P, the principal PI3-K enzyme product (Fig. 1), demonstrating that these antibodies led to the immunoprecipitation of enzymatically active PI3-K molecules. When non-immune rabbit IgG was used for immunoprecipitation instead of anti-PI3-K antibodies, there was no PI(3)P formation (Fig. 1). When BAEC lysates were immunoprecipitated with the anti-PI3-Kγ antibody, no PI(3)P formation was detectable. In order to avoid a false negative conclusion with the PI3-Kγ antibody, we transfected COS-7 cells with plasmid cDNA encoding PI3-Kγ, and we analyzed PI3-Kγ activity. We detected marked PI3-Kγ activity in the COS-7 cells transfected with PI3-Kγ but not in cells transfected with empty vector (Fig. 1). Taken together, these data demonstrate that BAEC contain significant PI3-K enzyme activity associated with the PI3-Kα and PI3-Kβ isoforms but not with PI3-Kγ.

Previous studies (15–21) have documented that only PI3-Kβ and PI3-Kγ, but not PI3-Kα, can be activated by G protein-coupled receptors. Since BAEC lack the activity of PI3-Kγ isoform (Fig. 1), we speculated that S1P would activate PI3-Kβ in this cell type. To test this possibility, we performed time course and dose-response experiments using S1P in BAEC and then analyzed lipid kinase assays following immunoprecipitation using antibodies specific to the PI3-Kβ isoform. S1P (100 nM) induced a marked increase in PI3-Kβ activation within 2 min, reaching a maximum ~3-fold after 5 min (Fig. 2*A*). PI3-Kβ activity gradually returned to base line by 60 min following S1P addition. Fig. 2*B* shows the dose response of PI3-Kβ activity in immunoprecipitates derived from BAEC lysates prepared from cells treated with increasing concentrations of S1P for 5 min. The degree of PI3-Kβ enzyme activation elicited by S1P was dose-dependent with an apparent EC_{50} value of ~30 nM (Fig. 2*B*), in good agreement with the EC_{50} of other responses elicited by S1P in endothelial cells (6, 9, 27, 33). These experiments establish that S1P induces rapid, reversible, and dose-dependent activation of PI3-Kβ in BAEC with an EC_{50} value in the physiologic range. Dihydro-S1P, an analogue of S1P that solely acts through EDG receptors (34), induced a similar degree of PI3-Kβ activation as did S1P (Fig. 2*C*). Conversely, when S1P was added not to cells, but only included in the reaction mixture of PI3-K activity assay *in vitro* (shown as "S1P post-hoc"), PI3-Kβ activity was not altered (Fig. 2*C*). These results suggest that PI3-Kβ activation by S1P is mediated by cell-surface (EDG) receptors, rather than by intracellular S1P targets (35). PI3-Kβ activation by S1P was abolished both by pertussis toxin and by the calcium chelator BAPTA (Fig. 2*D*), indicating that this pathway is dependent upon pertussis toxin-sensitive G protein pathways and involves an agonist-induced increase in intracellular calcium concentration. In contrast, PI3-Kβ activation by S1P was insensitive to genistein, an inhibitor of a wide spectrum of tyrosine kinases, suggesting that S1P activates PI3-Kβ independently of genistein-sensitive tyrosine kinase pathways (Fig. 2*D*).

These results (Fig. 2*D*) implicate pertussis toxin-sensitive G proteins in the S1P-mediated regulation of PI3-Kβ. As the G protein βγ subunit, rather than Gα, may play a major role in the activation of PI3-Kβ (17, 18, 20), we next explored the role of Gβγ subunits in S1P-modulated PI3-Kβ activation. We first studied the effects of purified G protein βγ subunits, derived from bovine brain, added *in vitro* to the immunoprecipitated PI3-Kβ derived from BAEC. Fig. 3 demonstrates that the addition of Gβγ dramatically augments the formation of PI(3)P by the BAEC-derived PI3-Kβ enzyme reaction *in vitro*. The apparent EC_{50} for Gβγ augmentation of PI3-Kβ activity is ~30 nM (Fig. 3). When Gβγ had been denatured by boiling, PI3-Kβ activity was not altered in comparison with its vehicle (Fig. 3). When PI3-Kα was immunoprecipitated from BAEC lysate instead of PI3-Kβ using isoform-specific antibodies (Fig. 1), Gβγ did not affect the degree of PI(3)P formation in an identically configured assay (data not shown). Together, these data suggest that G protein βγ subunits may play an important role for S1P-mediated PI3-Kβ activation and subsequent signal transduction events in BAEC.

After documenting that G protein βγ subunits activate BAEC-derived PI3-Kβ *in vitro*, we explored the functional role of Gβγ for S1P-mediated signal transduction in intact cells. We first exploited a heterologous expression system using transiently transfected COS-7 cells as a model. Previous reports have established that COS-7 cells express PI3-Kα and PI3-Kβ while lacking PI3-Kγ (20), a pattern of PI3-K isoform expression similar to what we observed in BAEC (Fig. 1). COS-7 cells were co-transfected with cDNA constructs encoding FLAG/EDG-1, Akt/Myc, and eNOS/HA. The cells were also co-transfected with plasmid cDNA encoding βARKct, a well characterized peptide scavenger of G protein βγ subunit (24). Co-transfected COS-7 cells were treated with S1P and subjected to immunoblot analysis. The *upper panels* of Fig. 4*A* indicate robust expression of heterologous proteins, concordant with the specific plasmid cDNAs used for co-transfection. We detected endogenous immunoreactivity of βARK-1 at ~80 kDa, whereas transfected βARKct proteins appeared at ~30 kDa with a high level of overexpression (Fig. 4*A, middle panels*). As shown in Fig. 4*A*, S1P mediates the phosphorylation of eNOS at Ser-1179 (the putative site for phosphorylation by kinase Akt) in transfected COS-7 cells. S1P also mediates phosphorylation of kinase Akt at Ser-473 (6, 8, 14). However, in cells co-transfected with βARKct, the phosphorylation of Akt, as well as that of eNOS, was markedly attenuated compared with cells transfected with empty vector instead of βARKct (Fig. 4*A*), suggesting that Gβγ plays a quantitatively important role in mediating these signaling pathways. To extend these findings further, we performed eNOS activation assays in these cells. COS-7 cells were co-transfected with FLAG/EDG-1, Akt/Myc, and eNOS/HA, either with or without βARKct, and treated with increasing concentrations of S1P. The degree of eNOS activation in the transfected cells was assessed by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline (5, 6, 32). S1P induced the dose-dependent activation of eNOS in cells transfected with FLAG/EDG-1, Akt/Myc, and eNOS/HA together with vector control for βARKct (Fig. 4*B, closed circles*). As shown in Fig. 4*B,* when βARKct was co-transfected along with these constructs,

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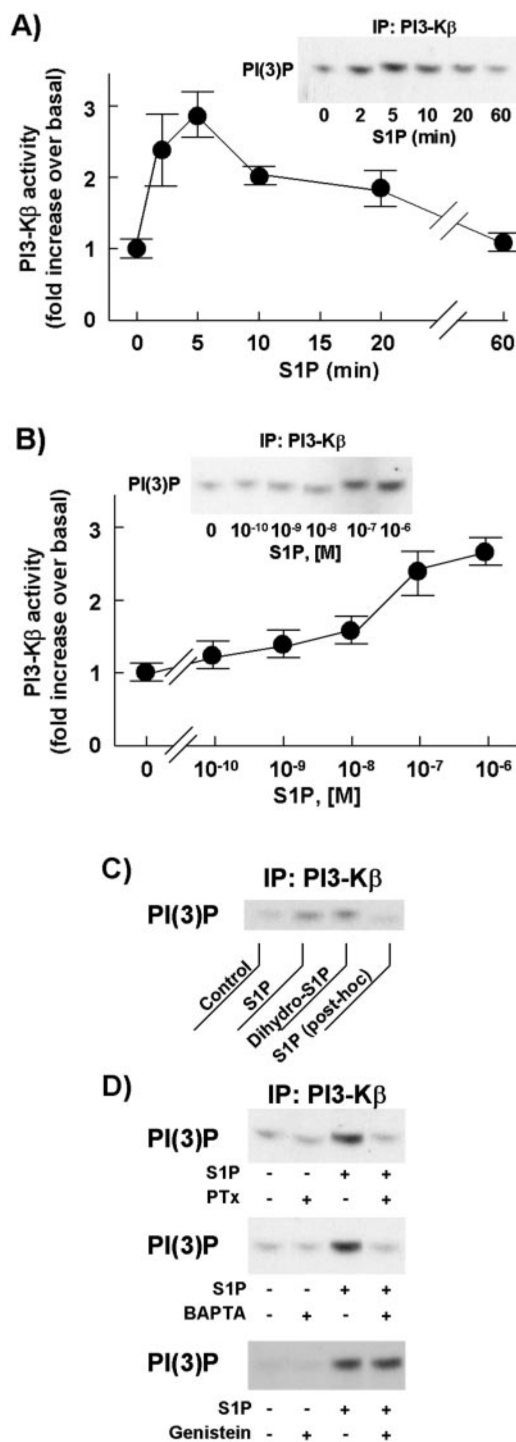

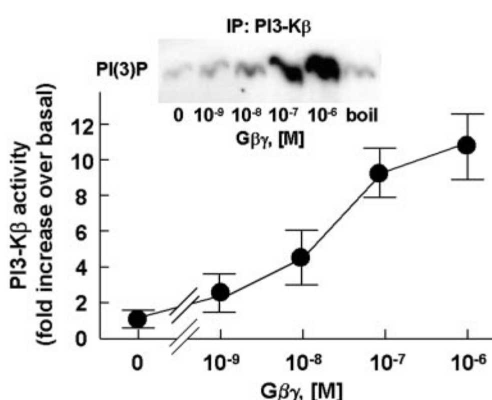

FIG. 3. **The effects of G protein βγ subunit for BAEC-derived PI3-Kβ.** Shown are the results of an *in vitro* PI3-Kβ activity assay performed in the presence of varying concentrations of G protein βγ subunit. Equal amounts of BAEC lysate were immunoprecipitated (*IP*) with an antibody specific for PI3-Kβ. Immunoprecipitates were then subjected to PI3-K assay as described in detail under "Experimental Procedures" in the presence of increasing concentrations of G protein βγ subunit (*Gβγ*) as indicated. *Inset* indicates the radiolabeled lipid spots corresponding to PI(3)P, the PI3-K product. In the *far right lane*, Gβγ (1 μM) had been boiled at 95 °C for 5 min before the addition to PI3-K reaction mixture. Shown are the representative data of three independent experiments. The signal intensity for each data point was analyzed by densitometry and normalized by the signal present in the absence of Gβγ. Each data point in the graph represents mean ± S.E. derived from three independent experiments.

the degree of eNOS activation elicited by S1P was significantly attenuated (69 ± 14% inhibition of eNOS activation relative to sham-transfected controls, $p < 0.01$, $n = 3$). Together, these data indicate that G protein βγ subunits mediate signal transduction pathways that couple EDG activation by S1P with eNOS activation via PI3-K/Akt and eNOS S1179 phosphorylation in transfected COS-7 cells.

We next investigated the roles of PI3-K isoforms in modulating signaling pathways elicited by S1P in comparison with other eNOS activators, bradykinin and VEGF, in endothelial cells. As shown in Fig. 5*A*, S1P and VEGF significantly increase phosphorylation of eNOS at Ser-1179 (the putative Akt site) in BAEC, whereas bradykinin does not induce Ser-1179-phospho-eNOS formation in this experimental setting. By performing immunoblot analysis using anti-phospho-Akt antibody, we also found a concomitant formation of phospho-Akt induced by S1P and VEGF but not by bradykinin (Fig. 5*A*). By contrast, mitogen-activated protein kinases ERK1/2 are similarly phosphorylated (activated) by all three eNOS activators, S1P, VEGF, as well as bradykinin. By using identical cell preparations, we also studied the degree of eNOS enzyme activation by these agonists by measuring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline (described above). As indi-

FIG. 2. **Activation of β isoform of PI3-K by S1P in BAEC.** Shown are the results of *in vitro* PI3-K activity assays in cell lysates derived from BAEC. Cell lysates were immunoprecipitated (*IP*) with an antibody specific to the β isoform of PI3-K (PI3-Kβ); immunoprecipitates were subjected to lipid kinase assay as described in detail under "Experimental Procedures." After enzyme reaction, the resulting radiolabeled lipid products were separated by TLC and subjected to autoradiography. The lipid spots corresponding to PI(3)P, the PI3-K product, are shown. *A* shows the results of time course experiments of PI3-Kβ activity in S1P-treated BAEC. Cells were treated with S1P (100 nM) up to 60 min as indicated. The *inset* shows the representative data of a PI3-Kβ activity assay, which was performed four times with equivalent results. The signal intensity of PI(3)P in each data point was analyzed with densitometry and normalized by the signal present at $t = 0$. Each data point in the graph represents the mean ± S.E. derived from four independent preparations. *B* shows the results of dose-response experiments of PI3-Kβ activity assay in S1P-treated BAEC lysates. Cells were treated for 5 min with increasing concentrations of S1P as indicated; BAEC-derived PI3-Kβ activity was measured and presented as described above. *Inset* indicates data from a representative experiment, which was performed three times with equivalent results. The signal intensity of PI(3)P in each data point was analyzed with densitometry and normalized by the signal present in vehicle-treated cell lysate. Each data point in the graph represents mean ± S.E. derived from three independent preparations. *C*, BAEC were treated with S1P (100 nM) or S1P-analogue dihydro-S1P (100 nM) for 5 min as indicated and subjected to PI3-Kβ activity assay. In some cultures, instead of treating cells before harvesting, S1P (1 μM) was included into the *in vitro* lipid kinase reaction mixture (shown as *S1P post-hoc*). Shown are the results of a representative experiment, which was repeated three times with equivalent results. *D* shows pharmacological characterization of PI3-Kβ activation by S1P. Prior to S1P treatment (100 nM for 5 min), BAEC were treated with pertussis toxin (*PTx*, 50 ng/ml for overnight), BAPTA (20 μM for 30 min), or genistein (10 μM for 30 min) and then subjected to PI3-Kβ activity assay as described above. Shown are results representative of experiments repeated independently three times with equivalent results.

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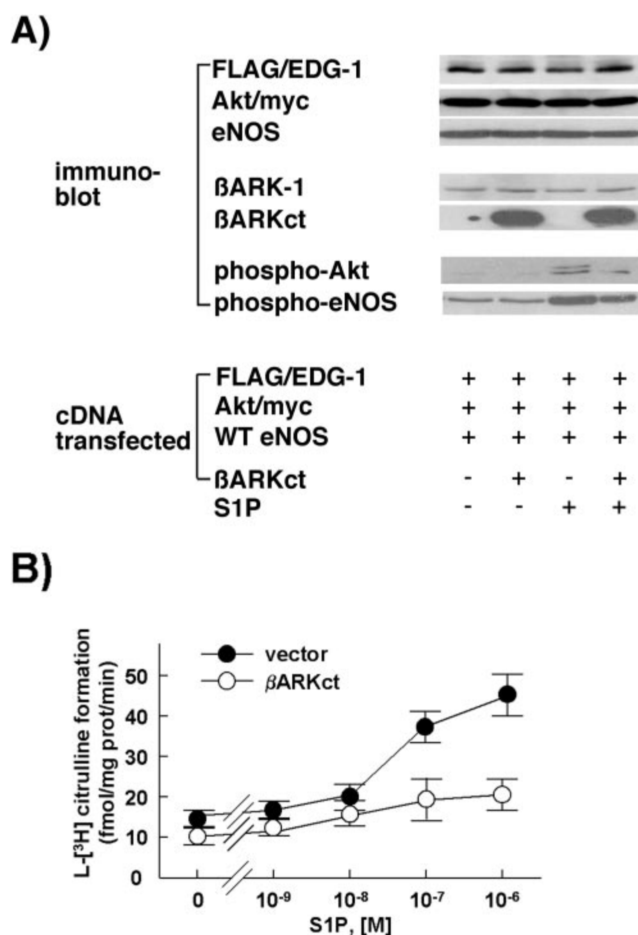

FIG. 4. **Effects of βARKct on S1P-mediated signal transduction of Akt and eNOS in COS-7 cells co-transfected with EDG-1, Akt, and eNOS.** *A,* effects of βARKct on S1P-mediated phosphorylation of Akt and eNOS. Shown are the results of Western blot analyses in COS-7 cells co-transfected with cDNA encoding FLAG/EDG-1, Akt/Myc, and eNOS. In some cells, βARKct, a peptide inhibitor of G protein βγ subunit was co-transfected, as indicated. The total DNA amount was normalized with "empty" vector plasmid DNA for each transfection. Cells were treated with S1P (100 nM for 5 min) or vehicle; cell lysates (20 μg/lane) were separated by SDS-PAGE and probed using antibodies directed against the FLAG epitope, Myc epitope, eNOS, βARK-1, phospho-Akt (Ser-473), or phospho-eNOS (Ser-1179) as indicated. Shown are the results of a representative experiment that was independently replicated three times with equivalent results. *B,* effects of βARKct on S1P-mediated eNOS activation. Shown are the results of eNOS activity assays performed in COS-7 cells co-transfected with FLAG/EDG-1, Akt/Myc, and eNOS together with/without βARKct. The total DNA amount was normalized with empty vector plasmid DNA for each transfection. Cells were treated with increasing concentrations of S1P and subjected to eNOS activation assay as described in detail under "Experimental Procedures." Each data point represents the mean ± S.E. derived from three independent cell preparations, each performed in duplicate. *Closed circles,* data derived from cells co-transfected with pRK5 vector plasmid DNA. *Open circles,* data derived from cells co-transfected with cDNA encoding βARKct.

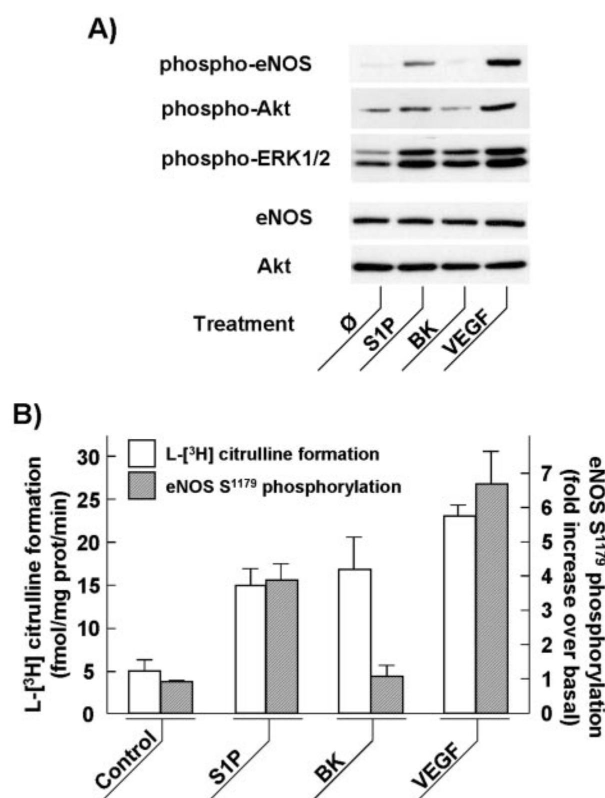

FIG. 5. **eNOS phosphorylation and enzyme activation by S1P, bradykinin, and VEGF in BAEC.** *A,* effects of S1P, bradykinin, and VEGF on phosphorylation of eNOS, Akt, and ERK1/2. Shown are the results of immunoblots probed with antibodies directed against phosphorylated forms of eNOS, Akt, and ERK1/2. BAEC were treated with S1P (100 nM), bradykinin (BK, 1 μM), or VEGF (20 ng/ml) for 5 min. An equal amount of cell lysate (20 μg/lane) was resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with antibodies directed against phospho-eNOS, phospho-Akt, and phospho-ERK1/2, as indicated. Equal loading of samples was confirmed by re-probing the immunoblots with antibodies against (total) eNOS and Akt. Shown are the results from a representative data from an experiment that was independently repeated three times with equivalent results. *B,* the effects of S1P, bradykinin, and VEGF on eNOS activation and phosphorylation. Shown are the results of eNOS activity assays performed in BAEC treated with S1P (100 nM), bradykinin (1 μM), or VEGF (20 ng/ml). Nitric-oxide synthase activity was quantitated as L-[^{3}H]citrulline formation from L-[^{3}H]arginine as described in detail under "Experimental Procedures." Each data point, shown as *open bars,* represents the mean ± S.E. derived from four independent cell preparations, each performed in triplicate. The degree of eNOS phosphorylation derived from immunoblot analyses shown in *A* was quantified using a chemiluminescence detector. The degree of eNOS Ser-1179 phosphorylation over basal was represented by the *shaded bars*. Each data point represents the mean ± S.E. derived from three independent cell preparations.

cated in Fig. 5*B*, S1P and bradykinin induced marked eNOS activation, and VEGF elicited an even higher degree of eNOS activation in BAEC. These results indicated that S1P, bradykinin, and VEGF activate eNOS, whereas the degree of eNOS Ser-1179 phosphorylation by kinase Akt differs importantly among these three agonists (Fig. 5*B*).

We next studied the role of the G protein βγ subunit in eNOS Ser-1179 phosphorylation in endothelial cells, using the same βARKct overexpression strategy as described above. Since these cells already express eNOS, and since only a small fraction of cells can be successfully transfected in primary cultures such as BAEC, we chose to transfect an eNOS construct epitope-tagged with the HA peptide (23). By this approach, we hoped to identify the effects of βARKct proteins only in the fraction of successfully transfected cells by using the HA antibody to immunoisolate HA epitope-tagged eNOS, thereby resolving the eNOS in transfected cells from endogenous eNOS in BAEC. BAEC were co-transfected with plasmid cDNAs encoding eNOS/HA as well as βARKct (or its control vector); the *upper panels* of Fig. 6 demonstrate the expression of transfected eNOS/HA and βARKct proteins in cell lysates derived from these cells, concordant with the plasmid DNAs transfected. The transfected cell cultures were treated with S1P, bradykinin, or VEGF using identical conditions as shown above (Fig. 5), and cell lysates were immunoprecipitated with an antibody directed against the HA epitope; immunoblots were probed with anti-phospho-eNOS antibody. As shown in Fig. 6, S1P as well as VEGF, but not bradykinin, induced marked

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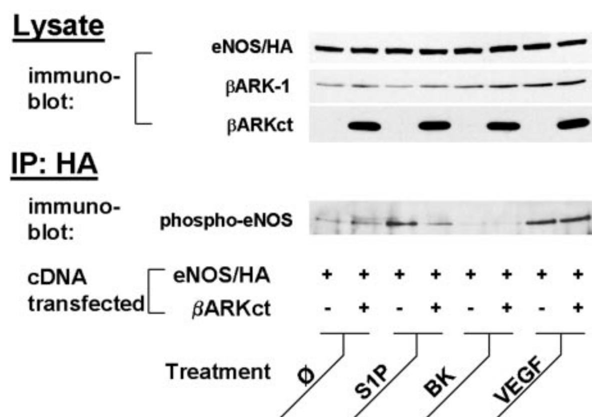

FIG. 6. **Effects of βARKct overexpression on eNOS Ser-1179 phosphorylation mediated by S1P, bradykinin, and VEGF in BAEC.** Shown are the results of Western blot analysis of cell lysates derived from BAEC that had been co-transfected with plasmid cDNAs encoding eNOS/HA together with βARKct (or its vector control), as indicated. Two days after transfection, cells were treated with S1P (100 nM), bradykinin (*BK*, 1 μM), or VEGF (20 ng/ml) for 5 min. An aliquot (20 μg/lane) of cell lysate was withdrawn and separated by SDS-PAGE, transferred to a nitrocellulose membrane, and subjected to immunoblot analysis (*top 3 panels*; indicated as *Lysate*), using polyclonal antibodies directed for HA epitope or for βARK-1. Signals corresponding to eNOS/HA (transfected), βARK-1 (endogenous), and βARKct (transfected) are shown. The remaining samples were immunoprecipitated with a monoclonal antibody directed against the HA epitope (*bottom panel*, indicated as *IP: HA*). Immunoprecipitates were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with an antibody specific to Ser-1179-phospho-eNOS (signals are indicated as *phospho-eNOS*). The membrane was re-probed with a polyclonal antibody specific to HA epitope to confirm the equal loading of total eNOS/HA (data not shown). The results shown are representative of experiments that were repeated independently three times with identical results.

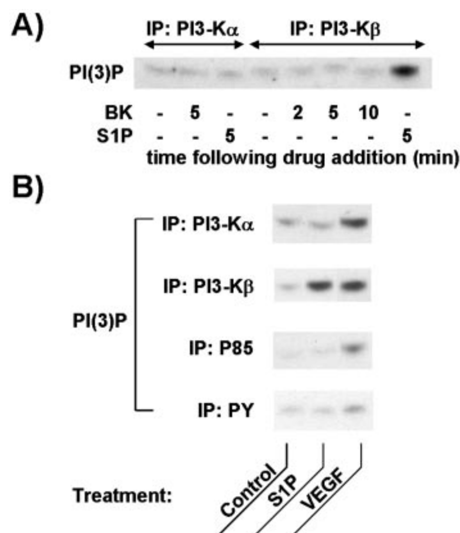

FIG. 7. **Differential regulation of PI3-K activity by S1P, bradykinin, and VEGF in BAEC.** Shown are the results of *in vitro* PI3-K activity assay in cell lysates derived from BAEC, immunoprecipitated with various antibodies as indicated. Immunoprecipitates were analyzed in lipid kinase assay as described in detail under "Experimental Procedures," using phosphatidylinositol as a substrate. After enzyme reaction, the resulting radiolabeled lipid products were separated by TLC and subjected to autoradiography. The lipid spots corresponding to phosphatidylinositol 3-monophosphate (PI(3)P), the PI3-K product, are shown. *A* indicates the results of PI3-K assays in cell lysates derived from BAEC treated with bradykinin (*BK*, 1 μM) or S1P (100 nM) for the times indicated. Cell lysates were immunoprecipitated with antibodies specific to PI3-Kα or PI3-Kβ; shown are representative results of experiments performed three times with equivalent results. *B* shows the results of PI3-K activity assays in BAEC treated with S1P (100 nM) or VEGF (20 ng/ml) for 5 min. Cell lysates were immunoprecipitated with antibodies directed against PI3-Kα, PI3-Kβ, p85 (a regulatory subunit of PI3-K), or phosphotyrosine (shown as *PY*), as indicated, and analyzed by lipid kinase assays as described above. Shown are results representative of experiments independently replicated three times with equivalent results.

eNOS Ser-1179 phosphorylation as detected in the transfected eNOS/HA immunoprecipitates. Significantly, the overexpression of βARKct attenuated S1P-mediated, but not VEGF-mediated, eNOS phosphorylation. Taken together, these experiments in BAEC strongly suggest that G protein βγ subunits play an important role in mediating eNOS Ser-1179 phosphorylation elicited by S1P, but not by VEGF, in native endothelial cells.

We next sought to explore the differences and similarities of PI3-K regulation elicited by S1P, bradykinin, and VEGF in BAEC. We first examined whether bradykinin mediates activation of any of the PI3-K isoforms in endothelial cells. BAEC were treated with bradykinin (1 μM) or S1P (100 nM) for the time indicated; cell lysates derived from these BAEC were immunoprecipitated with antibodies specific to PI3-Kα or PI3-Kβ (Fig. 7*A*). Bradykinin did not induce activation of PI3-Kα or PI3-Kβ, whereas under identical conditions S1P activated the PI3-K β isoform only (Fig. 7*A*). This is consistent with the fact that bradykinin does not activate kinase Akt or induce Ser-1179-phospho-eNOS formation in these cells while S1P does so (Fig. 5 and 6). Next, we compared the effects of S1P with those of VEGF on PI3-K regulation. BAEC were treated with S1P (100 nM) or VEGF (20 ng/ml) for 5 min. As shown in Fig. 7*B*, PI3-Kα was activated by VEGF but not by S1P. In contrast, PI3-Kβ was activated both by S1P and by VEGF, thus suggesting the presence of different regulatory pathways proximal to PI3-K. Previous reports have shown that p85, a regulatory subunit of PI3-K, mediates VEGF-induced PI3-K activation, associated with the activation of various tyrosine kinases (reviewed in Ref. 36). To assess the involvement of these mechanisms for S1P-mediated PI3-K regulation, cell lysates derived from BAEC treated with S1P or VEGF were immunoprecipitated with antibodies specific to p85 or phosphotyrosine and analyzed for PI3-K activity (Fig. 7*B*). Basal PI3-K activity in immunoprecipitates derived from resting cells was very low (Fig. 7*B*). VEGF, but not S1P, induced a significant augmentation of PI3-K enzyme activity associated with p85 or with phosphotyrosine.

DISCUSSION

These studies provide several lines of evidence that S1P modulates the isoform-specific activation of PI3-Kβ in BAEC, and they further suggest that G protein βγ subunits play a key role in S1P-mediated eNOS regulation via the PI3-Kβ/Akt pathway. We found that BAEC express significant PI3-K enzyme activity associated with the isoforms PI3-Kα and PI3-Kβ but not with PI3-Kγ (Fig. 1). This is consistent with previous observations that α and β isoforms of PI3-K are ubiquitously expressed, whereas the PI3-Kγ isoform shows a more restricted expression pattern (15). We documented that S1P modulates PI3-Kβ activation in BAEC (Fig. 2). S1P-mediated activation of PI3-Kβ was rapid, reversible (Fig. 2*A*), and dose-dependent (Fig. 2*B*). The time course and dose dependence of PI3-Kβ activation by S1P are in good agreement with those observed for S1P-mediated regulation of Akt activation as well as eNOS Ser-1179 phosphorylation (6), consistent with the hypothesis that PI3-Kβ is the upstream regulator of eNOS activation by S1P via protein kinase Akt.

Several lines of evidence help to establish that S1P activation of PI3-Kβ is mediated by cell-surface (EDG) receptors rather than by its intracellular or nonspecific membrane ac-

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tions. First, we found that S1P rapidly and potently induced PI3-Kβ activation with an EC_{50} of 30 nM, and we established that the *post hoc* addition of S1P to PI3-K reaction *in vitro* was without effect (Fig. 2). Furthermore, dihydro-S1P, which lacks intracellular actions while robustly activating cell-surface EDG receptors, mimicked S1P activation of PI3-Kβ in BAEC. Finally, pertussis toxin completely abolished PI3-Kβ activation by S1P (Fig. 2). It is noteworthy that BAEC appear to express several subtypes of EDG receptors, including EDG-1 and EDG-3 (27, 37), and each EDG subtype may be differentially coupled to G protein subunits (38). Although the overexpression of EDG-1 subtype was sufficient to mediate eNOS activation by S1P in heterologous expression systems (Fig. 4, also see Refs. 5 and 6), eNOS regulation by each EDG subtype in native endothelial cells is less well understood. However, the results of our own (current studies and Ref. 6) as well as those of other investigators (7, 8) demonstrate that pertussis toxin is capable of markedly attenuating S1P-mediated eNOS activation in several types of vascular endothelial cells. Thus, these EDG receptor responses are mediated by pertussis toxin-sensitive G proteins (namely $G\alpha_i$ or $G\alpha_i$-associated $G\beta\gamma$). These results suggest that only the subsets of EDG receptors that modulate pertussis toxin-sensitive responses are involved in mediating the effects of S1P on eNOS regulation.

Previous observations (17, 18, 20) have suggested that G protein βγ subunits, rather than Gα, mediate PI3-Kβ activation in some cellular systems. Our results indicate that BAEC-derived PI3-Kβ can be robustly activated by Gβγ *in vitro* (Fig. 3). We extended these findings by studying intact cells by overexpressing βARKct, a well established peptide inhibitor of G protein βγ subunit functions (24). In co-transfected COS-7 cells, the overexpression of βARKct markedly attenuated not only S1P-mediated phosphorylation of Akt as well as that of eNOS (Fig. 4*A*) but also S1P-elicited eNOS enzyme activation (Fig. 4*B*). In BAEC, βARKct overexpression markedly attenuated S1P-mediated, but not VEGF-mediated, eNOS Ser-1179 phosphorylation (Fig. 6), indicating the functional importance of G protein βγ subunits in mediating eNOS regulation by S1P in native endothelial cells. Given the importance of eNOS Ser-1179 phosphorylation for eNOS enzyme activation by S1P (6), the current results strongly suggest that G protein βγ subunits activate proximal signaling pathways that modulate eNOS stimulation by S1P in vascular endothelial cells. These results may also identify PI3-Kβ (activated by G protein βγ subunits) as a novel molecular locus for eNOS regulation, modulated by G protein-coupled EDG receptors in vasculature. The molecular basis whereby G protein βγ subunits regulate PI3-Kβ is not fully understood. For the γ isoform of PI3-K, the novel PI3-K regulatory subunit P101 activates PI3-Kγ following its binding with Gβγ (16). The role, if any, of PI3-K regulatory subunit remains less well understood in regulation of PI3-Kβ by Gβγ. It is possible that Gβγ directly binds to and activates PI3-Kβ, as has been observed for several other signaling proteins that are activated by G protein βγ subunits (reviewed in Ref. 39).

Bradykinin is a well established eNOS activator that acts through bradykinin B2 receptors that are coupled via G protein α_q subunit (for review see Ref. 40). Our results (Figs. 5, 6, and 7*A*) clearly demonstrate that bradykinin robustly activates eNOS under the same conditions in which bradykinin neither activates any PI3-K isoform nor activates kinase Akt nor phosphorylates eNOS at Ser-1179 in BAEC (also see Refs. 6 and 13). In contrast to these findings, Harris *et al.* (41) have recently presented data indicating that bradykinin may indeed induce eNOS Ser-1179 phosphorylation via PI3-K/Akt pathway. Although our data and the experiments reported by Harris *et al.* (41) utilize the same cell culture model (BAEC), the apparent discrepancy may derive from differences in cell culture methods, the utilization of different phospho-eNOS antibodies, or other differences in experimental conditions. Moreover, there is an internal inconsistency in the report of Harris *et al.* (41); these authors reported that the PI3-K inhibitor wortmannin did not alter eNOS enzyme activation by bradykinin, despite their observation that wortmannin abolished bradykinin-induced eNOS Ser-1179 phosphorylation. These results of Harris *et al.* (41) thus actually suggest that bradykinin does *not* activate eNOS via the PI3-K pathway, so in fact the basic conclusions are consistent with our previous (6) and current observations. We therefore believe that preponderance of data suggest that bradykinin-mediated eNOS Ser-1179 phosphorylation by kinase Akt has minimal, if any, effect on eNOS enzyme regulation by this nonapeptide hormone.

Our results also indicate important differences between S1P- and VEGF-induced PI3-K activation. S1P activates PI3-Kβ independently of tyrosine kinase pathways, but S1P does not modulate the activity of PI3-Kα isoform. In contrast, VEGF is capable of activating both PI3-Kα and PI3-Kβ isoforms, a pathway that appears to involve the recruitment of the PI3-K regulatory subunit p85 (Fig. 7*B*). Note that p85, which is regulated by tyrosine kinase pathways, can associate with and regulate both PI3-Kα and PI3-Kβ isoforms (15). It is interesting to speculate that the ability of VEGF to activate both α and β isoforms of PI3-K may explain the greater magnitude of VEGF-mediated Akt activation (and thereby eNOS Ser-1179 phosphorylation) compared with S1P (Fig. 5). The complexity of PI3-K/Akt pathway regulation, wherein multiple protein/lipid kinases/phosphates as well as a number of accessory proteins regulate and interact with one another (15, 36), provides many possible points of cross-talk within this pathway. Our present studies show several substantive differences and similarities of PI3-K regulation by these two important eNOS activators, S1P and VEGF, in vascular endothelial cells.

The physiological consequence of eNOS activation by S1P is less well understood. S1P inhibits apoptotic responses in endothelial cells (33), and the nitric-oxide synthase inhibitor *N*-monomethyl-L-arginine is able to abolish the anti-apoptotic actions of S1P (7). Thus eNOS activation may mediate survival signals of S1P in endothelial cells. It was also documented that eNOS activation by VEGF (42–44), but not that by S1P (8), induces chemotactic responses in endothelial cells. Based upon the current observations (Fig. 7*B*), we propose that this difference may be attributable to the divergence of regulatory mechanisms at a level proximal to eNOS regulation, possibly involving the distinct PI3-K isoforms activated by these different agonists (Fig. 7). Interestingly, Hobson *et al.* (45) recently reported that platelet-derived growth factor induces secretion of S1P, resulting in the subsequent trans-activation of EDG receptors in an autocrine or paracrine manner, leading to the augmentation of motility in the transfected HEK 293 cells. Since endothelial cell activation may be accompanied by an increase in intracellular S1P levels (46, 47), stimulation with VEGF or some other growth factors may lead to the secretion of S1P and subsequent trans-activation of EDG pathways in endothelial cells. If this is the case, the targeting of EDG receptors to the sphingolipid-enriched micro-compartment of the plasma membrane termed caveolae (5) may facilitate the interactions of these receptors with the S1P ligand upon secretion. Furthermore, effector molecules downstream of EDG receptors are also specifically compartmentalized to caveolae, which themselves are enriched in PI3-K (48), phosphatidylinositol 4,5-bisphosphate, the *in vivo* lipid substrate for PI3-K (49), as well as eNOS (50). Thus, caveolae may also serve to facilitate the interactions of EDG receptors with their downstream effec-

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tors as well as their sphingolipid ligands.

The isoform-specific regulation of PI3-K may also have implications for modulating the effects of S1P on eNOS expressed in cell types other than vascular endothelial cells. For example, both cardiac myocytes as well as blood platelets express eNOS (51, 52) as well as EDG receptors (53, 54). The role of the S1P/EDG/PI3-K/Akt pathways in eNOS regulation remains less well understood in these cell types. In cardiac myocytes, it is known that PI3-Kγ, rather than PI3-Kβ, seems to be the major PI3-K isoform regulated by G protein-coupled receptors (21, 55, 56). Thus, it is plausible that PI3-Kγ may mediate eNOS regulation by EDG receptors in cardiomyocytes. These considerations add another level of complexity to our understanding of the cell- and receptor-specific regulation of eNOS phosphorylation pathways.

In conclusion, we have provided evidence that S1P, a platelet-derived sphingolipid mediator, modulates the isoform-specific regulation of PI3-Kβ via G protein-coupled EDG receptors. The G protein $\beta\gamma$ subunit appears to play a major role in mediating proximal signaling pathways connecting the activation of EDG receptors by S1P to the activation of PI3-Kβ and ultimately leading to eNOS regulation by the protein kinase Akt. The classical eNOS agonist bradykinin does not activate any PI3-K isoform, while robustly activating eNOS via G protein-coupled bradykinin B2 receptors. VEGF activates both PI3-Kα and -β isoforms in BAEC through tyrosine kinase pathways, leading to even higher degree of eNOS activation. Thus, the signaling pathways elicited by these eNOS agonists diverge proximally following receptor activation but converge distally at the level of eNOS. We propose that the identification of novel points of control in nitric oxide-dependent signal transduction in the vasculature may derive from a deeper understanding of the divergence and convergence of the receptor-regulated kinase pathways.

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