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Bradykinin Stimulates the Tyrosine Phosphorylation and Bradykinin B2 Receptor Association of Phospholipase Cγ1 in Vascular Endothelial Cells

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Bradykinin (BK) B2 receptor signaling involves activation of phospholipase C (PLC). PLC activation by other receptors consists of either allosteric activation of PLCβ isoforms by G-proteins or tyrosine phosphorylation of PLCγ isoforms. Because the B2 receptor is a G-protein-coupled receptor, it has been assumed that the receptor signals through PLCβ. In the present study, however, we have found that BK stimulation of IP_3 production and the Ca^{2+} signal in endothelial cells is dependent on tyrosine phosphorylation. Furthermore, stimulation of B2 receptors in these cells is accompanied by a transient tyrosine phosphorylation of PLCγ1. Phosphorylation is correlated with increased IP_3 production and association of PLCγ1 with the C-terminal intracellular domain of the B2 receptor. The B2 receptor can thus physically associate with intracellular proteins other than G-proteins. Activation of PLCγ isoforms, rather than PLCβ isoforms, may, therefore, be primarily responsible for BK-stimulated IP_3 generation in endothelial cells.

The nonapeptide, bradykinin (BK) regulates many important biological processes including vascular endothelial cell release of nitric oxide (NO), prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factor (EDHF). NO, PGI_2, and EDHF exert their effects on the vascular smooth muscle cells underlying the endothelium and promote vascular relaxation (1). BK transmembrane signal transduction is mediated by receptors known as B2 receptors which, based on analysis of deduced amino sequences obtained from cDNA cloning (2,3), are classified as members of the G-protein-coupled receptor family. Receptors in this family are thought to contain seven membrane-spanning α-helices and to be directly coupled to guanine nucleotide-dependent regulatory G-proteins. In endothelial cells, as in other cell types, an essential early event in B2 post-receptor signaling is activation of phosphoinositide-specific phospholipase C (PLC) resulting in hydrolysis of inositol-containing membrane lipids and transient increases in levels of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (4-6). B2 receptor stimulation also produces a biphasic elevation in the intracellular concentration of free Ca^{2+}. An initial transient phase is due to IP_3-mediated Ca^{2+} release from intracellular stores whereas a more sustained second phase is due to Ca^{2+} influx across the plasma membrane (7,8).

Two alternate mechanisms for PLC activation have been described that involve two different families of PLC isoforms (9). PLCβ isoforms (of which there are four, termed PLCβ1, PLCβ2, PLCβ3, and PLCβ4) are regulated by direct allosteric activation by G-proteins (10). Because PLCβ isoforms are activated by G-proteins, it has been generally assumed that G-protein-coupled receptors, including the B2 receptor, signal exclusively, or at least predominately, through the β isoforms of PLC. In contrast, the γ isoforms of PLC (of which there are two, termed PLCγ1 and PLCγ2) are not activated by G-proteins but are activated instead by tyrosine phosphorylation. This mechanism of PLC activation is well-documented for the tyrosine kinase growth factor receptors. Growth factor-induced stimulation of PLC is independent of G-proteins and requires the intrinsic tyrosine kinase activity of the receptors.

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Abbreviations: BK, bradykinin; PGI_2, prostacyclin; EDHF, endothelial-derived hyperpolarizing factor; Ang II, angiotensin II; VSMC, vascular smooth muscle cells; BAEC, bovine aortic endothelial cells; NO, nitric oxide; IP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; PP1, 4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine; GST, glutathione *S*-transferase.

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Autophosphorylation of the activated receptors on specific tyrosine residues creates docking sites for binding by the SH2 domains of PLCγ, leading to enzyme tyrosine phosphorylation and activation (11).

PLC activation by tyrosine phosphorylation was initially thought to be limited to signaling by growth factor receptors. However, we have shown that the G-protein-coupled angiotensin II (Ang II) AT_1 receptor activates PLC by a signaling pathway that is similar to that of growth factor receptors. PLCγ1 is transiently tyrosine-phosphorylated in Ang II-stimulated vascular smooth muscle cells (VSMC) with a time-course that parallels that of Ang II-stimulated IP_3 production. Furthermore, Ang II-stimulated IP_3 formation is blocked by the tyrosine kinase inhibitor, genistein suggesting that PLC activation occurs through a tyrosine phosphorylation-dependent mechanism (12). Because the AT_1 receptor has no intrinsic tyrosine kinase activity, Ang II stimulation must activate one or more cytoplasmic tyrosine kinases in VSMC. A tyrosine kinase that appears to lie downstream from receptor activation and upstream from PLCγ1 phosphorylation is c-Src, as electroporation of neutralizing anti-c-Src antibodies into VSMC virtually eliminates Ang II-induced tyrosine phosphorylation of PLCγ1 and significantly reduces Ang II-stimulated IP_3 production (13). Recently, we have found that AT_1 receptor signaling also involves binding of PLCγ1 to the receptor in an Ang II- and tyrosine phosphorylation-dependent manner (14).

An important question arising from our studies of Ang II signal transduction in VSMC is whether the signaling mechanisms elucidated for the AT_1 receptor are also utilized by other members of the G-protein-coupled receptor family. Also of interest is whether this signaling pathway is unique to VSMC or whether similar signaling events occur in other cell types. Therefore, in the present study, we have examined the role of tyrosine phosphorylation and receptor binding in activation of PLCγ1 by the endothelial B2 receptor.

MATERIALS AND METHODS

Materials. Monoclonal anti-B2 receptor and monoclonal anti-phosphotyrosine (PY20) antibodies were purchased from Transduction Laboratories (Lexington, KY). Monoclonal anti-PLCγ1 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Protein A/G Plus agarose was obtained from Santa Cruz Biotechnology. Genistein was purchased from Life Technologies Inc. BK and sodium orthovanadate came from Sigma Chemical Co. PP1 was obtained from Calbiochem. Indo-1 AM came from Molecular Probes (Eugene, OR). IP_3 radioimmunoassay kit was obtained from Dupont NEN. ECL detection kits were purchased from Amersham. Protein molecular weight standards, detergent-compatible protein assay kit, and peroxidase-conjugated anti-IgG antibodies were purchased from Bio-Rad. Cell culture media and supplements were from Mediatech, Inc. (Herndon, VA). The glutathione *S*-transferase (GST) fusion protein cloning vector, pGEX-4T-1, glutathione Sepharose 4B, and anti-GST monoclonal antibody were purchased from Pharmacia Biotech Inc. The cDNA encoding the human B2 receptor was generously provided by Dr. Tanya MacNeil, Merck Research Laboratories, Rahway, NJ.

Cell culture. Bovine aortic endothelial cells (BAEC) were passaged from primary cultures. BAEC cultures were maintained in M199 medium supplemented with 10% fetal bovine serum, 5% iron supplemented calf serum, 20 μg/ml L-glutamine, 1$\times$ MEM amino acid and vitamin solutions, 0.6 μg/ml thymidine, 500 I.U./ml penicillin, and 500 μg/ml streptomycin. Cells were subcultured at 3-5 day intervals and used for experiments in passage two through passage six. Serum-containing medium was replaced by serum-free medium 24 hours prior to each experiment.

Measurement of inositol 1,4,5-trisphosphate. Following various timed exposures of BAEC (in 100 mM culture dishes) to BK only (10 μM), BK plus PP1 (1 μM, 30 min), BK plus genistein (100 μM, 1 h), or BK plus sodium orthovanadate (100 μM, 30 min), the incubation was stopped by the addition of 1 ml of 100% ice-cold trichloroacetic acid to the plates. The plates were placed on ice for 10 min and harvested by scraping. Cell extracts were sonicated on ice with a Cole Parmer ultrasonic homogenizer for approximately 1 min. Homogenate was then centrifuged for 10 min at 6,000 $\times$ *g*, the supernatant was removed, and warmed to room temperature for 15 min. IP_3 levels were measured using a radioimmunoassay kit as previously described (15,16).

Measurement of intracellular calcium by confocal fluorescent imaging. BAEC were grown on collagen-coated glass coverslips and preincubated with 5 μM of Indo-1 AM at room temperature for 30 min and washed three times in phosphate buffered saline. Cells were visualized with a Merician ACAS 570/Ultima laser scanning confocal microscope. Cells loaded with Indo-1 AM were excited by UV at 351-364 nm. Cytoplasmic free calcium was read as the fluorescence ratio at 405 and 530 nm. Intracellular calcium concentrations were determined by standard curves of fluorescence ratio vs. free Ca^{2+} in Ca^{2+}-EGTA buffers as described previously (17).

Immunoprecipitation and immunoblotting. BAEC (in 100 mM culture dishes) were stimulated with BK (10 μM) for various times following preincubation in the presence or absence of PP1 (1 μM, 30 min), genistein (100 μM, 1 h), or sodium orthovanadate (100 μM, 30 min). The reaction was terminated by washing the cells twice with ice-cold Tris-buffered saline containing 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4, 136 mM NaCl, 2.6 mM KCl, 1 mM Na_3VO_4, pH 7.4. Cells were then lysed in ice-cold buffer containing 20 mM Tris-HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 50 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotonin, 10 μg/ml leupeptin. Lysates were scraped from the plates and centrifuged for 25 min at 10,000 $\times$ g at 4° C. Protein concentrations were measured in the cleared supernatant by Bio-Rad detergent-compatible protein assay. To immunoprecipitate tyrosine-phosphorylated proteins from the cleared lysate, 10 μg/ml of anti-phosphotyrosine monoclonal antibody was added to the lysate. Antibodies were allowed to equilibrate with the lysate for 2 h at 4° C followed by the addition of protein A/G plus agarose (50 μl) overnight with rocking at 4° C. The immunoprecipitates were then recovered by centrifugation and washed 3 times in 1 ml of ice-cold wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 % Triton X-100, 1 mM Na_3VO_4). The immunoprecipitated proteins were eluted from beads in 100 μl of SDS sample buffer, boiled for 5 min, and separated by SDS polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane for 16 h at 100 mA. The membrane was then blotted with anti-PLCγ1 antibody. Immunoreactive proteins were visualized using a horseradish-peroxidase conjugated goat anti-mouse antibody and an enhanced chemiluminescence detection kit. In other experiments, BAEC proteins were either initially immunoprecipitated with anti-B2 receptor antibody (4 μg/ml) and then probed with anti-PLCγ1 antibody or were immunoprecipitated with anti-PLCγ1 antibody (2 μg/ml) and then probed with anti-phosphotyrosine antibody.

Construction and purification of a GST-B2 receptor fusion protein. A cDNA construct encoding a GST fusion protein of the human B2 receptor intracellular domain 4 (residues 310-364) (3) was created by subcloning into the GST-fusion protein cloning vector, pGEX-4T-

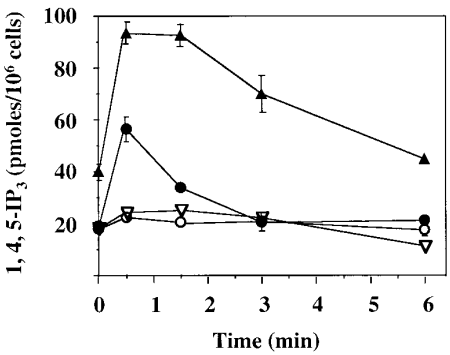

FIG. 1. Effect of tyrosine kinase inhibition and tyrosine phosphatase inhibition on BK-stimulated IP_3 production in BAEC. BAEC were stimulated with BK (10 μM) in the absence (●—●) or presence of genistein (100 μM, 1 h) (○—○), or PP1 (1 μM, 30 min) (▽—▽), or sodium orthovanadate (1 mM, 30 min) (▲—▲) for the times indicated. BK stimulation was terminated by addition of ice-cold trichloroacetic acid, cells were scraped, sonicated, and centrifuged to remove insoluble material. IP_3 levels were determined by radioimmunoassay of the cleared supernatant (results shown are mean ± S.E., n = 3).

1. B2 receptor cDNA sequence encoding residues 310-364 was generated by PCR amplification of the full-length human cDNA subcloned into the pcDNA3 vector (Invitrogen). Primers for PCR contained 5′ *Eco* RI and *Sal* I restriction sites for subcloning. The cDNA encoding the fusion protein was sequenced in the Molecular Biology Core Facility of the Medical College of Georgia to confirm that it contained no PCR-associated nucleotide incorporation errors. The GST fusion protein and GST alone were expressed in *Escherichia coli* and purified as described previously (18,19).

In vitro binding of PLCγ1 to the GST-B2 receptor fusion protein. BAEC in 100 mM culture dishes were stimulated with BK (10 μM) for various times. Cells were washed two times with ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4 and lysed in 2.5 ml of lysis buffer containing 25 mM Tris-HCl, pH 7.6, 0.15 M NaCl, 1% Triton X-100, 10% glycerol, 50 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl flouride, and 1 μg/ml aprotinin. Cells were scraped off the plates and gently sonicated. Lyastes were then used in in vitro binding assays with GST alone or the GST-B2 receptor fusion protein as described previously (14).

RESULTS AND DISCUSSION

To determine whether tyrosine phosphorylation has a role in BK-stimulated PLC activation in endothelial cells, we investigated the effects of tyrosine kinase inhibitors and a tyrosine phosphatase inhibitor on BK-induced IP_3 formation in cultured BAEC. Cells were either treated or not treated (control) with the broad spectrum tyrosine kinase inhibitor, genistein (100 μM for 1 h) or the broad spectrum tyrosine phosphatase inhibitor, sodium orthovanadate (100 μM for 30 min) prior to subsequent treatment for various times with BK (10 μM). In additional experiments, cells were pretreated with the Src family tyrosine kinase-selective inhibitor, PP1 (1 μM for 30 min). This inhibitor has been shown to be highly selective for Src family tyrosine kinases relative to other known tyrosine kinases (20). Following treatment, cells were lysed, and IP_3 levels in cell lysates were quantitated by radioimmunoassay. As shown in Fig. 1, BK stimulated a rapid and transient increase in IP_3 levels of BAEC. Genistein almost completely blocked BK-stimulated IP_3 production, suggesting that PLC activation in response to BK requires tyrosine phosphorylation. Furthermore PP1 also almost completely blocked BK-stimulated IP_3 production, suggesting that a phosphorylation event or events mediated by one or more Src family kinases is required for BK-induced PLC activation. In contrast, sodium orthovanadate increased both the peak levels and the duration of the BK-stimulated IP_3 signal, suggesting that PLC activity in BAEC is regulated by the combined actions of both tyrosine kinases and tyrosine phosphatases. Thus, following PLC activation by tyrosine phosphorylation, PLC activity appears to be down-regulated by a process involving tyrosine dephosphorylation.

Blockade of BK-induced IP_3 formation by PP1 suggests that one or more Src family tyrosine kinases may be required for generation of the BK-induced Ca^{2+} signal in endothelial cells. To determine whether c-Src or other Src family tyrosine kinases contribute significantly to BK-stimulated increases in intracellular Ca^{2+}, BAEC were grown on glass coverslips and pretreated with the flourescent dye, Indo-1 AM. Cells were then either treated or not treated with PP1 (1 μM for 30 min) prior to stimulation with BK (10 μM). Intracellular Ca^{2+} levels were assessed by measuring the Indo-1 flourescence of individual cells by confocal microscopy. BK stimulated a transient increase in intracellular Ca^{2+} with a time-course that was similar to that of BK stimulation of IP_3 production. BK-induced increases in intracellular Ca^{2+} levels, however, were completely blocked by pretreatment of cells with PP1 (Fig. 2) implicating Src family kinases in generation of the BK-stimulated Ca^{2+} signal in BAEC.

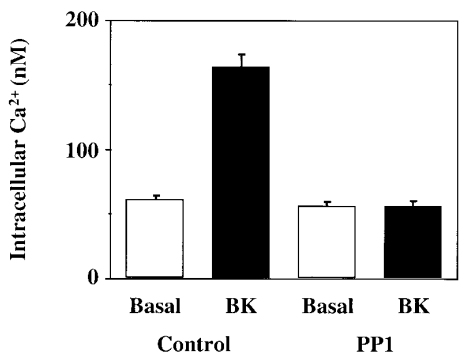

FIG. 2. Effect of PP1 on peak BK-stimulated intracellular calcium levels in BAEC. BAEC were grown on collagen-coated coverslips and preloaded with Indo-1 AM. Cells were then treated or not treated (control) with PP1 (1 μM, 30 min) prior to stimulation with BK (10 μM). Cells were excited by UV at 351-364 nm. Cytoplasmic free calcium was read as the fluorescence ratio at 405 and 530 nm. Peak intracellular calcium concentrations were determined by standard curves of fluorescence ratio vs. free Ca^{2+} in Ca^{2+}-EGTA buffer (results shown are mean ± S.E., n = 8 individual cells for each condition).

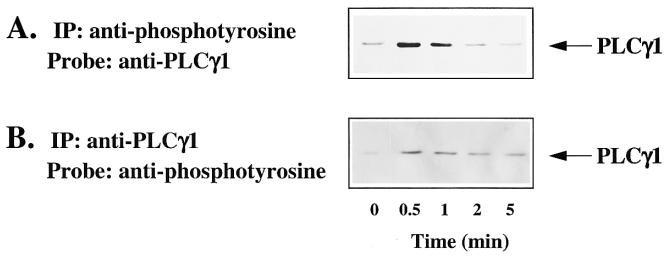

FIG. 3. Time-course of BK-stimulated tyrosine phosphorylation of PLCγ1 in BAEC. BAEC were stimulated for the times shown with BK (10 μM). Cells were lysed and cleared supernates were immunoprecipitated *(IP)* with anti-phosphotyrosine or anti-PLCγ1 antibodies. Immunoprecipitated proteins were separated by SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with anti-PLCγ1 or anti-phosphotyrosine antibodies. Results shown are representative of three separate experiments.

Tyrosine phosphorylation appears to be essential for generation of the BK-stimulated IP_3 and Ca^{2+} signals in endothelial cells, suggesting that PLC activity may be regulated by a tyrosine phosphorylation event or events. Because tyrosine phosphorylation of PLCγ1 itself is known to activate the enzyme, we examined whether BK treatment of BAEC might promote PLC activation due to PLCγ1 tyrosine phosphorylation. Cells were either treated or not treated with PP1 (1 μM for 30 min) or sodium orthovanadate (100 μM for 30 min) and then treated with BK (10 μM) for various times prior to lysis. Tyrosine-phosphorylated proteins in lysates were then immunoprecipitated with anti-phosphotyrosine antibody. Immunoprecipitated proteins were separated on SDS polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with anti-PLCγ1 antibody. As shown in Fig. 3A, BK treatment resulted in a significant and transient increase in the amount of tyrosine-phosphorylated PLCγ1 (140 kDa). Furthermore, the time-course of PLCγ1 phosphorylation was temporally correlated with that of the BK-stimulated increase in IP_3 production (Fig. 1). When the order of antibody addition was reversed, and anti-PLCγ1 immunoprecipitates were probed with anti-phosphotyrosine antibody, a similar time-course of PLCγ1 tyrosine phosphorylation was detected (Fig. 3B). To verify that phosphorylation did not influence the amount of PLCγ1 precipitated by the anti-PLCγ1 antibody in these experiments, immunoblots were stripped and reprobed with anti-PLCγ1 antibody. Equal amounts of PLCγ1 were confirmed to be precipitated by the antibody for each of the time points. BK-stimulated phosphorylation was completely blocked by pretreatment with PP1, suggesting that PLCγ1 is either phosphorylated directly by a Src family kinase or is phosphorylated downstream from Src activation in a phosphorylation cascade. By comparison, following pretreatment with sodium orthovanadate, dephosphorylation was blocked, and the BK-stimulated increase in PLCγ1 phosphotyrosine content was maintained out to at least 10 min.

Signaling by the G-protein-coupled AT_1 receptor involves both a transient increase in tyrosine phosphorylation of PLCγ1 (12) and a temporally correlated increase in the amount of PLCγ1 associated with the receptor (14). To examine whether BK stimulation of BAEC involves a similar BK-dependent association of PLCγ1 with the B2 receptor, we immunoprecipitated the B2 receptor from BK-treated cells with a monoclonal antibody that recognizes the 15 amino acid residue sequence at the C-terminus of the human B2 receptor (residues 310-364 in intracellular domain 4). This antibody reacts with two different polypeptides in immunoblots of BAEC lysates. These immunoreactive proteins have apparent molecular masses of 45 kDa (major band) and 69 kDa (minor band). These molecular weights correspond closely to the sizes reported previously for the nonglycosylated and glycosylated forms of the B2 receptor, respectively (21). The specificity of the antibody for the B2 receptor was confirmed in two different ways. First, preincubation of the antibody with the B2 receptor peptide (residues 350-364) blocked reactivity of the antibody with both of the two proteins in immunoblots of BAEC lysates. Incubation of the antibody with other nonantigen peptides had no effect. Second, the antibody also reacted with a GST fusion protein containing the B2 receptor intracellular domain 4 (residues 310-364) when the fusion protein was expressed and purified from *E. coli.*

BAEC were either treated or not treated with PP1 (1 μM for 30 min) prior to stimulation with BK (10 μM) for various times. The B2 receptor in cell lysates was then immunoprecipitated with anti-B2 receptor antibody. Immunoprecipitated proteins were separated by SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-PLCγ1 antibody. As shown in Fig. 4, minimal complex formation between the B2 receptor and PLCγ1 (140 kDa) was detected for nonstimulated cells (time zero). However,

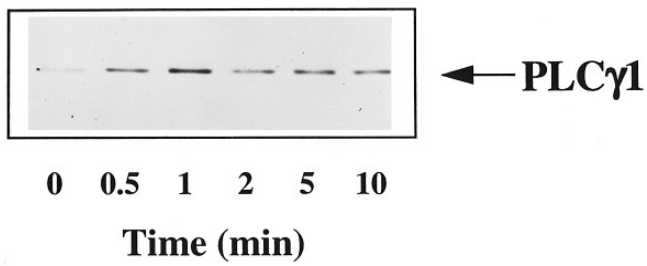

FIG. 4. Time-course of BK-stimulated association of PLCγ1 with the B2 receptor in BAEC. BAEC were stimulated for the times shown with BK (10 μM). Cells were lysed and cleared supernates were immunoprecipitated with anti-B2 receptor antibody. Immunoprecipitated proteins were separated by SDS polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with anti-PLCγ1 antibody. Results shown are representative of three separate experiments.

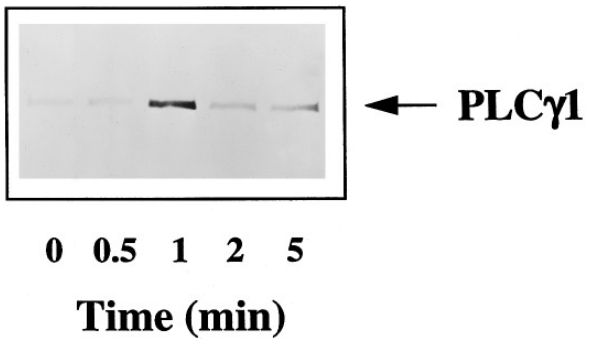

FIG. 5. Time-course of BK-stimulated association of PLCγ1 with a GST-B2 receptor fusion protein. BAEC were stimulated with BK (10 μM) for the times indicated and then lysed. Lysates were subjected to an *in vitro* binding assay with a GST-B2 receptor fusion protein containing human B2 receptor residues 310-364. Binding was quantitated by immunoblotting with anti-PLCγ1 antibody. Similar results were obtained in three separate experiments.

within 1 min of exposure to BK, a significant increase was observed in the amount of PLCγ1 associated with the receptor. Differences in the amount of PLCγ1 coimmunoprecipitated with the anti-B2 receptor antibody were not due to differences in the amount of the receptor immunoprecipitated. Thus, when blots were stripped and reprobed with anti-B2 receptor antibody, equal amounts of the receptor were detected for each time point. PP1 pretreatment completely blocked the association of PLCγ1 with the receptor. Blockade of PLCγ1-receptor association by PP1 suggests that tyrosine phosphorylation of either PLCγ1 or of the receptor (or both) may be required for enzyme docking to the receptor. Moreover, this phosphorylation event appears to be mediated either directly by a Src family kinase or lies downstream from Src activation in a phosphorylation cascade.

In order to confirm that PLCγ1 associates with the B2 receptor in a BK- and tyrosine-phosphorylation-dependent manner and to determine further whether PLCγ1 binds to the B2 receptor C-terminal intracellular domain, we utilized a GST-B2 receptor fusion protein containing the C-terminal 55 amino acids of the human B2 receptor (residues 310-364). These residues comprise the predicted intracellular domain 4 (3). The GST-B2 receptor fusion and GST alone were expressed in *E. coli* and purified to homogeneity on glutathione-Sepharose beads. BAEC were stimulated with BK (10 μM) for various times, cell lysates were prepared, and used in *in vitro* binding assays with the GST-B2 receptor fusion protein prebound to beads. After a 2 h incubation at 4° C, the beads were washed twice in lysis buffer and bound proteins were eluted. The amount of PLCγ1 eluted was then quantitated by immunoblotting with anti-PLCγ1 antibody. As shown in Fig. 5, minimal complex formation was detected for nonstimulated cells (time zero). Exposure of cells to BK, however, resulted in a transient and significant increase in association of PLCγ1 with the GST-B2 receptor fusion proteins. No association was detected for GST alone either basally or in stimulated cells.

In summary, the present study demonstrates several previously unrecognized features of endothelial bradykinin B2 receptor signaling. Stimulation of B2 receptors in endothelial cells involves a transient tyrosine phosphorylation of PLCγ1 which is temporally correlated with an increased association of the enzyme with the C-terminal tail of the receptor and with increased IP_3 formation. The B2 receptor can thus physically associate with intracellular proteins other than G-proteins in a manner similar to that of growth factor receptors. Furthermore, BK-stimulated IP_3 formation can be blocked by tyrosine kinase inhibitors, which suggests that tyrosine phosphorylation of PLCγ1 may be a major mechanism for PLC activation by BK in endothelial cells. Activation of the PLCγ isoforms, rather than the PLCβ isoforms may, therefore, be primarily responsible for endothelial bradykinin B2 post-receptor IP_3 generation.

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