Requirement of Phosphatidylinositol 3-Kinase Activity for Bradykinin Stimulation of NF-κB Activation in Cultured Human Epithelial Cells*

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The signaling mechanisms utilized by bradykinin (BK) to activate the transcription factor nuclear factor κB (NF-κB) are poorly defined. We previously demonstrated that BK-stimulated NF-κB activation requires the small GTPase RhoA. We present evidence that BK-induced NF-κB activation both activates and requires phosphatidylinositol 3-kinase (PI 3-kinase) in A549 human epithelial cells. Pre-treatment with the PI 3-kinase-specific inhibitors, wortmannin, and LY294002 effectively blocked BK-induced PI 3-kinase activity from A549 cells. Wortmannin and LY294002 also abolished BK-induced NF-κB activation, as did transient transfection with a dominant negative mutant of the p85 subunit. BK-stimulated PI 3-kinase activity and NF-κB activation were sensitive to pertussis but not cholera toxin, suggesting that the B2 BK receptors transducing the response were coupled to Gai or Gao heterotrimeric G proteins. Tumor necrosis factor α (TNFα) also stimulated increased PI 3-kinase activity, however TNFα-stimulated NF-κB activation was not affected by the PI 3-kinase inhibitors or the p85 dominant negative mutant. These findings provide evidence that BK-induced NF-κB activation utilizes a signaling pathway that requires activity of both RhoA and PI 3-kinase and is distinct from the signaling pathway utilized by TNFα. Furthermore, we show that the p85 regulatory subunit is required for activation of PI 3-kinase activity by this G protein-coupled receptor.

Bradykinin (BK)1 is rapidly generated following inflammation or injury. The released BK is known to mediate multiple proinflammatory effects including smooth muscle contraction, pain, vasodilatation, increased vascular permeability, eicosanoid synthesis, and neuropeptide release. We recently reported that BK can also activate the transcription factor NF-κB and stimulate proinflammatory cytokine synthesis in human fibroblasts and epithelial cells (1, 2). BK-stimulated NF-κB activation was shown to be mediated through the G protein-coupled B2 BK receptor and was pertussis toxin sensitive (1). The small GTPase RhoA was also shown to be both necessary and sufficient for BK-stimulated NF-κB activation (2).

Phosphatidylinositol 3-kinase (PI 3-kinase) is a ubiquitous lipid kinase that phosphorylates the 3-position of the inositol ring of inositol phospholipids to generate such lipid messengers as phosphatidylinositol 3-phosphate (PtdIns(3)P), phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P2), and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3). The exact role and signal molecular targets of these lipid products has not yet been determined, however, increasing evidence suggests that they may serve as intracellular second messengers (3–5). PI 3-kinase is a heterodimer consisting of a p85 regulatory subunit with SH2 domains and a p110 catalytic subunit (5, 6). In the regulation of receptor-mediated intracellular pathways, including G-protein-coupled receptor, PI 3-kinase has been reported to be directly activated by βγ subunits released from activated G proteins (7).

We therefore investigated the role of PI 3-kinase in the signaling events that lead to NF-κB activation in BK-stimulated A549 human transformed epithelial cells. In this report we show that BK stimulates PI 3-kinase activity in A549 cells and that inhibition of PI 3-kinase blocks BK-stimulated NF-κB activation. These results indicate that PI 3-kinase is a novel signal transducer for BK-induced NF-κB activation in airway epithelial cells.

EXPERIMENTAL PROCEDURES

Reagents—BK was obtained from Peninsula Laboratories (Belmont, CA). The human lung adenocarcinoma cell line, A549, (distal respiratory epithelium-like) was obtained from the American Type Culture Collection (Manassas, VA). A549 cells were maintained in Ham’s F12K medium containing 10% fetal bovine serum at 37 °C in a humidified 5% CO2 environment. Immediately before stimulation, A549 cells were changed into serum-free RPMI 1640 (Irvine Scientific). Oligonucleotides and their complementary strands for electrophoretic mobility shift assays (EMSA) were from Promega (Madison, WI) and Santa Cruz Biotechnology. The sequences were a consensus κB site (underlined), 5′-AGTTAGGAGGGACTTTCCAGGC-3′ (NF-κB) (8), and a mutant κB site with the G to C substitution (underlined) in the κB binding motif, 5′-AGTTAGGAGCGCTTTCCAGGC-3′. γ32P]ATP (>5000 Ci/mmol) was from Amer sham Pharmacia Biotech. The plasmid pmTNFα (a gift from Vladimir Kravchenko, The Scripps Research Institute La Jolla, CA) was used for preparation of recombinant murine TNFα from Escherichia coli. The specific activity of TNFα purified by ion-exchange chromatography was 7 × 107 units/mg protein. The p85 dominant negative plasmid, p85N-C478–514, was constructed using a previously published method (9).

EMSA—Nuclear extracts were prepared from A549 cells plated at a density of 1 × 106 cells in a 6-well plate using a modified method of Dignam et al. (10), and EMSA were performed using 2.5 μg of the nuclear extract as described previously (2).

PI 3-Kinase Assay—Aliquots of cell lysates normalized for protein

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‡ The abbreviations used are: BK, bradykinin; AP-1, activator protein-1; BH, breakpoint cluster region-homology domain; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; NF-κB, nuclear factor κB; PI 3-kinase, phosphatidylinositol 3-kinase; PKC, protein kinase C; PtdIns, phosphatidylinositol; TNFα, tumor necrosis factor α; phOx, 4-ethoxymethylene-2-phenyl-2-oxazolin-5-one.

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content were incubated for 3 h with anti-PI 3-kinase antibodies directed against the 85-kDa regulatory subunit (Upstate Biotechnology, Lake Placid, NY). The immune complexes were absorbed onto protein A-Sepharose and washed as described (11). PI 3-kinase assays were performed directly on beads. Briefly, the reaction was carried out for 10 min in a buffer containing 40 mM HEPES, pH 7.2, 6 mM MgCl₂, 1 mM EDTA, 10 µg of PI (Avanti Polar Lipids, Alabaster, AL), 10 µg ATP, and 10 µCi [γ-32P]ATP (6,000 Ci/mmol; NEN Life Science Products). Adenosine (0.2 mM) was added to the reaction mixture to inhibit residual PI 4-kinase activity. After the incubation, the reaction was stopped with methanol plus 2.4 N HCl (1:1, v/v), and lipids were extracted and analyzed by thin-layer chromatography.

**RESULTS**

**BK-induced Activation of NF-κB Is Blocked by PI 3-Kinase Inhibitors**—To assess the role of PI 3-kinase in BK-induced NF-κB activation, we examined the consequences of pre-incubating cells with PI 3-kinase inhibitors. Wortmannin and LY294002 have both been shown to specifically inhibit PI 3-kinase activity in multiple cell types with distinct and different modes of action (13). Following pretreatment with wortmannin, LY294002, or media control, A549 cells were stimulated with BK or TNFα, and NF-κB activation was assessed by EMSA. Whereas the DNA binding activity of NF-κB was potently induced by BK and TNFα (Fig. 1, lanes 2-3), BK-induced NF-κB activation was completely inhibited in A549 cells pretreated with wortmannin (Fig. 1, lane 4) or LY294002 (Fig. 1, lane 6). In contrast, neither wortmannin nor LY294002 had an effect on TNFα-induced NF-κB activation (Fig. 1, lanes 5 and 7). These results suggest that PI 3-kinase activity is required for BK- but not TNFα-induced NF-κB activation.

We next examined the dose-response and timing of the inhibitory effect of wortmannin on BK-induced NF-κB activation. As shown in Fig. 2A, wortmannin at doses greater than or equal to 50 nM significantly inhibited BK-induced NF-κB activation. To effectively inhibit BK-induced NF-κB activation, wortmannin needed to be added to the A549 cells at least 5 min prior to stimulation with BK (Fig. 2B).

**BK Stimulates a Rapid but Transient Increase in PI 3-Kinase Activity**—The results presented above demonstrate that inhibition of PI 3-kinase activity abrogates BK-induced NF-κB activation. We next examined whether BK would induce increased PI 3-kinase activity in A549 cells. PI 3-kinase activity was measured using an in vitro kinase assay using phosphatidylinositol as the substrate (11). A549 cells were lysed at varying times following stimulation with BK, and the cellular extracts were collected for analysis of PI 3-kinase activity. BK stimulated a time-dependent increase in PI 3-kinase activity (Fig. 3). The BK-induced increase of PI 3-kinase activity was seen within 5 min of stimulation and peaked at 20 min. The kinetics of BK-induced PI 3-kinase activation preceded that of

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**FIG. 1**. The PI 3-kinase inhibitors wortmannin and LY294002 abolish BK-induced NF-κB activation. A549 cells were pre-incubated with media alone (lanes 1–3), 100 nM wortmannin (lanes 4–5), or 50 nM LY294002 (lanes 6–7) for 15 min, then stimulated with 20 nM BK (lanes 2, 4, and 6), or 40 ng/ml TNF (lanes 3, 5, and 7) for 40 min. Nuclear extracts were prepared, and NF-κB activation was measured by EMSA as described under “Experimental Procedures.” The EMSA autoradiograph is shown with the DNA-protein complex marked with a bracket, and the unbound probe is indicated by an arrow. These results are representative of two separate experiments. Med, media; WM, wortmannin; LY, LY294002.

**FIG. 2**. Wortmannin inhibits BK-stimulated NF-κB activation in a dose- and time-dependent manner. A, A549 cells were pre-incubated with varying concentrations of wortmannin (WM) as shown for 15 min, then stimulated with BK (20 nM) for 40 min. NF-κB activation was determined as described for Fig. 1 with the DNA-protein complex marked with a bracket and the unbound probe indicated by an arrow. B, A549 cells were preincubated with 100 nM wortmannin for the indicated times, then stimulated with BK (20 nM) for 40 min. NF-κB activation was determined as described for Fig. 1 with the DNA-protein complex marked with a bracket and the unbound probe indicated by an arrow. These results are representative of three separate experiments.

**FIG. 3**. Wortmannin inhibits BK-stimulated NF-κB activation in a dose- and time-dependent manner. The PI 3-kinase inhibitors did not affect TNFα-induced NF-κB activation, TNFα, like BK, stimulated increased PI 3-kinase activity (Fig. 4, lane 6). Wortmannin and LY294002 each abolished the PI 3-kinase activity stimulated by either BK (Fig. 4, lanes 1 and 4) or TNFα (Fig. 4, lanes 2 and 5). Thus BK stimulates PI 3-kinase activity, and this activity is required for subsequent NF-κB activation.
PI 3-Kinase Is Required for BK-induced NF-kB Activation

FIG. 3. BK stimulates PI 3-kinase in a time-dependent manner. A549 cells were treated with BK (20 nM) for the indicated times. The whole cell lysates were subjected to immunoprecipitation with a rabbit polyclonal antibody against the p85α subunit of PI 3-kinase. PI 3-kinase activity in the immunoprecipitated fraction was determined using an in vitro kinase assay as described under “Experimental Procedures.” The products of the kinase assay were separated by thin-layer chromatography as described under “Experimental Procedures.” Activity of PI 3-kinase is presented as production of phosphatidylinositol phosphate (PIP), indicated by an arrow. Experiments were repeated twice with essentially identical results.

FIG. 4. Wortmannin and LY294002 inhibit BK- and TNF-α-stimulated PI 3-kinase activity. A549 cells were preincubated with 100 nM wortmannin (WM, lanes 1 and 2), media alone (lanes 3 and 6), or LY294002 (LY, lanes 4 and 5) for 15 min, then stimulated (Stim.) with 20 nM BK (lanes 1, 3, and 4), or 40 ng/ml TNF (lanes 2, 5, and 6) for 40 min. Inhib., inhibitor. Activity of PI 3-kinase, measured as described for Fig. 3, is presented as production of phosphatidylinositol phosphate (PIP), indicated by an arrow.

bran G protein-coupled receptor superfamily (14). We also showed that the B2 BK receptor is coupled to a Gi protein in WI38 cells, based on the sensitivity of BK-induced NF-κB activation and interleukin-1β gene expression to pertussis toxin treatment (1). The identity of the heterotrimeric G proteins coupling the B2 BK receptor to PI 3-kinase activity and NF-κB activation in A549 epithelial cells has not been elucidated. We therefore examined the effect of pertussis toxin and cholera toxin on BK-induced NF-κB activation and PI 3-kinase. A549 cells were pre-treated with pertussis and cholera toxins separately, then stimulated with BK. Pertussis toxin (0.5 μg/ml) markedly reduced BK-stimulated NF-κB activation (Fig. 5A, lane 5), whereas cholera toxin had no such inhibitory effect in A549 cells (Fig. 5A, lane 2). Neither of the toxins inhibited TNF-α-induced NF-κB activation in the same cells (Fig. 5A, lanes 3 and 6). BK-induced PI 3-kinase activity was also blocked by pertussis toxin (Fig. 5B, lane 5) but not cholera toxin (Fig. 5B, lane 2), whereas neither pertussis nor cholera toxin inhibited TNF-α-induced PI 3-kinase activity (Fig. 5B, lanes 3 and 6). Thus, our results indicate that BK stimulates both PI 3-kinase and NF-κB activation through B2 BK receptors that are coupled to pertussis toxin-sensitive heterotrimeric G proteins.

PI 3-Kinase Activity Is Required for BK-induced NF-κB Activation—Further demonstration of the necessity for PI 3-kinase activity in BK-induced NF-κB activation was obtained by overexpressing a dominant negative PI 3-kinase (p85αN-CA478–514) in A549 cells. The deletion of codons 478–514 from the regulatory p85 component of PI 3-kinase has been shown to confer PI 3-kinase dominant negative activity (9). A549 cells were co-transfected with p85αN-CA478–514 together with an IκB promoter-CAT reporter construct. Overexpression of the dominant negative PI 3-kinase mutant protein abolished BK-induced IκB-mediated CAT activity but had no effect on TNF-α-mediated CAT activity (Fig. 6, A and B). To confirm that overexpression of p85αN-CA478–514 mutant protein inhibited PI 3-kinase activity in A549 cells, transfected cells were recovered using the plasmid phOx-2, which encodes a single-stranded cell surface antibody that can be bound to magnetic beads coated with antigen (phOx). Co-transfection of A549 cells with the p85αN-CA478–514 plasmid inhibited BK-stimulated PI 3-kinase 83% compared with A549 cells transfected with phOx-2 plasmid alone (Fig. 6C).

DISCUSSION

BK has recently been shown to stimulate activation of the transcription factor NF-κB (1), an effect that may be an important contributor to the inflammatory actions of BK (15). The cellular signaling pathways required for BK-induced NF-κB activation, however, are not completely understood. We recently demonstrated that nanomolar concentrations of BK stimulated NF-κB activation in A549 epithelial cells and that the small G protein RhoA was necessary to mediate this effect (2). The present study provides evidence that the lipid products of PI 3-kinase are an important part of the signaling pathway leading to activation of NF-κB by BK. Our results demonstrate, for the first time, that BK stimulates increased PI 3-kinase
PI 3-Kinase Is Required for BK-induced NF-κB Activation

**Fig. 6. PI 3-kinase is necessary for BK-stimulated NF-κB activation.** A, A549 cells were co-transfected with 2.5 μg of the WT-1xB-CAT plasmid (lanes 1–6), 0.5 μg of pCMVβ (lanes 1–6), and 2.0 μg of either p85α-N-CA478–514 (lanes 1–3) or empty vector (lanes 4–6). After a 48-h incubation in normal culture media, the transfected cells were stimulated with media alone (lanes 1 and 4), 20 nM BK (lanes 2 and 5), or 40 ng/ml TNFα (lanes 3 and 6) for 1 h and then harvested. CAT activity was measured in the crude cell lysates using [14C]chloramphenicol as a substrate, separated by thin-layer chromatography as described under “Experimental Procedures.” All results were normalized for transfection efficiency using the expression of β-galactosidase. A PhosphorImager screen was exposed, and the autoradiograph of the separated native and acetylated [14C]chloramphenicol is shown. B, relative CAT activity of the samples shown in panel A is expressed as the percentage of acetylated [14C]chloramphenicol in each lane. These results are representative of two separate experiments. C, A549 cells were co-transfected with 1 μg of the pHook-2 plasmid (lanes 1–3) and 1.5 μg of either empty vector (lanes 1–2) or p85α-N-CA478–514 (lane 3). After a 60-h incubation in normal culture media, the transfected cells were isolated using the Capture-Tec pHook-2 system, then stimulated (Stim.) with media alone (Med, lane 1) or 100 nM BK (lanes 2–3) for 5 min and harvested. Activity of PI 3-kinase, measured as described for Fig. 3, is presented as production of phosphatidylinositol phosphate (PIP), indicated by an arrow.

Activity in A549 epithelial cells and further that PI 3-kinase activity is essential for BK-induced activation of NF-κB. Compared with BK-induced NF-κB activation, BK-stimulated PI 3-kinase activity occurred earlier (5 versus 15 min after adding BK) and peaked earlier (20 versus 60 min after adding BK). To test the possibility that PI 3-kinase is a component of the BK-stimulated signaling pathway leading to NF-κB activation, we assessed the effect of inhibiting PI 3-kinase activity on subsequent BK-induced NF-κB activation. Wortmannin and LY294002 have been shown to be specific PI 3-kinase inhibitors. Wortmannin irreversibly inactivates PI 3-kinase by binding to its p110 catalytic subunit (13); LY294002 is a competitive inhibitor, binding to the ATP-binding site of the PI 3-kinase (16). Pre-incubation of A549 cells with either wortmannin or LY294002 completely abrogated BK-induced NF-κB activation. Additional proof that PI 3-kinase was required for BK-induced NF-κB activation was provided by the ability of a dominant negative mutant form of the p85 subunit of PI 3-kinase to block BK-induced NF-κB activation.

The role of PI 3-kinase in NF-κB activation has been frequently addressed in several other reports with variable results. Like BK, interleukin-1-mediated activation of NF-κB and activator protein-1 (AP-1) was shown to require PI 3-kinase in human hepatoma (HepG2) and epidermoid carcinoma (KB) cell lines (17). In this system, overexpression of the p110 catalytic subunit of PI 3-kinase was necessary but not sufficient to activate NF-κB, however it was both necessary and sufficient to activate AP-1. Interestingly, we found that TNF-α-induced NF-κB activation was unaffected by pre-incubation with PI 3-kinase inhibitors or expression of the dominant negative p85 PI 3-kinase mutant. Wortmannin also failed to inhibit activation of NF-κB in human T-cell blasts following CD28 receptor ligation, although it did inhibit AP-1 activation (18).

A number of distinct forms of PI 3-kinase have been described in mammalian cells. The classic PI 3-kinase is linked to receptors with intrinsic or associated tyrosine kinase activity and is a heterodimer consisting of a p110 catalytic subunit and a p85 regulatory subunit containing one SH3 and two SH2 domains. At least 3 isoforms of the p110 and p85 subunits have been described in mammalian cells. The p110/p85 form of PI 3-kinase 3-phosphorylates PtdIns, PtdIns(4)P, and PtdIns(4,5)-P2 in vitro, although its primary product in vivo appears to be PtdIns(3,4,5)P3 (19). A p110 PI 3-kinase that does not bind to p85 has also been described (5) as well as a p170 form of PI 3-kinase that preferentially 3-phosphorylates PtdIns and to a lesser extent PtdIns(4)P and is relatively insensitive to wortmannin (20). A mammalian counterpart to the yeast Vps34p kinase that only phosphorylates PtdIns has been found and is associated with a 150-kDa protein but not p85 (19, 21). Our results indicate that the PI 3-kinase involved in BK-induced NF-κB activation is the p110/p85 heterodimer. This conclusion is based on: 1) its sensitivity to low concentrations of wortmannin, 2) the ability of a dominant negative p85 mutant to inhibit the response, and 3) the capacity of an anti-p85 antibody to immunoprecipitate BK-induced PI 3-kinase.

BK is an agonist for the B2 BK receptor, and we have previously shown that B2 BK receptor antagonists completely block BK-induced NF-κB activation. The B2 BK receptor is a member of the heptahedral superfamily of receptors that are coupled to heterotrimeric G proteins. To assess the type of heterotrimeric G protein coupling of the B2 BK receptor to PI 3-kinase and NF-κB, we analyzed the effects of pertussis and cholera toxins. Pertussis toxin ADP ribosylates Goi and Gai proteins, whereas cholera toxin ADP ribosylates Gai proteins (22). BK-stimulated PI 3-kinase activity and NF-κB activation were both inhibited by pertussis toxin but not by cholera toxin, indicating that both responses are transduced through the Goi or Gai class of heterotrimeric G proteins.

Previous studies suggested that G protein-coupled receptors may activate PI 3-kinase through the p110 γ, which is independent of p85 independent (5, 23). Other studies, however, provided indirect evidence that some G protein-coupled receptors activate PI 3-kinase through the p85/p110 heterodimer (24, 25). By transfecting the A549 cells with a p85 dominant negative mutant, we showed that the p85/p110 form of PI 3-kinase was activated and required for NF-κB activation following stimulation with BK, a ligand that acts through a G protein-coupled receptor.

The mechanisms linking PI 3-kinase activation to NF-κB activation are unknown. D3-phosphorylated phosphatidylinositol bisphosphates are known to play important roles in cell growth and survival, although the exact role and immediate downstream molecular targets of PtdIns(3)P, PtdIns(3,4)P2, and PtdIns(3,4,5)P3 are only now emerging (26, 27). PtdIns(3,4,5)P3 has been shown to activate several of the Ca2⁺-independent iso-
types of protein kinase C (PKC) (28) as well as the atypical ζ isotype (PKCζ) (29). Interestingly, PKCζ has been reported to be important in NF-κB activation (30), an effect that was dependent on p85/p110 PI 3-kinase and protein phosphatase 2A activity (31). PI 3-kinase was also shown to be required for NF-κB activation induced by interleukin-1 in transformed human B cells (32).

Based on our current results and our previous report that RhoA is required for BK-induced NF-κB activation (2), the relationship between PI 3-kinase activation and activation of the Rho family of small GTPases appears to be an important issue. Several studies have demonstrated that PI 3-kinase may be activated downstream of the small Rho GTPases (33–35). GTP-loaded Rac (but not RhoA) was shown to directly bind PI 3-kinase (36). Furthermore, inactivation of Rho using bacterial GTP-loaded Rac (but not RhoA) was shown to directly bind PI 3-kinase in Swiss 3T3 cells (37). Other studies, however, have suggested that PI 3-kinase may activate the small Rho GTPases. Expression of a constitutively active PI 3-kinase mutant in Swiss 3T3 cells induced a subset of Rac and Rho-mediated cellular responses (38). The PI 3-kinase product PtdIns(3,4,5)P3 has been shown to bind the pleckstrin homology domain of guanine nucleotide exchange factors, providing a potential mechanism for PI 3-kinase-mediated regulation of Rho activation (38, 39). Additionally, the p85 regulatory subunit of PI 3-kinase contains a breakpoint cluster region homology domain (BH) that has been shown to have GTPase activating protein activity (40).

In summary, we have shown that BK rapidly activates the p85/p110 heterodimeric PI 3-kinase in A549 cells. BK-stimulated PI 3-kinase activity and NF-κB activation were both inhibited by pertussis toxin but not cholera toxin, suggesting that the B2 BK receptor mediating both responses is coupled to the Gi/Go class of G proteins. Utilizing both specific inhibitors as well as transient expression of a dominant negative p85 PI 3-kinase mutant, we further showed that BK-induced NF-κB activation required PI 3-kinase activity. Although TNFα also stimulated PI 3-kinase activity, TNFα-stimulated NF-κB activation was not affected by inhibition of PI 3-kinase activity. These findings provide evidence that BK-induced NF-κB activation utilizes a signaling pathway that requires activity of both RhoA and PI 3-kinase and is distinct from the signaling pathway utilized by TNFα. The relationship between BK-mediated activation of PI 3-kinase and RhoA, however, remains to be determined.

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