Bradykinin Stimulates NF-κB Activation and Interleukin 1β Gene Expression in Cultured Human Fibroblasts

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Abstract

Bradykinin (BK), a pluripotent nonameric peptide, is known for its proinflammatory functions in both tissue injury and allergic inflammation of the airway mucosa and submucosa. To understand the mechanisms by which BK serves as an inflammatory mediator, the human lung fibroblast cell line WI-38 was stimulated with BK and the expression of IL-1β gene was examined. BK at nanomolar concentrations induced a marked increase in immunoreactive IL-1β, detectable within 2 h in both secreted and cell-associated forms. BK-induced IL-1β synthesis was inhibited by a B2-type BK receptor antagonist and by treatment of the cells with pertussis toxin, indicating the involvement of a BK receptor that couples to the G_i/G_o class of heterotrimeric G proteins. Whereas cycloheximide and actinomycin D both inhibited BK-induced IL-1β synthesis, results from Northern blot and nuclear run-on assays suggested that BK acted primarily at the transcription level which led to the accumulation of IL-1β message in stimulated cells. Gel mobility shift assays were used with nuclear extracts from stimulated WI-38 cells to examine the transcription mechanism for BK-induced IL-1β expression. A DNA binding activity specific for the decameric κB enhancer was detected and was found to contain the p50 and p65 subunits of the NF-κB/rel protein family. BK-induced NF-κB activation correlated with IL-1β message upregulation with respect to agonist concentration, time course, sensitivity to bacterial toxins, and blockade by the B2 receptor antagonist. After BK stimulation, a significant increase in the activity of chloramphenicol acetyltransferase was observed in WI-38 cells transfected with a reporter plasmid bearing the κB enhancers from the IL-1β gene. Deletion of the κB enhancer sequence significantly reduced BK-induced chloramphenicol acetyltransferase activity. These findings suggests a novel function of BK in the activation of NF-κB and the induction of cytokine gene expression. (*J. Clin. Invest.* 1996. 98:2042–2049.) Key words: bradykinin • inflammation • gene expression • NF-κB • G proteins

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Introduction

Tissue damage and inflammatory stimuli can trigger the activation of plasma and tissue kallikreins, resulting in the generation of biologically active kinins by limited proteolysis of kininogens (for reviews see references 1 and 2). Bradykinin (BK)[1] is a major kinin of nine amino acids with well documented pharmacological properties. A potent paracrine mediator, BK is involved in physiological and pathological processes including smooth muscle contraction, vasodilation, increased vascular permeability, and induction of pain (for reviews see references 2 and 3). BK serves a sentinel function as an early and ubiquitous mediator of inflammation after injury (4). It stimulates eicosanoid synthesis in several types of cells including fibroblasts and endothelial cells (5, 6). BK is also involved in allergic inflammation of the airway mucosa and submucosa. Allergen challenge in asthmatic volunteers has been shown to lead to the generation of kinins in the airway (7), whereas administration of a B2 receptor antagonist has been shown to significantly abrogate the late-phase inflammatory response to allergen inhalation in an allergic model (8, 9). These findings suggest a pivotal role of BK in several inflammatory conditions.

Two mammalian BK receptor subtypes, B1 and B2, have been pharmacologically characterized. The B1 receptor is not expressed at significant levels in normal tissues, but its synthesis can be induced after tissue injury and by inflammatory factors such as lipopolysaccharide (10). Recent studies indicate that the B1 receptor mediates hyperalgesia in animals with induced chronic inflammation (11). The B2 receptor is constitutively expressed in many types of cells including smooth muscle cells, certain neurons, fibroblasts, and epithelial cells of the lung. Activation of the B2 receptor can cause pronounced hypotension, bronchoconstriction, and inflammatory pain (2, 11). BK is a potent agonist for the B2 receptor, but it interacts with the B1 receptor only with low affinity. Recent cloning of the cDNAs for the B1 and B2 BK receptors (12–15) reveals that these receptors belong to the seven membrane–span G protein–coupled receptor family, which currently has hundreds of members that mediate a multitude of physiological functions. Several studies have demonstrated that the BK receptors can couple to at least G_i, G_q/G_{11}, and G_{13} proteins depending on the types of cells and effector systems present in the cells (16, 17). Furthermore, it has been shown that BK activates protein kinase C (18) and stimulates tyrosine phosphorylation (19) in

the Swiss 3T3 fibroblasts. However, although much is known about the physiological effects of BK, very little is understood concerning the mechanisms by which BK serves its proinflammatory functions. To address this issue, we investigated the effect of BK on the expression of proinflammatory cytokines. We report here that in the human WI-38 fibroblast cells, BK stimulates the activity of nuclear factor κB (NF-κB) and the expression of IL-1β. This novel function of BK involves the coupling of the B2 receptor to a pertussis toxin–sensitive signaling pathway and may contribute to the expression of other proinflammatory cytokines at the sites of tissue injury and inflammation.

Methods

Reagents. BK and the BK antagonists [Des-Arg⁹,Leu⁸]-BK and [D-Arg⁰,Hyp³,Thi⁵,⁸,D-Phe⁷]-BK were obtained from Peninsula Laboratories (Belmont, CA). Cholera and pertussis toxins were purchased from List Laboratory (Campbell, CA). The human lung fibroblast cell line WI-38 was from American Type Culture Collection (Rockville, MD). WI-38 cells were maintained in DMEM supplemented with 5% fetal bovine serum, 5% newborn bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Oligonucleotides for electrophoretic mobility shift assay (EMSA) were purchased from Promega (Madison, WI) and contain the murine intronic κ chain κB site (NF-κB site, underlined), 5′AGTTGAGGGGACTTTCCCAGGC-3′ (20). Actinomycin, cycloheximide (CHX), phorbol myristate acetate, and pyrrolidine dithiocarbamate (PDTC) were obtained from Sigma Chemical Co. (St. Louis, MO).

Detection of immunoreactive IL-1β. WI-38 cells in 6-well plates were stimulated with BK at concentrations ranging from 0.1 to 1,000 nM, or at 10 nM for various times up to 8 h. The conditioned media were collected and secreted IL-1β was measured by ELISA using a commercially available kit (Genzyme Corp., Cambridge, MA) with manufacturer recommended protocol. Quantitation of secreted IL-1β was accomplished by normalization of the ELISA data with a standard IL-1β dose curve.

Northern blot analysis. WI-38 cells (1×10^7) were stimulated with BK and other agonists as indicated in figures, and total RNA was prepared using the guanidinium/acidic phenol method (21). 20 μg of the total RNA from each sample was separated on a 1.1% agarose gel containing formaldehyde, stained with ethidium bromide, and transferred to Hybond-plus nylon membrane (Amersham, Arlington Heights, IL). The blot was hybridized with probes derived from the coding sequence of the human IL-1β cDNA, which was randomly labeled with α-[³²P]dATP to a specific activity of 2–5×10^8 cpm/μg DNA. Hybridization was carried out at 62°C for 2 h in a rapid hybridization buffer (Amersham). The blot was then washed at 62°C with 1× SSC, 0.1% SDS until background was sufficiently low, before it was exposed to a PhosphorImage screen. For standardization, the same blot was probed again with a 0.45-kb DNA fragment of the glyceraldehyde 3-phosphate dehydrogenase (G3PDH) housekeeping gene. In some experiments, the cells were treated for 1 h with the transcription inhibitor actinomycin D (ActD, 2.5 μg/ml) or the protein synthesis inhibitor CHX (5 μg/ml) before stimulation as indicated in the text and legends for figures.

Nuclear run-on experiment. WI-38 cells were treated with or without BK (10 nM) for 1 h. The cells were harvested by scraping and resuspended in 0.5 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 3 mM MgCl₂, 10 mM NaCl, 0.5% Nonidet P-40) and incubated for 5 min on ice. The samples were then centrifuged at 400 *g* at 4°C and the nuclei-containing pellet was resuspended in 0.25 ml of ice-cold freezing buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM MgCl₂, 0.1 mM EDTA, pH 8.0). To 230 μl of the nuclei suspension were added 60 μl of 5× nuclear run-on buffer (25 mM Tris-HCl, pH 8.0, 12.5 mM MgCl₂, 750 mM KCl, 1.25 mM each of dGTP, dCTP, and dATP) and

100 μCi of α-[³²P]UTP (3,000 Ci/mmol; Amersham). The samples were incubated at 30°C for 30 min and elongated transcripts were isolated using the guanidinium/acidic phenol method as above, with 50 μg yeast tRNA added to each sample as a carrier. The precipitated RNA was dissolved in 180 μl of ice-cold TNE (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA) and denatured with 20 μl of 2 N NaOH on ice for 10 min. The solution was neutralized by the addition of Hepes, pH 7.2 (0.48 mM final concentration), precipitated with 880 μl of ethanol, and the RNA pellet was resuspended in 100 μl of hybridization solution (10 mM TES, 2% SDS, 10 mM EDTA, 300 mM NaCl). DNA samples (0.2 μg each) were denatured with 0.3 mM NaOH at 65°C for 60 min, neutralized with equal amount of 2 M ammonium acetate, and transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH) with a slot blot device (GIBCO-BRL, Gaithersburg, MD). The immobilized DNA on membrane was prehybridized in the above solution for 24 h at 65°C. Hybridization was initiated by addition of radiolabeled RNA probe in a volume of 2 ml/membrane and was maintained at 65°C for 72 h. The membrane was then washed in 0.2× SSC at 65°C with several changes of the buffer solution. The dried membrane was exposed to a PhosphorImager screen.

Nuclear extract preparation. Nuclear extracts were prepared by a modified method of Dignam et al. (22). WI-38 cells in T25 flasks were stimulated with BK or other ligands as indicated in the figures. After stimulation, the cells were washed three times with PBS, harvested, and resuspended in 0.4 ml of buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EDTA, 1 mM DDT, 0.5 mM PMSF). Nonidet P-40 (23 μl of 10% solution) was added and the mixture was incubated on ice for 10 min. Nuclei were separated from cytosol by centrifugation at 13,000 *g* for 10 s and were resuspended in 50 μl buffer B (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EDTA, 0.1 mM PMSF). After 30 min of incubation on ice, lysates were separated by centrifugation (13,000 *g*, 30 s) and supernatant containing nuclear proteins were transferred to new vials. The protein concentration of extracts was measured using a protein dye reagent (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as standards and samples were diluted to equal concentration in buffer B for use directly or storage at −80°C.

EMSA. Double-stranded oligonucleotide (5 pmol) was labeled with T4 polynucleotide kinase. EMSA was performed by incubating nuclear proteins (2.5 μg) in 12 μl of binding buffer (5 mM Hepes, pH 7.8, 5 mM MgCl₂, 50 mM KCl, 0.5 mM dithiothreitol, 0.4 mg/ml poly(dI-dC) (Pharmacia Biotech Inc., Piscataway, NJ), 0.1 mg/ml sonicated double-stranded salmon sperm DNA, and 10% glycerol) for 10 min at room temperature. Then, 25–50 fmol of ³²P-labeled oligonucleotide probe (30,000–50,000 cpm) was added and the reaction mixture was incubated for 10 min at room temperature. For competition assays, unlabeled oligonucleotides were added to various concentrations with the radiolabeled probe. For supershift experiments, antibodies (0.4 μg each) against p50, p65, and c-rel (Santa Cruz Biotechnologies, Santa Cruz, CA) were added to the samples and incubated for 10 min. Labeled oligonucleotide probe was then added and incubation continued for another 10 min. All samples were analyzed on 6% acrylamide gels, which were made in 50 mM Tris-borate buffer containing 1 mM EDTA (TBE) or 50 mM Tris/380 mM glycine/2 mM EDTA (TGE) and were preelectrophoresed for 2 h at 12 V/cm. Electrophoresis was carried out at the same voltage for 2–2.5 h. Gel contents were transferred onto Whatman DE-81 papers, dried, and exposed to a PhosphorImager screen.

Chloramphenicol acetyltransferase (CAT) assay. The plasmids p0.2-kb(WT)CAT and p0.2kb(Mu)CAT, which contain the wild-type and mutant κB enhancers from the IκB gene, respectively, were obtained from P. Chiao (23). The plasmid pIL-1(-4000)CAT contains the three κB-like-enhancers in a 4-kb fragment from the IL-1β gene. These enhancers were deleted in the plasmid pIL-1(-133)CAT. Both constructs were kindly provided by J.P. Cogswell and were described previously (24). pTri-SVo-CAT was the parent vector for the IL-1β constructs, and pBLCAT2 was the parent vector for the IκB con-
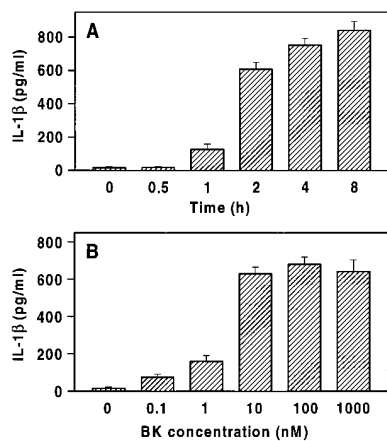
structs. The plasmid pCMVβ (Clontech, Palo Alto, CA) was used as a control for monitoring the transfection efficiency by the expression of β-galactosidase. 10 μg of each plasmid DNA was used for transient transfection of WI-38 cells with the cationic lipid DOTAP (Boehringer Mannheim, Indianapolis, IN), using procedures recommended by the manufacturer. After 6 h, the DNA-containing medium was removed, and the cells were washed with PBS and incubated with normal medium for 16 h. The cells were then stimulated with agonists for 2 h and harvested by scraping. Crude cell extracts were prepared for the measurement of CAT activity with the use of [^{14}C]chloramphenicol (Amersham) as substrate and thin layer chromatography for the separation of the native from the acetylated forms as described (25). After development, the extent of CAT activity was measured using the ImageQuant software.

Data analysis. Quantitation of the Northern blot data was accomplished with a PhosphorImager, using the ImageQuant software (version 3.3; Molecular Dynamics, Mountain View, CA). Results are expressed as mean±SEM.

Results

BK induces the production of immunoreactive IL-1β. IL-1β, a prototypic proinflammatory cytokine, induces the expression of a variety of genes, the products of which are involved in acute and chronic inflammatory conditions (26). Unstimulated WI-38 fibroblast cells produced little IL-1β. Addition of BK (10 nM) resulted in a time-dependent production of IL-1β as measured by ELISA with a specific anti–IL-1β antibody (Fig. 1 *A*). A notable increase in secreted IL-1β was detected 1 h after BK stimulation and continued for at least 8 h. The time course was similar when BK was applied at 100 nM (data not shown). Immunoreactive IL-1β was also detected in cell extracts prepared from BK-stimulated cells, and CHX treatment (5 μg/ml) before BK stimulation abolished IL-1β synthesis (data not shown). These results suggest de novo protein synthesis in WI-38 cells in response to BK stimulation. BK was able to induce IL-1β production at subnanomolar ligand concentrations, consistent with the range of BK concentrations for most of its biological functions. The effect of BK reached its peak at 10 nM and higher concentrations of BK did not produce significant increases in IL-1β production (Fig. 1 *B*).

BK increases the steady state level of IL-1β mRNA. Concomitant with the synthesis of IL-1β protein, an accumulation of cytosolic mRNA for IL-1β was detected by Northern blot

analysis of RNA samples from stimulated cells (Fig. 2). The concentrations of BK required for the upregulation of the IL-1β mRNA were similar to those for IL-1β protein synthesis. Upregulation of the IL-1β mRNA level was detectable 30 min after BK stimulation, reached maximum in 2 h (Fig. 2), and subsided gradually within the next 6 h (data not shown). Unstimulated WI-38 cells had a very low background level of the IL-1β mRNA. PMA (100 nM), which is known to initiate IL-1β synthesis in other cell systems by activation of protein kinase C, also induced a sizable increase in the IL-1β mRNA in WI-38 cells.

To determine whether BK-induced upregulation of IL-1β mRNA was due to the activation of transcription, WI-38 cells were treated with the transcription inhibitor ActD (2.5 μg/ml) before BK stimulation. It was found that ActD reduced BK-induced IL-1β message by 97% and inhibited IL-1β protein synthesis to a similar extent. Interestingly, although CHX blocked IL-1β protein synthesis, it augmented the effect of BK on IL-1β mRNA level by an additional 1.7-fold compared with BK alone. CHX by itself also induced a twofold increase of the IL-1β mRNA compared with untreated cells. CHX associated superinduction is believed to result from enhanced NF-κB activity due to blocking of the synthesis of the inhibitory protein IκB (27). To further confirm the effect of BK on IL-1β gene transcription, nuclear run-on experiments were performed. WI-38 cells were incubated in the absence or presence of BK (10 nM) for 1 h and the nuclei were harvested. Elongation of the transcripts was conducted in the presence of α-[^{32}P]UTP,
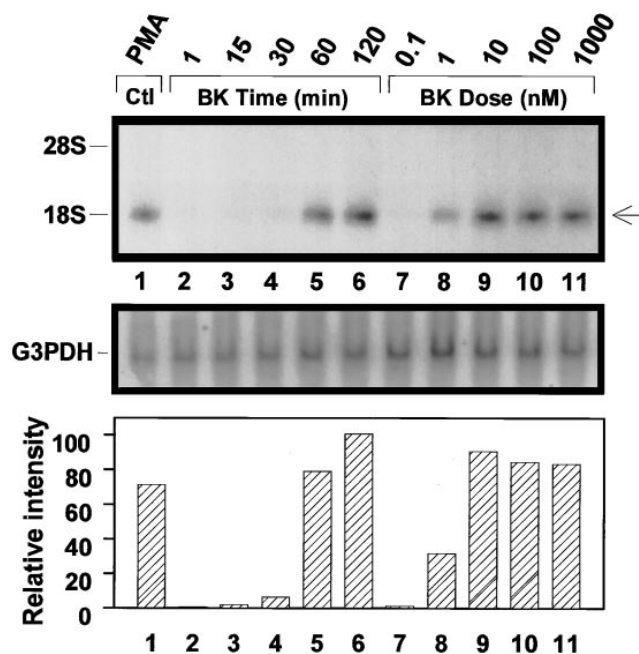
Figure 2. Effect of BK on the steady state level of IL-1β mRNA. WI-38 cells were stimulated with BK at different concentrations for 1 h, or at 10 nM for various times, and total RNA was prepared. Samples (20 μg each) were separated by gel electrophoresis, transferred to nylon membranes, and hybridized with a ^{32}P-labeled IL-1β cDNA probe as described in Methods. *Ctl*, cells treated with PMA (100 nM, 1 h). For normalization, the same blot was hybridized again with a ^{32}P-labeled housekeeping gene (G3PDH). The relative intensity of the IL-1β mRNA was determined as the ratio of IL-1β message over G3PDH message with the same sample and is shown in the bar chart.
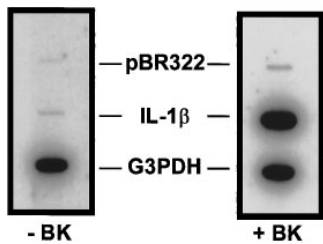
Figure 1. Induction of immunoreactive IL-1β in supernatant from BK-stimulated WI-38 cells. WI-38 cells were stimulated with BK (10 nM) for various times as indicated (*A*), or for 2 h with BK at different concentrations (*B*). The supernatants were collected and the secreted IL-1β was measured by ELISA as described in Methods. Results shown are mean±SEM from three separate measurements.

Figure 3. BK-stimulated transcription of IL-1β gene in WI-38 cells. The cells were incubated without (*left*) or with (*right*) BK at 10 nM for 1 h. Nuclei were prepared and incubated in the presence of α-[³²P]UTP before RNA isolation. Transcription assays were performed as described in Methods. Radiolabeled nuclear RNA was hybridized with the DNA samples of pBR322 plasmid, IL-1β, and G3PDH housekeeping gene that were immobilized on nitrocellulose membranes. The autoradiograph of one representative experiment is shown.

as described in Methods. Results of hybridization experiments shown in Fig. 3 indicated a nearly 50-fold increase in the level of IL-1β transcripts following BK stimulation. Taken together, these results clearly indicate that BK stimulates the transcription of the IL-1β gene in WI-38 cells.

*BK stimulates the activation of NF-κB.* The transcription activator NF-κB regulates the expression of a large number of proinflammatory immediate-early genes. Previous studies have indicated a role of NF-κB in regulating IL-1β gene expression (24). We investigated whether BK could induce the activation of NF-κB and thereby regulate the expression of IL-1β. Nuclear extracts were prepared from BK-stimulated WI-38 cells and incubated with radiolabeled DNA probe containing the κB sequence (GGGACTTTCC). EMSA demonstrated a dose- and time-dependent elevation of the DNA binding activity, which peaked at 40 min after stimulation with 10 nM BK (Fig. 4 *A*). The specificity and composition of BK-induced κB binding activity were next examined. In competition studies, unlabeled oligonucleotide (Fig. 4 *B*, lanes *1* and *2*) dose-dependently reduced the binding of the labeled oligonucleotides to base level (lane *3*). A nonfunctional mutant oligonucleotide (with a G→C switch at the second position in the decameric sequence) had no inhibitory effect on the binding of the la-

beled probe (compare lanes *4* and *5*, Fig. 4 *B*). Thus, BK-induced DNA binding activity is specific for the decameric κB sequence. In electrophoretic mobility supershift assays, antibodies against the p50 and p65 subunits of the NF-κB/rel proteins were able to reduce the mobility of the DNA–protein complexes in the respective samples, suggesting that the p65/p50 heterodimer was present in the complexes as expected with a canonical κB site (date not shown). No effect on gel mobility was observed with antibodies against the c-rel, relB, or p52 proteins (data not shown).

BK induces the expression of IL-1β–specific κB reporter gene. The above results suggested a correlation between BK-stimulated NF-κB activation in IL-1β gene expression. To further examine this possibility, WI-38 cells were transiently transfected with chimeric reporter plasmids before BK stimulation. In the first experiment, BK was found to stimulate the expression of the bacterial CAT gene in cells transfected with a reporter plasmid containing the κB enhancer from the IκB gene (WT-CAT; Fig. 5, *top*). Only a weak background CAT activity was detected in cells transfected with a plasmid containing the mutated κB enhancer (Mu-CAT; Fig. 5, *top*). Three CAT reporter constructs were used in the second experiment (Fig. 5, *bottom*), the wild-type pIL-1(-4000)-CAT, which contains three κB-like enhancers in a 4-kb DNA fragment from the IL-1β gene (WT-CAT); the deletion mutant pIL-1(-133)-CAT with all three κB-like enhancers removed (Mu-CAT); and the parent vector, pTri-SVo-CAT (24). Results from this experiment indicated that only the wild-type reporter construct was able to mediate the expression of CAT in response to BK stimulation. PMA, a known inducer of NF-κB activation and IL-1β expression, also stimulated CAT expression directed by the wild-type reporter constructs. These results suggest that BK-induced NF-κB activities contribute to the expression of IL-1β. Further supporting evidence for a causal relationship was obtained by the use of PDTC, an antioxidant inhibitor of NF-κB activation. PDTC at concentrations of 1 and 10 μM inhibited BK-stimulated NF-κB activation (Fig. 6 *A*) and correspondingly reduced the level of IL-1β message
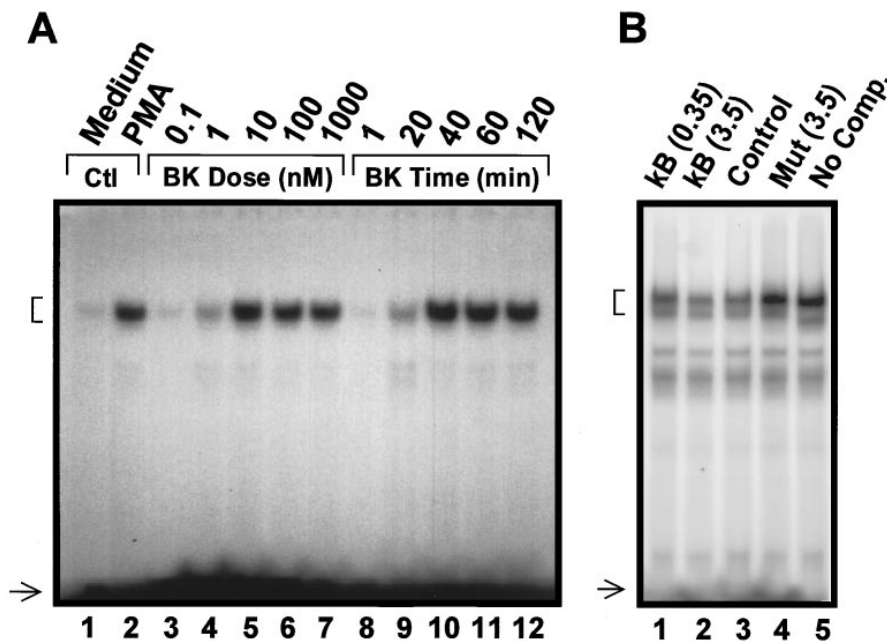
Figure 4. Induction of NF-κB binding activity by BK. (*A*) WI-38 cells were stimulated with PMA (positive control; 100 nM) and BK at various concentrations for 1 h, or at 10 nM for various time periods. Nuclear extracts were prepared as described in Methods and incubated with a ³²P-labeled nucleotide containing the consensus κB site (GGGACTTTCC). The EMSA autoradiograph is shown. The DNA–protein complex is marked with a bracket, and the unbound probe is indicated by an arrow. (*B*) Competition with unlabeled oligonucleotide probes. Nuclear extracts prepared from BK-stimulated (10 nM, 1 h) WI-38 cells were incubated with the radiolabeled κB probe in the absence (lane *5*) or presence of identical but unlabeled oligonucleotide (lanes *1* and *2*, with concentration indicated in picomoles), or a mutated nonfunctional oligonucleotide (lane *4*). Nuclear extracts from unstimulated cells were used as control (lane *3*). The samples were subsequently analyzed by EMSA and the autoradiograph is shown.
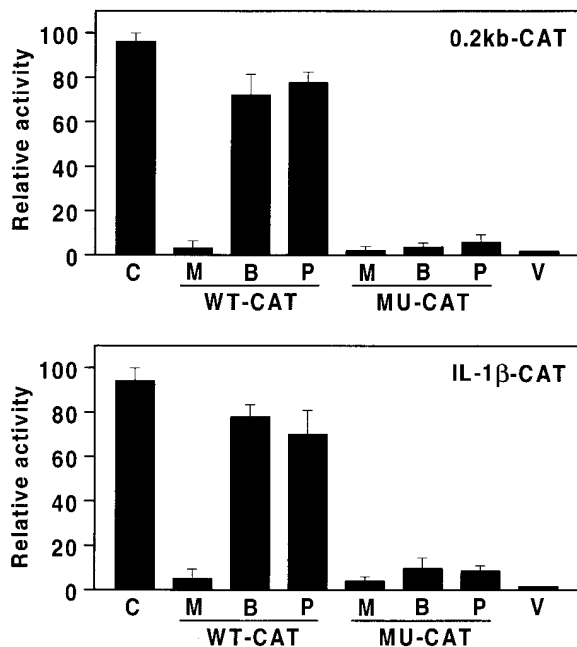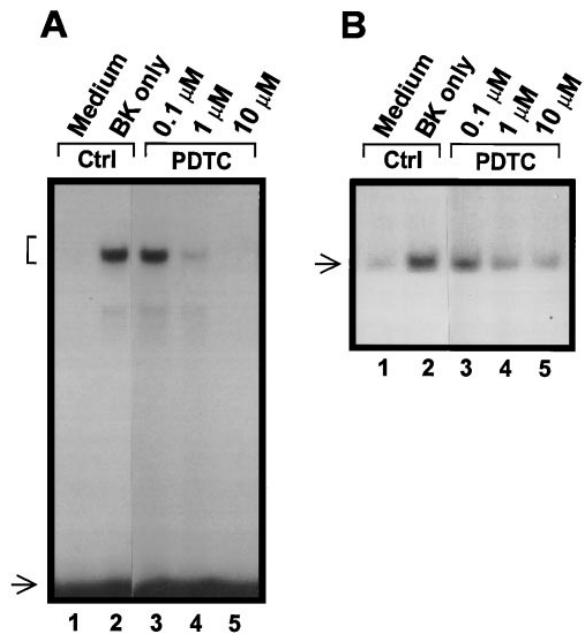
Figure 5. NF-κB regulation of reporter gene expression in BK-stimulated cells. (*Top*) WI-38 cells were transfected with the p0.2kb(WT)CAT (*WT-CAT*) or the p0.2kb(Mu)CAT (*MU-CAT*) plasmids, which contain the wild-type and nonfunctional mutant κB sites in a 0.2-kb DNA fragment from the IκBα promoter, respectively. (*Bottom*) The cells were transfected with pIL-1(-4000)CAT, containing the three κB sites from the IL-1β promoter (WT-CAT), or with pIL-1(-133)CAT, a deletion mutant construct of the above (MU-CAT). *C*, cells transfected with a constitutively active reporter construct; *V*, cells transfected with the parent vectors. After overnight incubation, the WT-CAT and MU-CAT transfected cells were incubated for 2 h with the following: *M*, medium only; *B*, BK (10 nM); *P*, PMA (100 nM). CAT activity was measured from crude cell extracts and the results are shown as means±SD (*n* = 3) of relative CAT activities as compared with the positive controls (100%).

Figure 6. Inhibition of BK-induced NF-κB activation and IL-1β message by PDTC. WI-38 cells were preincubated for 1 h with PDTC at three different concentrations before BK stimulation (10 nM, 1 h). Nuclear extracts (*A*) and total RNA (*B*) were then prepared and analyzed by EMSA and Northern blotting, respectively. *A* is an autoradiograph of EMSA, with the bracket indicating the DNA–protein complex and the arrow marking the unbound radiolabeled probe. *B* is an autoradiograph of the Northern blot after hybridization with a ^{32}P-labeled IL-1β probe. The arrow indicates the IL-1β message.

(Fig. 6 *B*). These results suggest that BK-stimulated NF-κB activation may involve the production of reactive oxygen intermediate as observed with other NF-κB activators (28).

BK-induced IL-1β gene expression is primarily mediated by the B2 receptor coupling to a pertussis toxin–sensitive G protein. WI-38 cells have been shown to express both the B1 and the B2 types of BK receptors (29, 30). BK interacts with the B2-type receptor with a high affinity and binds the B1-type receptor with a lower affinity (1, 3). Consistent with the difference in binding affinities, a reduction of 94% on BK-induced IL-1β message RNA was observed when the cells were treated with the B2 antagonist [D-Arg^0,Hyp^3, Thi^{5,8},D-Phe^7]-BK (Fig. 7). The B1 antagonist [Des-Arg^9,Leu^8]-BK produced a much smaller inhibitory effect (23%) under the same experimental conditions. Neither of the two antagonists had a significant effect on IL-1β mRNA level in PMA-stimulated cells, indicating that the inhibition was specific to the BK receptors. In parallel experiments, the B2 antagonist nearly completely blocked BK-induced NF-κB activation (data not shown) and reduced IL-1β secretion by 87±3.4% compared with a 11±2.6% reduction by the B1 antagonist (*n* = 3). Thus, the B2 receptor serves as a primary mediator for BK-induced IL-1β expression.

To determine which type of G_α protein is involved in BK-

induced IL-1β gene expression, we treated the WI-38 cells with either pertussis toxin (PTX, 100 nM) or cholera toxin (CTX, 1 μM) for 4 h before stimulation with BK. Northern blot analysis indicated that treatment with PTX, which ADP-ribosylates G_i and G_o proteins, reduced BK-induced IL-1β gene expression by > 80%. In contrast, treatment with CTX,
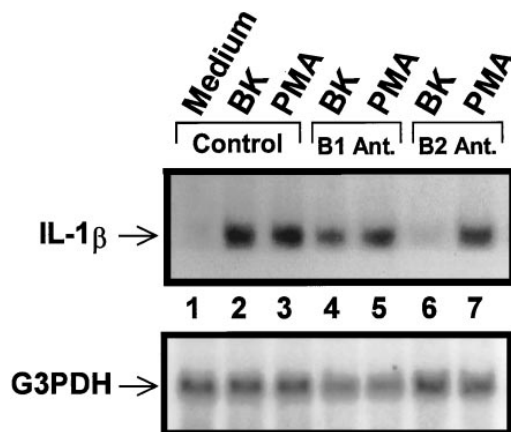
Figure 7. Effect of kinin receptor antagonists on BK-induced upregulation of IL-1β message. The WI-38 cells were stimulated with either BK (10 nM) or PMA (100 nM) for 1 h, in the absence (lanes *2* and *3*) or presence of the B1 receptor antagonist [Des-Arg^9,Leu^8]-BK (200 nM; lanes *4* and *5*) or the B2 receptor antagonist [D-Arg^0,Hyp^3,Thi^{5,8},-D-Phe^7]-BK (200 nM; lanes *6* and *7*). Autoradiographs of Northern blots probed with the IL-1β or G3PDH cDNA probes are shown.

A

B
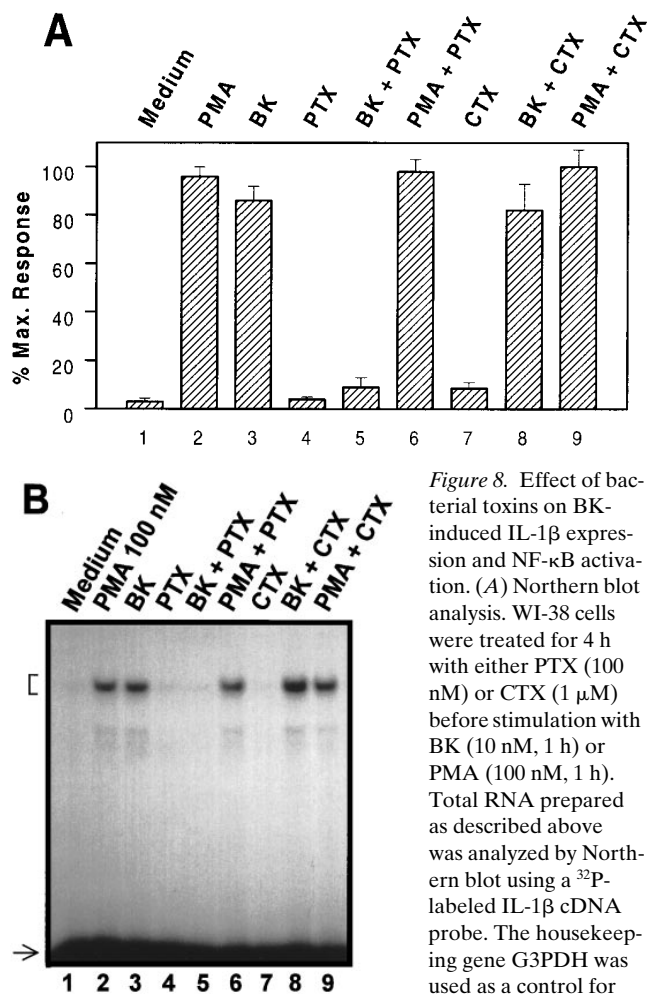
Figure 8. Effect of bacterial toxins on BK-induced IL-1β expression and NF-κB activation. (*A*) Northern blot analysis. WI-38 cells were treated for 4 h with either PTX (100 nM) or CTX (1 μM) before stimulation with BK (10 nM, 1 h) or PMA (100 nM, 1 h). Total RNA prepared as described above was analyzed by Northern blot using a ^{32}P-labeled IL-1β cDNA probe. The housekeeping gene G3PDH was used as a control for each sample. The levels of IL-1β message, determined as described in the legend to Fig. 2, are shown as means±SD based on data from two separate experiments. Maximal IL-1β message level (100%) was that induced by PMA (lane *2*). (*B*) EMSA showing κB binding activity. The cells were treated with PTX and CTX as described above. Nuclear extracts from the treated cells were incubated with a radiolabeled κB probe as described in Methods. Autoradiograph of one representative EMSA experiment is shown.

which potentiates the activation of adenylyl cyclase by ADP-ribosylation of G_s, had a much smaller inhibitory effect (Fig. 8 *A*). As with BK-induced IL-1β gene expression, BK-stimulated NF-κB activation was also sensitive to PTX treatment (Fig. 8 *B*). CTX had no inhibitory effect on this function of BK, but appeared to potentiate the effect of BK on NF-κB activation. Neither of the toxins inhibited PMA-induced NF-κB activation. We conclude that a G_i/G_o protein-mediated signaling pathway is involved in BK stimulation of NF-κB activation and IL-1β synthesis. The potential involvement of adenylyl cyclase in these cellular responses remains to be investigated.

Discussion

After inflammation or injury, BK is rapidly generated through a series of proteolytic reactions involving cleavage of kininogen by plasma or tissue kallikreins. The released autacoid, BK, is known to mediate multiple proinflammatory effects including smooth muscle contraction, pain, vasodilatation, increased vascular permeability, eicosanoid synthesis, and neuropeptide release. However, little is known about the role of BK in regulating cytokine gene expression. A recent study demonstrated that BK stimulates the synthesis of IL-1β, IL-2, and IL-6 from lung strip explants, an effect susceptible to blockade by a B2 BK receptor antagonist (31). Results presented in this report provide the first evidence that BK can stimulate cytokine gene expression through activation of the transcription factor NF-κB, a potentially important mechanism for the inflammatory effects of kinins.

Our data suggest a correlation between BK-stimulated NF-κB activation and IL-1β production in the WI-38 fibroblasts. We first showed that IL-1β synthesis in BK-stimulated WI-38 cells correlated with an increase in the steady state level of IL-1β mRNA, which was the consequence of transcription activation as indicated by the result of nuclear run-on experiment. The kinetics of BK-stimulated IL-1β transcription was closely paralleled by NF-κB activation, suggesting that the mechanism of BK-stimulated IL-1β synthesis may directly involve activation of NF-κB, a transcription factor known to be important for inflammatory cytokine synthesis. Using chimeric reporter plasmids containing the wild-type and mutated κB enhancer sites from either the IκB or the IL-1β genes, we further demonstrated that BK could functionally activate NF-κB. Although NF-κB may not be the only transcription factor responsible for IL-1β gene expression, it appears to be important for the initiation of the transcription of IL-1β after BK stimulation. Based on the ability of NF-κB to induce synthesis of multiple cytokines, we predicted that BK would stimulate synthesis of other cytokines since fibroblasts are known to be capable of synthesizing both IL-6 and IL-8 in addition to IL-1 (32). In preliminary studies, we found that BK induced an increase in the steady state mRNA levels for both IL-8 and IL-6 in stimulated WI-38 cells (data nor shown). Induction of additional cytokines may extend the proinflammatory function of BK, as IL-8 is known for its chemotactic and angiogenic effects that can contribute to inflammation and wound healing. BK rapidly loses its activity in plasma due to cleavage by the carboxypeptidase known as kininase II. Thus, BK-induced cytokine production is likely a local response to inflammation, and targeted application of B2 antagonists may be an effective means to block this function of the kinin.

WI-38 cells are known to express both the B1 and B2 BK receptors. The B1 BK receptor preferentially binds des-Arg^9-BK and induces collagen formation, protein synthesis, and cell proliferation (33); the B2 BK receptor preferentially binds BK and mediates many of the classic inflammatory effects of BK (34). Whereas our results suggest that the B2 receptor is a primary mediator of the transcription activation effect of BK in WI-38 cells, IL-1β production by BK-stimulated cells may also exert a positive regulatory effect on the expression of both types of BK receptors. IL-1β has been known to be one of the most potent inducers for the B1 receptor (35, 36), and it may also increase the expression of functional B2 BK receptors (37–39). Collectively, these findings suggest the likelihood that BK initiates an inflammatory cycle by stimulating cytokine synthesis and thereby induces an increased expression of both B1 and B2 BK receptors. Such a cycle would maximize the effects of kinins and their metabolites at the site of inflammation.

In addition to the WI-38 fibroblasts, BK was found to stim-

ulate NF-κB activation and IL-1β secretion in two other types of cells tested, the human lung fibrosarcoma cell line Hs913T and the type II lung epithelial cell line A549. In both cases the NF-κB activation and IL-1β secretion peaked at 10 nM BK. The two cell lines differ from the WI-38 cells in that maximal IL-1β was observed at 4 h after stimulation and additional incubation with BK induced no further IL-1β production (data not shown).

The BK receptor joins a growing number of G protein–coupled receptors that have been shown recently to activate NF-κB upon agonist binding (40–43). Our studies suggest that BK-induced IL-1β gene expression and NF-κB activation are mediated by a receptor-coupled G protein system that involves the G_i/G_o class of G_α proteins. The mechanism for the potentiation of BK induced NF-κB activation by CTX is currently unknown, and the same phenomenon has been observed in EMSA with the platelet-activating factor receptor (data not shown). It is possible that G_s and adenylyl cyclase might also function in the signaling mechanism governing NF-κB activation in these systems, but this will require further investigation. While signaling mechanisms for TNFα- and IL-1β–induced NF-κB activation have been studied in some detail, little is known about the signal transduction pathways for G protein–coupled receptor-stimulated NF-κB activation. Likewise, BK stimulation may activate other transcription factors that contribute to the expression of proinflammatory cytokine genes. Current efforts are focused on these studies as well as the investigation of the signaling pathways leading to BK-induced gene expression.

Acknowledgments

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