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Research Article

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The B_1-agonist [des-Arg^{10}]-kallidin Activates Transcription Factor NF-κB and Induces Homologous Upregulation of the Bradykinin B_1–receptor in Cultured Human Lung Fibroblasts

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Abstract

The bradykinin B_1-receptor is strongly upregulated under chronic inflammatory conditions. However, the mechanism and reason are not known. Because a better understanding of the mechanism of the upregulation will help in understanding its potential importance in inflammation, we have studied the molecular mechanism of B_1-receptor upregulation in cultured human lung fibroblasts (IMR 90) in response to IL-1β and the B_1-agonist [des-Arg^{10}]-kallidin. We show that treatment of human IMR 90 cells by IL-1β stimulates the expression of both B_1-receptor mRNA and protein. The latter was studied by Western blot analysis using antipeptide antibodies directed against the COOH-terminal part of the human B_1-receptor. We furthermore report the novel observation that the B_1-receptor is upregulated by its own agonist which was completely blocked by the specific B_1-antagonist [des-Arg^{10}-Leu^9]-kallidin, indicating an upregulation entirely mediated through cell surface B_1-receptors. The increased population of B_1-receptors was functionally coupled as exemplified by an enhancement of the B_1-agonist induced increase in free cytosolic calcium. Upregulation by the B_1-agonist was blocked by a specific protein kinase C inhibitor. B_1-agonist–induced upregulation was correlated to the induction of transcription factor nuclear factor κB (NF-κB) which efficiently bound to the NF-κB–like sequence located in the promoter region of the human B_1-receptor gene. This correlation was further confirmed by reporter gene assays which showed that this NF-κB–like sequence, in the B_1-receptor promoter context, could contribute to IL-1β and DLBK-induced B_1-receptor transcription activation, and by the effect of NF-κB inhibitor pyrrolidinedithiocarbamate which diminished both B_1-receptor upregulation and NF-κB activation. NF-κB is now recognized as a key inflammatory mediator which is activated by the B_1-agonist but which is also involved in B_1-receptor upregulation. (*J. Clin. Invest.* 1998. 101:2080–2091.) Keys words: bradykinin • B_1-receptor • NF-κB • gene expression • inflammation

Introduction

It is now well known that the responses to vasoactive kinin peptides are mediated through the activation of two receptors termed B_1 and B_2, which have been first defined on the basis of the structure–function relationships of their agonists and antagonists (1). The natural agonists of the B_2-receptor are the nonapeptide bradykinin (BK)[1] and the decapeptide Lys-BK (kallidin) which are generated by the proteolytic action of the serine protease kallikrein from the protein precursor kininogen (2). BK and Lys-BK are weak B_1-receptor agonists, but the cleavage of these two B_2-agonists by arginine carboxypeptidases produces the high affinity B_1-receptor agonists, [des-Arg^9]-BK and [des-Arg^{10}]-kallidin (DLBK), respectively (1). Cloning studies confirmed the existence of B_1- and B_2-receptors (3–8), which showed that they belong to the superfamily of seven transmembrane domain G protein–coupled receptors. Up- and downregulation has been reported for the B_2-receptor (9–11). The B_1-receptor, which is only weakly expressed under physiological conditions, is strongly induced in injured tissue, which can be mimicked in vitro by inflammatory mediators such as bacterial lypopolysaccharides and IL-1β (12).

BK is involved in physiological and pathological processes including vasodilatation, diuresis and natriuresis, cell contraction or relaxation, increased vascular permeability, pain, and inflammation (2, 13–15). Several observations support also an involvement of BK in allergic airway inflammation (16–20). Furthermore, in cultured human lung fibroblasts, BK activates nuclear factor κB (NF-κB) and a concomitant gene expression of the proinflammatory cytokine IL-1β (21). NF-κB appears to be a key mediator in allergic inflammation as it activates a large number of inducible genes that encode inflammatory proteins (cytokines, enzymes, receptors, etc.; reference 22).

On the contrary, inhalation of nebulized B1-agonist [des-Arg^9]-BK in asthmatic volunteers is without effect, suggesting

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1. *Abbreviations used in this paper:* ACE, angiotensin converting enzyme; BK, bradykinin; $[Ca^{2+}]_i$, intracellular calcium concentration; CHX, cholera toxin; DLBK, [des-Arg^{10}]-kallidin; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HB_1, human B_1; NF-κB, nuclear factor κB; PKC, protein kinase C; PTX, pertussis toxin; PDTC, pyrrolidinedithiocarbamate.

no role of this carboxypeptidase metabolite of BK in allergic airway inflammation (19, 20). However, several observations suggest that the B_1-receptor may play a role under such chronic inflammatory conditions: (*a*) the B_1-receptor is strongly induced (sevenfold) in cultured human lung fibroblasts upon treatment with the proinflammatory cytokine IL-1β (3) and in several other tissues after injury or inflammation (12); (*b*) it was suggested recently that B_1-receptors can amplify and take the relay of B_2-receptors in chronic pathologies (12) which is now supported by the observation that the B_1-receptor, unlike the B_2-receptor, does not desensitize (23); and (*c*) the accumulation of B_1-agonist during sepsis, where the disappearance of angiotensin converting enzyme (ACE) from the pulmonary circulation favors the formation of B_1-agonist DLBK by arginine carboxypeptidases from Lys-BK (24). Furthermore, the absence of an effect of [des-Arg^9]-BK in the earlier studies of Polosa and colleagues (19, 20) is now attributed to the fact that [des-Arg^9]-BK is not the natural human B_1-receptor agonist since recent studies have clearly demonstrated that the B_1-agonist [des-Arg^9]-BK has a 1000-fold lower affinity for the human B_1-receptor than the carboxypeptidase product of Lys-BK, DLBK (3, 23).

Taken together, these findings suggest that the B_1-receptor plays a role under chronic inflammatory conditions. However, until now, only hypotheses have been formulated to explain why and how the B_1-receptor is induced (12). Because a better knowledge of the molecular mechanism involved in the induction of the B_1-receptor will help in understanding its potential importance in chronic inflammation, we have evaluated the molecular mechanism of the upregulation of the B_1-receptor in cultured human lung fibroblasts in response to IL-1β and DLBK to mimic inflammation and B_1-agonist accumulation, respectively. We report here that upregulation of the B_1-receptor by IL-1β is controlled at the transcriptional level and is strongly correlated to the activation of transcription factor NF-κB. Further, we report that the novel observation that the B_1-receptor is upregulated by its own agonist involving activation of protein kinase C (PKC) and NF-κB through pertussis and cholera toxin-insensitive pathways.

Methods

Materials

FCS and DME were purchased from GIBCO BRL (Gaithersburg, MD). Penicillin, streptomycin, and glutamine were from Biochrom (Berlin, Germany). DLBK and [des-Arg^{10}-Leu^9]-kallidin were from Neosystem (Strasbourg, France), and human recombinant IL-1β was from Promega Corp. (Madison, WI). Fura-2/AM was purchased from Molecular Probes (Interchim-Montluçon, France). Pyrrolidinedithiocarbamate (PDTC), pertussis toxin (PTX), cholera toxin (CHX), actinomycin D, and PMA were from Sigma Chemical Co. (St. Louis, MO). PKC inhibitor GF 109203X was obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA). IMR 90 cells (ATCC[R]186-CCL) were purchased from American Type Culture Collection (ATCC, Rockville, MD) and were used between passage six and nine. [α-^{32}P]UTP (800 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Irvine, CA). T7 RNA polymerase was from Eurogentec (Seraing, Belgium). p65 (A)-G, p50 (N-19), and c-Rel (N)-G were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell culture and cell treatment

IMR 90 cells were grown in DME (4.5 g/liter glucose) supplemented with 15% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin. Before mRNA extraction, membrane protein preparation, intracellular calcium concentration ($[Ca^{2+}]_i$) determination, nuclei isolation, or nuclear extract preparation, IMR 90 cells were washed with PBS and then exposed to the drugs, in the same culture medium without FCS. Cells were isolated as indicated in each specific section.

Human B_1-receptor expression cell line

The putative human B_1-receptor coding region (−5 to 1,081 bp relative to the ATG start codon) was subcloned in the expression vector RcRSV (Invitrogen Corp., San Diego, CA) and transfected into COS-7 cells using Lipofectamin (GIBCO BRL). 48 h later, selection for transfectants was initiated by addition of 1 mg/ml G418 (GIBCO BRL) and resistant colonies were isolated after 2 wk of treatment.

Selection, production, and testing of antipeptide antibodies

Peptide selection. The transmembrane topology of the primary sequence of the B_1-receptor (3) was predicted using the TMAP program (25), available at the EMBL Web site: http://www.embl-heidelberg.de/tmap/tmap_info.html. The hydrophobicity scale was determined according to Engelman et al. (reference 26; see Fig. 1 *A*), and compared with that of the B_2-receptor. The primary structures of the B_1- and B_2-receptor were also compared and three sequences of lesser homology were selected in putative nontransmembrane regions. The corresponding peptides, all derived from intracellular loops, were synthesized and used for immunization.

Peptide synthesis. Amino acids were of the L-configuration, and were purchased from NovaBiochem (Meudon, France) and Neosystem. Peptides derived from the human B_1-receptor sequence were synthesized by solid phase peptide synthesis using the Applied Biosystems 431 A peptide synthesizer after a Fmoc chemistry (27). After deprotection and cleavage, peptides were purified by gel-filtration on a Sephadex G-25 column (Pharmacia Fine Chemicals, Uppsala, Sweden), equilibrated in 5% acetic acid, and their homogeneity checked by reverse-phase chromatography on a C18 OD300 cartridge (Brownlee, Touzart and Matignon, Paris, France), using a 40-min linear (0–80%) acetonitrile gradient in 0.075% trifluoroacetic acid. Elution was monitored by an Applied Biosystems 1000S diode array detector (Perkin Elmer, Roissy, France). Peptide molecular weight was determined by MALDI TOF mass spectroscopy (Brüker, Bremen, Germany).

Immunizations and antibodies testing. Peptides Y18C and A15C were coupled to BSA using sulfo m-maleimidobenzoyl *N*-hydroxysuccinimidyl ester as a linker (28). For this purpose, a cysteinyl residue was added at the COOH-terminal end of peptide Y18C to direct coupling to the carrier protein. K21N was conjugated to succinylated BSA using 1-ethyl-3 (3-dimethylaminopropyl) carbodiimide as a coupling agent (29). About 1 mg of peptide conjugate was suspended in Freund's complete adjuvant and injected subcutaneously to rabbits. Boosters were administered at least twice at monthly intervals with 0.25 mg of conjugate in incomplete Freund's adjuvant. Boosters were repeated until the antibody titer was high enough as judged from indirect ELISAs using the immobilized cognate peptide antigen. Typically, rabbits were bled as soon as antisera were used at 10^{-4} dilutions for ELISA assays. IgG were purified from rabbit serum by 40% ammonium sulfate precipitation and extensive dialysis against PBS at 4°C.

ELISAs. Microtiter plates (microtest three flexible assay plates; Falcon Labware, Cockeysville, MD) were coated with peptides (100 ml of a 10 mg/ml solution) according to the procedure described in reference 29. Serial dilutions of crude antisera or purified IgG were then reacted, followed by a peroxidase-conjugated secondary antibody to rabbit IgG. Peroxidase activity was measured using *O*-phenylenediamine.

Western blot analysis

After treatment, IMR 90 cells were washed three times with ice-cold PBS, scraped and lysed in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 50 mM NaF, 5 mM EDTA, and 20 mM sodium pyrophosphate). Lysates were centrifuged for 30 min at 25,000 rpm and

4°C, and the pellet was dissolved in ice-cold lysis buffer. The resulting mixture containing the membrane fraction was sonicated for 10 s. To concentrate the proteins below 50 kD, the samples were passed through a microsep microconcentrator with a molecular mass cutoff of 50 kD (Filtron-France, Colgnieres, France). The protein concentration was determined using Coomassie Brilliant Blue with BSA as the standard.

Western blot analysis was essentially performed as described previously (30). Briefly, after addition of Laemmli's SDS-PAGE sample buffer (final concentration: 0.16 M Tris-HCl, pH 6.8, 5% SDS [wt/vol], 25% glycerol [vol/vol], 0.0025% Bromophenol Blue [wt/vol], 13% 2-mercaptoethanol [vol/vol]), the lysates were heated for 5 min at 100°C. Lysates containing equal amounts of protein (20 μg) were separated on a 12% (wt/vol) polyacrylamide gel followed by electrotransfer to ECL nitrocellulose membrane (Amersham Corp., Arlington Heights, IL). The membrane was blocked by incubation for 60 min in 5% nonfat dry milk and 0.1% Tween-20 in PBS (136.8 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.76 mM KH_2PO_4) (PBS-T), washed twice with PBS-T and incubated with B_1-receptor antipeptide antibody (dilution 1/5,000) overnight at 4°C. After three washes with PBS-T, blots were incubated 1 h with a 1/5,000 dilution of a peroxidase-coupled donkey anti–rabbit antibody followed by washing three times with PBS-T. Revelation of the antibody–protein complex was done using the ECL-detection reagent (ECL-kit; Amersham Corp.) followed by exposition to film (Hyperfilm-ECL; Amersham Corp.) during 1–5 min. Molecular weight markers were from Bio-Rad Laboratories, Richmond, CA (Kaleidoscope prestained standards). In inhibition experiments, the antibodies were incubated with the corresponding peptide at a concentration of 10 μg/ml at 4°C overnight.

RNA extraction, [^{32}P]riboprobe synthesis, and RNase protection assay

Total RNA was extracted from confluent IMR 90 cells using the acid guanidinum thiocyanate-phenol-chloroform extraction method as described previously (31). B_1-receptor mRNA abundance in IMR 90 cells was determined using RNA–RNA solution hybridization as outlined by Sambrook et al. (30). In brief, an [α-^{32}P]-labeled B_1-receptor antisense RNA-probe around 500 bp was prepared by in vitro transcription using the T7-promoter from pBluescript (Stratagene, Inc., La Jolla, CA). In this construct the human B_1-receptor cDNA was cloned in the reverse orientation behind the T7-promoter and linearized by BglII (located at 545 bp from the start codon) before in vitro transcription. The template DNA was digested by RNase-free deoxyribonuclease I (GIBCO BRL). 50 μg of dried cellular RNA was used in overnight hybridization experiments at 55°C in a 30-μl vol of hybridization buffer (80% deionized formamide, 0.4 M NaCl, 0.5 mM EDTA, 40 mM Pipes, pH 6.7) containing the [^{32}P]-riboprobe. The unhybridized portion of the probe was digested with 0.3 ml RNase A (40 μg/ml) and RNase T1 (700 U/ml). After 2 h at 37°C, digestion was stopped by addition of 5 μl of proteinase K (10 mg/ml) and the samples were further incubated for 15 min at 37°C. Carrier tRNA (10 μg) and 0.3 ml buffer (38 mM sodium citrate, pH 7.0, 4.2 M guanidine isothiocyanate, and 20 mM *N*-lauroylsarcosine) were added to each tube and protected hybrids were precipitated with isopropyl alcohol. After washing with 70% ethanol, RNA pellets were dissolved in 10 μl of sample buffer (97% deionized formamide, 0.1% SDS, 10 mM Tris-HCl, pH 7.0). [^{32}P]RNA-RNA hybrids were analyzed on a 6% polyacrylamide/7 M urea gel. Dried gels were exposed to a Kodak X-Omat film for 24 h at −80°C. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control.

Measurement of the $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ was determined, as currently done in the laboratory (32), on adherent cells. After treatment with the IL-1β (200 pM) and DLBK (0.1 μM) during the indicated time, IMR 90 cells were washed with Krebs-Ringer buffer (10 mM Hepes, pH 7.4, 145 mM NaCl, 2.5 mM KH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM glucose, and 0.1% BSA) and then loaded for 45 min in a 5 μM solution of Fura-2/AM in Krebs-Ringer buffer at 37°C. Before measuring the $[Ca^{2+}]_i$ in response to 0.1 μM DLBK the cells were washed twice with Krebs-Ringer buffer. Fluorescence measurements were done using a Spex Fluorilog spectrofluorometer, set for alternative dual wavelength excitation at 340 nm and 380 nm. Light-emitted at 505 nm was collected by a photomultiplier and passed to a Spex system microcomputer which averaged the emission collected over a 0.5-s period at each excitation wavelength. Autofluorescence of unloaded cells was found to be about 18% of the emitted signal and was subtracted from the Fura-2/AM loaded fluorescence at each excitation wavelength before calculating the fluorescence ratio R (340/380). As previously described, $[Ca^{2+}]_i$ was calculated from the equation of Grynkiewicz et al. (33): $[Ca^{2+}]_i = K_d \times (R - R_{min}/R_{max} - R) \times l$, where K_d (224 nM) is the dissociation constant of the complex Fura-2-Ca^{2+} and R_{min}, R_{max}, and l are constant parameters depending on the optical system used. In our experimental conditions they were R_{min}, 0.8; R_{max}, 16; and l , 4.

Nuclear run-on experiment

Confluent IMR 90 cells (2×10^6) were treated during 4 h with IL-1β (200 pM), DLBK (0.1 μM) or with medium only. They were washed twice with ice-cold PBS, harvested by trypsinization, and resuspended in ice-cold 5 ml medium with 15% FCS. Medium was removed by centrifugation (10 min at 400 *g*) and the cells were resuspended in 1 ml of ice-cold lysis buffer (15 mM Hepes, pH 7.5, 0.3 mM sucrose, 60 mM KCl, 15 mM NaCl, 0.5 mM EGTA, 2 mM EDTA, 14 mM β-mercaptoethanol, 15 mM, 0.15 mM spermine [Sigma Chemical Co.], 0.5 mM spermidine [Sigma Chemical Co.], 1 mM PMSF [Sigma Chemical Co.], 10 μg/ml leupeptine [Sigma Chemical Co.], 10 μg/ml aprotinine [Sigma Chemical Co.], and 0.04% Nonidet P-40 [Sigma Chemical Co.]) and homogenized by gentle in- and out-pipetting. Cell-lysis and the integrity of the nuclei was examined under a microscope using Trypan-Blue stain (Sigma Chemical Co.). Nuclei were collected by centrifugation (5 min at 800 *g*) and washed twice with ice-cold lysis buffer. The pelleted nuclei were resuspended in a 190 μl ice-cold solution containing 50 mM Tris-HCl, pH 8.0, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA and rapidly frozen in liquid nitrogen and stored at −80°C until further use. To the 190 μl of the nuclei suspension were added 50 μl of 5× nuclear run-on buffer (150 mM Tris-HCl pH 8.0, 750 mM KCl, 25 mM $MgCl_2$, 1 mM EDTA, 12.5 mM DTT, and 2.5 mM each of ATP, CTP, and UTP) and 100 μCi of α-[^{32}P]GTP (800 Ci/mmol; ICN Biomedicals, Inc.). The samples were incubated at 30°C for 30 min and the elongated transcripts were isolated as follows. DNA was degraded by addition of 5 U of DNase-RNase free (Promega Corp.) for 5 min at 30°C. 200 μg yeast tRNA (Boehringer Mannheim Biochemicals, Indianapolis, IN) was then added as the carrier as well as 300 μl of TES-buffer (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 0.1% SDS). Proteins were eliminated by proteinase K (50 μg; Boehringer Mannheim Biochemicals) digestion for 30 min at 37°C followed by an extraction with phenol (pH 4.3; Sigma Chemical Co.) and precipitation overnight at −20°C (0.2 M NaCl [final concentration] and 2.5 vol of ethanol). The RNAs were precipitated by centrifugation (30 min at 12.000 *g*), resuspended in 200 μl TES buffer and precipitated twice with 0.2 M ammonium acetate (final concentration) and 2.5 volumes of ethanol. The final pellet was resuspended in 500 μl TES-buffer. DNA-samples (1 μg each) were denatured at 100°C for 5 min in 0.2 M NaOH and placed on ice. 10 vol of 5 M NaCl were added and the samples were blotted on a nitrocellulose membrane (Oncor Appligene, Illkirch, France) pre-wetted with 2× SSC (sodium chloride/sodium citrate) using a slot-blot device (Hoefer Scientific Instruments, San Francisco, CA). Every slot was washed three times with 2× SSC followed by fixation of the DNA by UV-crosslinking (Stratagene, Inc. cross-linker). Prehybridization was performed at 42°C overnight in 4 ml of a 20 mM phosphate buffer, pH 7.4, containing 50% deionized formamide, 1 mM EDTA, 750 mM NaCl, 0.1% SDS, 5× Denhardt's reagent, 10% dextran sulfate (Sigma Chemical Co.), 150 μg/ml yeast tRNA, and 100 μg/ml denaturated salmon sperm DNA (Sigma Chemical Co.). Hybrid-

ization was initiated by addition of the denatured (2 min at 90°C) radiolabeled RNA and continued for 15 h at 42°C. The membranes were then washed with 2× SSC/0.1% SDS, twice at room temperature (15 min) and twice at 60°C (30 min). Autoradiography was performed at −80°C using a Kodak XAR-5 film and intensifying screens.

Nuclear extract preparation and electrophoretic mobility shift assay

Confluent cultures of IMR 90 cells were harvested by trypsinization, resuspended in PBS, and washed once with PBS (centrifugation at 3,000 rpm for 5 min). Nuclear extracts were prepared as described (34) with the following modifications: cells were suspended in a hypotonic buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 300 mM sucrose, 0.1 mM EGTA, 0.5 mM PMSF, and 0.5 mM DTT) followed by homogenization by gentle in- and out-pipetting. The homogenates were centrifuged at 3,000 rpm for 5 min and the pellets containing the crude nuclei were resuspended in high-salt buffer (20 mM Hepes, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1 mM EGTA, 0.5 mM PMSF, and 0.5 mM DTT) to extract the nuclear proteins. After centrifugation at 14,000 rpm for 15 min, the supernatants were dialyzed for 4 h against a buffer containing 20 mM Hepes, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, and 0.1 mM DTT. Protein concentrations were determined using Coomassie Brilliant Blue with BSA as the standard.

The electrophoretic mobility shift assay (EMSA) was done with a consensus double stranded NF-κB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′; Promega Corp.) end-labeled with [γ-^{32}P]ATP (7000 mCi/mmol; ICN Biomedicals, Inc.) using T4-polynucleotide kinase (GIBCO BRL). A typical binding reaction was performed with 10 μg of nuclear protein, 2 μg poly dIdC (Sigma Chemical Co.), and 0.5 ng of labeled oligonucleotide (specific activity 50,000–200,000 cpm/ng DNA) in binding buffer (10 mM Tris-HCl [pH 7.5], 5% glycerol, 100 mM NaCl, 1 mM EDTA, and 1 mM DTT). The binding reaction was carried out at 25°C for 45 min. After the reaction, the samples were electrophorized at 4°C for 3.5 h on a 4% nondenaturating polyacrylamide gel (30:1) in a Tris-Borate-EDTA buffer system. Dried gels were exposed to a Kodak X-Omat film at −80°C. The specificity of the binding reaction was tested using excess of unlabeled consensus NF-κB oligonucleotide to compete with the labeled probe for binding to the nuclear protein. Nonspecific AP-1 oligonucleotide (Promega Corp.) was used as a control. The nuclear proteins that bound to the NF-κB probe were identified by preincubation of the nuclear extracts for 45 min at 25°C with an antibody (1.5 μg) directed to different protein subunits of NF-κB (p65, p50, and c-Rel).

The NF-κB-like sequence located about 1,000–1,200 bp upstream from the transcription initiation sites of the human B_1-receptor gene (NF-κB–HB_1, 5′-GGCTGGAGAGACCCCCGCCTA-3′; NF-κB–like sequence underlined; reference 35) was used to compete with the consensus NF-κB sequence. To obtain the double stranded oligonucleotide, single stranded oligonucleotides (sense and antisense) were synthesized by Eurogentec (Seraing, Belgium). These were dissolved in buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 150 mM NaCl) to a final concentration of 50 ng/μl, mixed in equal amounts, denaturated (15 min at 70°C), and slowly cooled down to room temperature. These double stranded NF-κB–HB_1 oligonucleotides were directly used in competition assays or end-labeled as described above.

Gene-reporter studies

The human B_1-promoter was cloned with and without the NF-κB–HB_1 into pGL3-Basic (Promega Corp.) to verify its ability to drive reporter gene-expression in IMR 90 cells upon IL-1β and DLBK stimulation. The human B_1-promoter with the NF-κB–HB_1 sequence was PCR-amplified from chromosomal DNA using a sense oligonucleotide 5′-AACGCTTCTCAGAAACAGTATAG-3′ (oligo-1; position 49–71; reference 35) with an additional SacI restriction-site added to the 5′-end and an antisense oligonucleotide 5′-TGAGGACTCGAGAGTGAGAGCTGCTTTTGGAGGAGT-3′ (oligo-2; position 1,299–1,322; reference 35) with an additional HindIII restriction-site added to the 5′-end. The human B_1-promoter without the NF-κB–HB_1 sequence was PCR-amplified using the same antisense oligonucleotide above and as sense oligonucleotide was used 5′-TCAGATCTATCCCGGGAGTAATGG-3′ (oligo-3; position 175–198; reference 35) with an additional SacI restriction-site added to the 5′-end. The amplification products were cut with SacI and HindIII and cloned in SacI/HindIII-cut pGL3-Basic. The amplification products were also cloned in the reverse orientation upstream from the reporter gene by addition of a SacI restriction-site to the 5′-end of oligo-2 and a HindIII restriction-site to the 5′-end of oligo-1 and oligo-3. To verify the absence of errors introduced during the amplification process the inserts were sequenced using an automated sequencer (Applied Biosystems, Inc., Foster City, CA). The plasmid pRL-CMV (Promega Corp.) was used as a control for monitoring the transfection efficiency by the expression of the *Renilla* luciferase. 2 μg of each plasmid DNA was used for transient transfection of IMR 90 cells with Lipofectamin (GIBCO BRL) using the procedures recommended by the manufacturer. The DNA-containing medium was removed after 6 h and replaced by normal medium for 48 h. Cells were stimulated for 2 h by 200 pM IL-1β and 0.1 μM DLBK and harvested using Promega's passive lysis buffer. The remainder of the experiment was performed as described in the technical manual of the Dual-Luciferase™ Reporter Assay System (Promega Corp.).

Statistical analysis

Results are means±SEM. The differences were tested using the non parametric Mann-Whitney U test. Differences were considered significant at $P < 0.05$.

Results

Design and testing of human B_1-receptor antipeptide antibodies. Three fragments in the cDNA-deduced B_1-receptor pri-

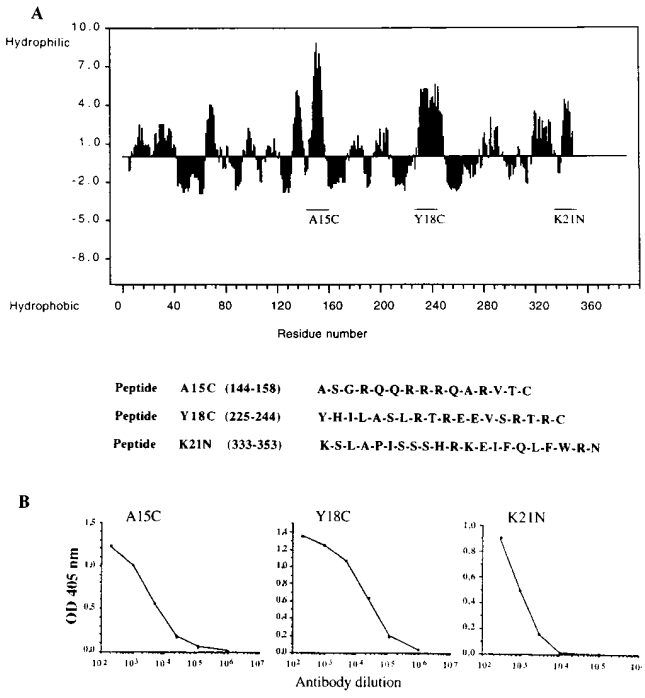

Figure 1. Design and testing of B_1-receptor antipeptide antibodies. (*A*) Engelman human B_1-receptor hydrophobicity scale. The average was calculated over seven residues. Selected hydrophilic regions are indicated and the sequences of corresponding peptides are shown. (*B*) Specificity of antibodies probed by ELISA. Titer plates were coated at 10 mg/ml. The ordinate gives the absorbance at 405 nM and the abscissa indicates the antibody dilution.

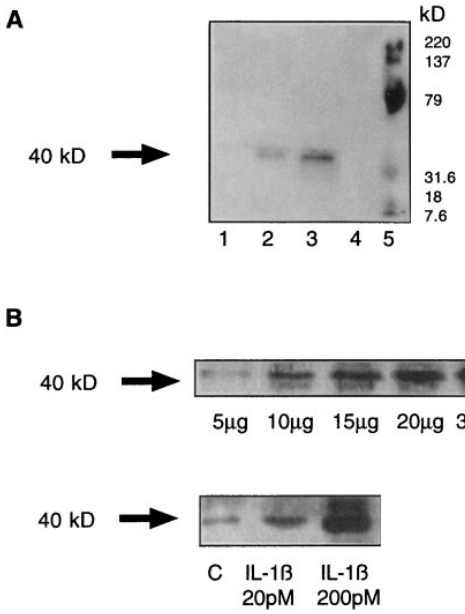

Figure 2. Specificity of antipeptide antibodies as analyzed by Western blot. Western blot analysis of the membrane proteins from cells expressing the human B_1-receptor using the IgG purified from the K21N antipeptide antibody (dilution 1/5,000). (*A*) Membrane proteins (20 μg per lane) from mock transfected COS-7 cells (lane *1*), untreated IMR 90 cells (lane *2*), and COS-7 cells transfected with a plasmid expressing the human B_1-receptor (lane *3*). Lane *4* shows the inhibition of the detection of the 40 kD band in COS-7 cells expressing the human B_1-receptor when the antibody is preincubated (overnight at 4°C) with the K21N peptide at a concentration of 10 μg/ml. Molecular mass marker proteins were run simultaneously (lane *5*). Their molecular masses (*kD*) are shown on the right-hand side of the figure. (*B*) Western blot analysis of increasing amount of membrane proteins (from 5 to 30 μg) from IMR 90 cells treated during 6 h with IL-1β (100 pM), (*top*). Western blot analysis (*bottom*) of membrane proteins (20 μg per lane) from IMR 90 cells—untreated (*C*) and treated 6 h with either 20 pM or 200 pM IL-1β.

mary sequence (3) were selected based on their hydrophilicity, their positioning in non-transmembrane domains, and their maximal sequence variability upon comparison with the B_2-receptor. Fragments belonging to intracellular domains answered the best to these criteria and were therefore selected to raise antisera that could specifically react with the B_1-receptor. Peptides A15C, Y18C, and K21N corresponded to the amino acid sequences within intracellular loops 3, 4, and to the COOH-terminal domain of the B_1-receptor, respectively (Fig. 1 *A*). Peptide Y18C was substituted COOH-terminally by a cysteinyl residue to direct coupling to the carrier protein.

All three conjugates were injected to elicit antipeptide antibodies that could possibly cross react with the B_1-receptor. Antisera with high antibody titers were obtained for each peptide as assessed by ELISA (Fig. 1 *B*). All antipeptide antibodies also recognized a single band of ~ 40 kD upon electrophoresis of membrane proteins from IMR 90 cells. However, anti-A15C and anti-Y18C–purified IgGs generated a higher background staining than anti-K21N upon Western blotting, so only the latter was used in this study.

Western blot analysis of membrane proteins of IMR 90 cells and COS cells transfected with the human B_1-receptor, using the IgG-purified K21N antibody, showed a band with an apparent molecular mass of ~ 40 kD that was absent in mock transfected COS cells (Fig. 2 *A*). 40 kD is close to the calculated molecular mass of the human B_1-receptor (38 kD; reference 3). The specificity of the response of the K21N antibody was further demonstrated by the ability of the free K21N peptide to inhibit the presence of the 40-kD band (Fig. 2 *A*, lane *4*). These results shows that the 40-kD band identified by the polyclonal K21N antipeptide antibody is most likely the human B_1-receptor. Furthermore, the 40-kD band was found to be dependent both on the protein concentration of the extract (Fig. 2 *B*, *top*) and on the IL-1β dose (Fig. 2 *B*, *bottom*). Thus, the K21N antipeptide antibody is a useful analytical tool to study B_1-receptor protein upregulation.

Upregulation of the B_1-receptor by IL-1β and B_1-agonist DLBK. Treatment with IL-1β was used as a first approach to study the mechanism of upregulation of the B_1-receptor in IMR 90 cells. In a preliminary experiment, the time effect (2, 4, and 6 h) on the induction of the B_1-receptor showed that a maximum expression of the B_1-receptor was achieved after 6 h of treatment with 200 pM IL-1β (data not shown). RNase protection analysis of B_1-receptor mRNA from IMR 90 cells treated for 6 h with 200 pM IL-1β showed a significant increase in the concentration of B_1-receptor mRNA (Fig. 3 *A*). The concentration of B_1-receptor protein, evaluated by Western blot analysis, was also clearly increased by this treatment (Fig. 3 *B*).

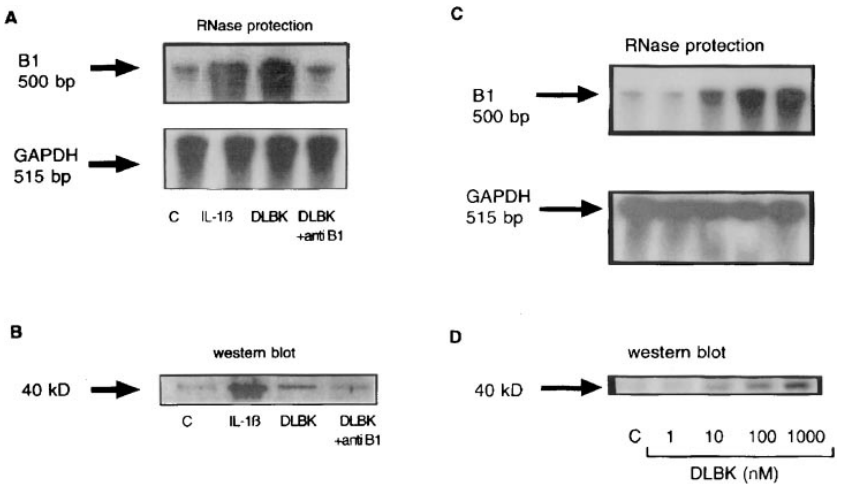

Figure 3. Upregulation of the B_1-receptor by IL-1β and DLBK. (*A*) Detection of the B_1-receptor mRNA by RNase protection. (*B*) Western blot analysis of the expression of the human B_1-receptor using the IgG purified from K21N (dilution 1:5,000) on membrane proteins (20 μg per lane) from IMR 90 cells. *C*, IMR 90 cells untreated; *IL-1β*, IMR 90 cells treated 6 h with 200 pM IL-1β; *DLBK*, treatment for 6 h with 0.1 μM DLBK; *DLBK + anti B1*, DLBK treatment (6 h, 0.1 μM) in presence of the specific B_1-antagonist [des-Arg^{10}-Leu^9]-kallidin (1 μM). (*C* and *D*) DLBK induces B_1-receptor mRNA and protein expression in a dose-dependent manner. Cells were treated for 6 h with medium only (*C*) or increasing concentrations of DLBK (1–1000 nM). The autoradiographs shown are representative of four independent experiments.

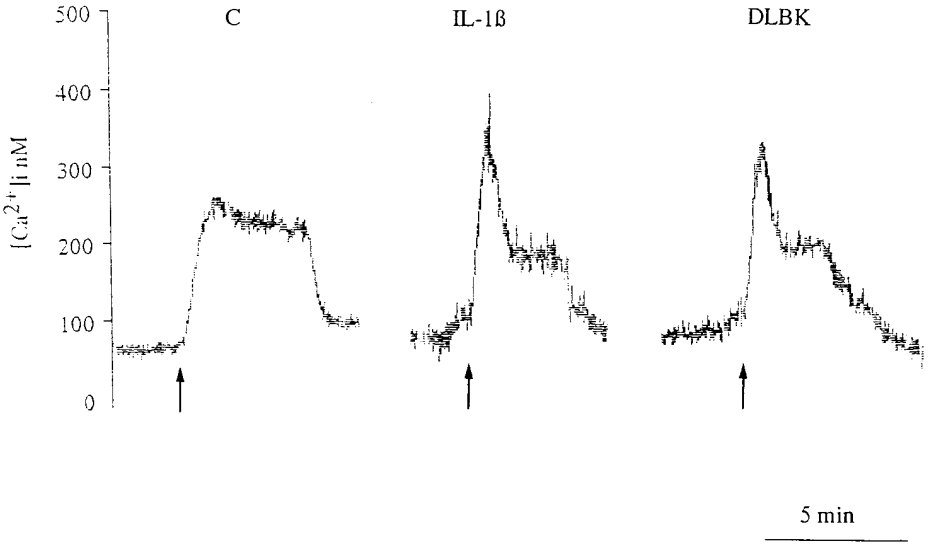

Figure 4. Upregulation by IL-1β and agonist DLBK results in functional B_1-receptors. Increase in intracellular calcium $[Ca^{2+}]_i$ induced in adherent IMR 90 cells by 0.1 μM DLBK on untreated cells (*C*); cells treated for 6 h with 200 pM IL-1β (*IL-1β*); cells treated 6 h with 0.1 μM DLBK (*DLBK*). Arrows indicate the stimulation with DLBK. B_1-receptor stimulation was performed in a calcium containing medium (1 mM). The profiles are representative for four independent experiments (see Table I).

Interestingly, similar results were found by treatment of IMR 90 cells with the B_1-agonist DLBK. DLBK increased both B_1-receptor mRNA and protein in a dose-dependent manner from 1 nM to 1 μM (Fig. 3, *C* and *D*). The observed upregulation of the B_1-receptor by DLBK was completely prevented by the use of the high affinity B_1-antagonist [des-Arg^{10}-Leu^9]-kallidin, indicating that the upregulation of the B_1-receptor by its own agonist acts via the present population of cell surface B_1-receptors (Fig. 3, *A* and *B*). Treatment of the cells with the antagonist alone did not induce any effect on B_1-receptor gene expression (not shown).

Effect of the induction of the B_1-receptor on the $[Ca^{2+}]_i$ mobilization induced by DLBK. Functional B_1-receptors were synthesized during the upregulation by IL-1β and DLBK since a significant increase in the mobilization of $[Ca^{2+}]_i$ induced by DLBK (0.1 μM) compared with the control was observed (Fig. 4). DLBK induced the classical biphasic increase in $[Ca^{2+}]_i$ mobilization (32). However, only the first, transient phase was increased, whereas the sustained phase was not affected by the treatment with IL-1β or DLBK (Table I). Moreover, treatment of IMR 90 cells either with IL-1β or DLBK was without effect on the B_2-receptor stimulation since the $[Ca^{2+}]_i$ increase induced by BK (0.1 μM) was unchanged (Table I). The increase in the mobilization of $[Ca^{2+}]_i$ by DLBK was selectively inhibited by the B_1-antagonist [des-Arg^{10}-Leu^9]-kallidin (not shown).

Transcriptional activation of B_1-receptor expression. To determine whether the B_1-receptor upregulation was due to the activation of transcription or an increase in stabilization of B_1-receptor mRNA, IMR 90 cells were stimulated with medium only, IL-1β (200 pM), or DLBK (0.1 μM) for 4 h before addition of transcriptional inhibitor actinomycin D (6 μg/ml). B_1-receptor mRNA expression was examined by RNase-protection at $t = 0$, 30, 60, 120, and 240 min after addition of actinomycin D (Fig. 5 *A*). Both IL-1β and DLBK did not significantly alter the stability of B_1-receptor mRNA, their half-lives were comparable to the control (half-lives: 121±15, 115±13, and 109±16 min for medium only, IL-1β, and DLBK, respectively). To further confirm the effect of IL-1β and DLBK on B_1-receptor mRNA induction, nuclear run-on experiments were performed. IMR 90 cells were treated for 4 h with medium only, IL-1β (200 pM), or DLBK (0.1 μM) followed by isolation of the nuclei. Fig. 5 *B* shows the results of a typical run-on assay in which IL-1β and DLBK clearly stimulated transcriptional activation of the B_1-receptor. Taken together, these results show that both IL-1β and DLBK induce transcriptional activation of the B_1-receptor.

IL-1β and DLBK induce transcription factor NF-κB. For more insight into the transcription mechanism of B_1-receptor upregulation, we decided to study the activation of transcription factor NF-κB. Transcription factor NF-κB (36, 37) was chosen as the transcription factor to be studied for the following two reasons: (*a*) IL-1β often induces transcription factor NF-κB (36, 37, 38); and (*b*) computer analysis of the promoter region of the human B_1-receptor revealed the presence of a NF-κB–like sequence (35).

The EMSA was used to study the involvement of transcription factor NF-κB in B_1-receptor upregulation (Fig. 6). Nuclear extracts harvested from untreated (control) IMR 90 cells demonstrated little binding of a double stranded oligonucleotide containing the consensus NF-κB recognition sequence

Table I. Effect of 0.1 μM DLBK and 0.1 μM BK on the Transient Phase (Peak) and Sustained Phase (Plateau) of Increase in $[Ca^{2+}]_i$ from IMR 90 Cells Treated either with 200 pM IL-1β or with 0.1 μM DLBK. B_1 and B_2-receptor Stimulation was Performed in a Calcium Containing Medium (1 mM)

		Cell treatment		
Stimulation	Phase	Control	IL-1β	DLBK
DLBK (0.1 μM)	Transient	270±20 nM	395±25 nM*	390±15 nM*
	Sustained	210±15 nM	205±20 nM	212±10 nM
BK (0.1 μM)	Transient	430±20 nM	415±15 nM	420±12 nM
	Sustained	150±10 nM	135±10 nM	152±15 nM

The results are the mean±SEM of four independent experiments. *$P <$ 0.05 compared to the respective control value.

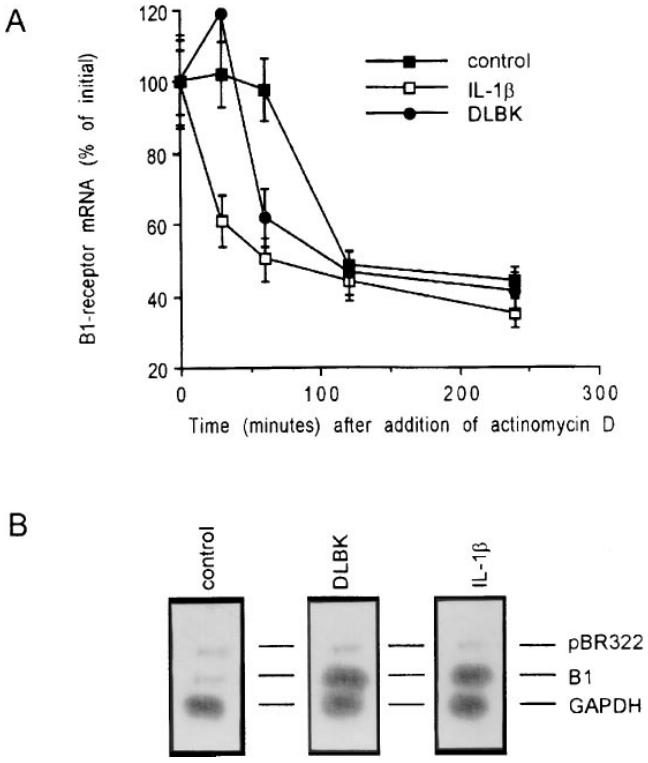

Figure 5. (*A*) Effect of IL-1β and DLBK on B_1-receptor mRNA stability. IMR 90 cells were stimulated for 4 h with medium only, IL-1β (200 pM) and DLBK (0.1 μM). Cells were harvested for RNA-extraction at various time intervals after addition of actinomycin D (6 μg/ml). Total RNA (50 μg) was analyzed with the RNase protection assay using ^{32}P-labeled B_1-receptor and GAPDH probes. The densitometric values of each band were evaluated using the Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA). B_1-receptor signals were corrected for differences in total RNA quantities using GAPDH. The corrected density of each time point was then divided by that of the B_1-receptor mRNA density at the time of actinomycin D addition (initial). Results are mean ± SEM from three separate experiments. (*B*) IL-1β and DLBK activate B_1-receptor gene transcription. Cells were stimulated with medium only, IL-1β (200 pM) and DLBK (0.1 μM) for 4 h followed by nuclei isolation. Transcripts were elongated in the presence of α-[^{32}P]GTP before RNA isolation. Radiolabeled nuclear RNA was hybridized with DNA samples of pBR322 plasmid, human B_1-receptor and GAPDH housekeeping gene immobilized on nitrocellulose membranes. The autoradiograph of one representative experiment is shown.

(36), with low interassay variations (Fig. 6, *control*). However, after treatment for 1 h 45 min with IL-1β (200 pM), NF-κB–DNA binding activities were identified (Fig. 6 *A*, *bracket*). The specificity of the observed DNA–protein interaction was confirmed by the ability of excess unlabeled NF-κB oligonucleotide (specific competitor) to inhibit binding, whereas the addition of nonspecific oligonucleotide (a tandem repeat of the AP-1 consensus motif) did not block the binding (Fig. 6 *A*). Further characterization of the nuclear proteins binding to the NF-κB motif was obtained by preincubation of the nuclear extracts with antibodies against the p65, p50, and c-Rel protein subunits of NF-κB. This resulted in retardation (supershift) of the NF-κB–DNA binding activity in case of p65. Antibodies against the p50 and cRel subunits of NF-κB were also able to reduce the NF-κB–DNA binding activity. This suggests that in IMR 90 cells, IL-1β induced nuclear binding proteins containing p65, p50, and c-Rel forms of NF-κB (Fig. 6 *B*). Stimulation of cells with 40 pM IL-1β for 1 h 45 min gave similar results (not shown).

Treatment of IMR 90 cells with the B_1-agonist DLBK (0.1 μM) for 1 h 45 min resulted in a significant increase in the density of a NF-κB–DNA binding activity, indicating the accumulation of NF-κB protein in the nucleus (Fig. 6 *C*, bracket). Competition experiments with unlabeled specific and nonspecific oligonucleotides confirmed the specificity of the binding reaction (Fig. 6 *C*). Preincubation of the nuclear extracts with antibodies against the p65, p50, and c-Rel NF-κB subunits resulted in inhibition of the NF-κB–DNA binding activity with all antibodies (Fig. 6 *D*). Inhibition of the NF-κB–DNA binding activity was not observed when the extracts were preincubated with an unrelated nonspecific antipeptide antibody directed against the human B_1-receptor K21N (1.5 μg, not shown). The disappearance of the NF-κB–DNA binding activ-

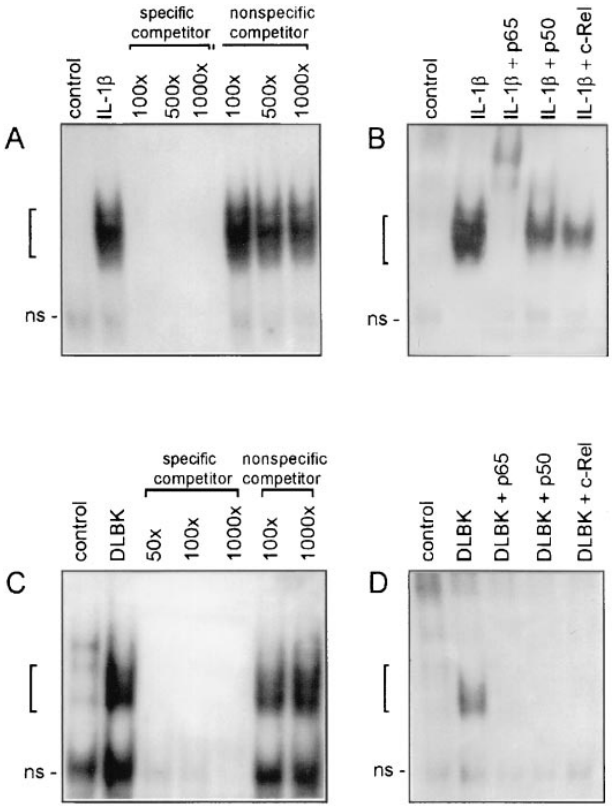

Figure 6. Induction of transcription factor NF-κB by IL-1β and DLBK evaluated by EMSA of nuclear extracts. The EMSA was performed with ^{32}P-labeled consensus NF-κB oligonucleotide and with IMR 90 nuclear extracts as described in Methods. Cells were treated for 1 h 45 min with 200 pM IL-1β (*A* and *B*) or 0.1 μM DLBK (*C* and *D*); *control*, untreated cells; *specific competitor*, unlabeled consensus NF-κB oligonucleotide added (the fold molar excess to labeled NF-κB oligonucleotide is indicated); *nonspecific competitor*, unlabeled AP-1 consensus motif added (the fold molar excess to labeled NF-κB is indicated); *p65*, *p50*, and *c-Rel*, preincubation of the nuclear proteins with antibody directed against the p65, p50, and c-Rel subunits of NF-κB, respectively (1.5 μg); *ns*, nonspecific. A bracket indicates the NF-κB–DNA binding activity.

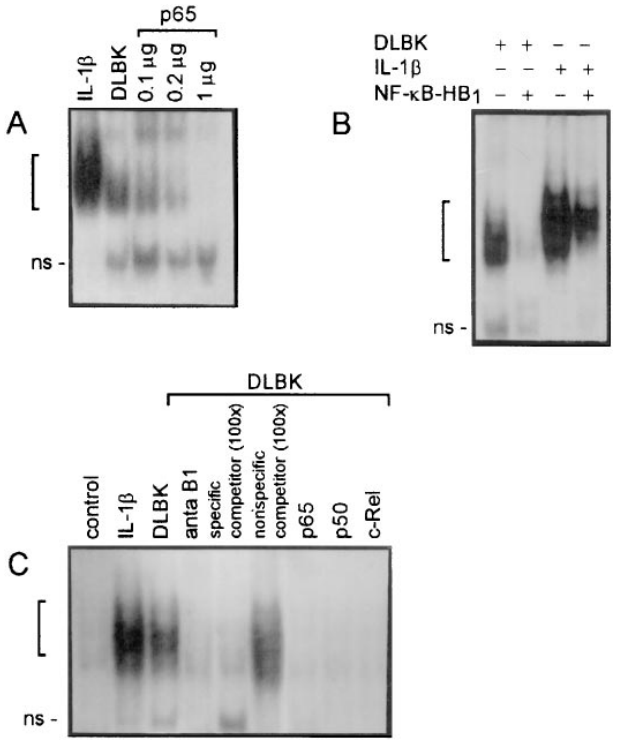

Figure 7. NF-κB–DNA binding activities in extracts of cells treated for 1 h 45 min with IL-1β (200 pM) and DLBK (0.1 M). (*A*) Competition between labeled NF-κB oligonucleotide and p65 antibody in DLBK treated cells (different concentrations of p65 antibody used are indicated). (*B*) Competition of ^{32}P-labeled consensus NF-κB oligonucleotide and unlabeled NF-κB–like sequence, located in the promoter region of the human B_1-receptor gene (*NF-κB–HB$_1$*), between NF-κB complexes induced by DLBK and IL-1β treatment. Unlabeled NF-κB–HB_1 oligonucleotide was added in ~ 200-fold molar excess to the consensus NF-κB oligonucleotide. (*C*) Analysis of NF-κB–DNA binding activities using radiolabeled NF-κB–like sequence (NF-κB–HB_1). *anta B1*, DLBK treatment (1 h 45 min, 0.1 μM) in presence of the specific B_1-antagonist [des-Arg^{10}-Leu^9]-kallidin (1 μM); *specific competitor*, unlabeled NF-κB–HB_1 oligonucleotide added (100-fold excess); *nonspecific competitor*, unlabeled AP-1 consensus motif added (100-fold excess); *p65*, *p50*, and *c-Rel*, preincubation of the nuclear proteins with antibody directed against the p65, p50, and c-Rel subunits of NF-κB, respectively (1.5 μg); *ns*, nonspecific. A bracket indicates the NF-κB–DNA binding activity.

ity upon incubation with antibodies directed against the different NF-κB subunits was specific since an antibody concentration dependent decrease in NF-κB–DNA binding activity was observed (Fig 7 *A*, shown for p65; p50 and c-Rel not shown) which did not significantly affect the non-specific binding (Fig. 7 *A*, *ns*). These results suggested that the NF-κB complexes activated by DLBK contain the p65, p50, and cRel subunits of NF-κB (Fig. 7 *A*).

The recent publication of a NF-κB–like sequence in the promoter region of the human B_1-receptor gene (NF-κB–HB_1; reference 35) allowed us to perform competition experiments between the commercially available consensus NF-κB and the NF-κB–HB_1 sequence located in the promoter region of the B_1-receptor (Fig. 7 *B*). Interestingly, binding of the consensus NF-κB oligonucleotide to the NF-κB complex induced by DLBK was clearly displaced by 200-fold molar excess of the NF-κB–HB_1 oligonucleotide. We only reduced the binding of the consensus NF-κB oligonucleotide to the IL-1β–induced NF-κB complexes with a 200-fold molar excess of NF-κB–HB_1 oligonucleotide (Fig. 7 *B*, *bracket*). A complete displacement was obtained by using a 500-fold molar excess of NF-κB–HB_1 oligonucleotide (not shown). To prove direct binding of the NF-κB–like sequence to NF-κB proteins, the NF-κB–HB_1 oligonucleotide was labeled and used in EMSA analysis (Fig. 7 *C*). As for the consensus NF-κB oligonucleotide, NF-κB–DNA binding activity was observed using the NF-κB–like sequence in IL-1β and DLBK treated IMR 90 cells. DLBK-induced NF-κB–DNA binding activity was prevented by pretreatment with the high affinity B_1-antagonist [des-Arg^{10}-Leu^9]-kallidin. Competition experiments with unlabeled specific and nonspecific oligonucleotides confirmed the specificity of the binding reaction. Preincubation of the nuclear extracts with antibodies against the p65, p50, and c-Rel NF-κB subunits resulted in inhibition of the NF-κB–DNA binding activity with all antibodies.

IL-1β and DLBK induce reporter gene-expression directed by the human B_1-promoter. The above results suggest a correlation between NF-κB activation and B_1-receptor upregulation. This relationship was further studied by the ability of IL-1β and DLBK to drive reporter gene-expression under control of the human B_1-receptor promoter. The human B_1-receptor promoter with and without the NF-κB–like sequence was cloned upstream from the luciferase gene of pGL3-Basic. These constructs were used to transiently transfect IMR 90

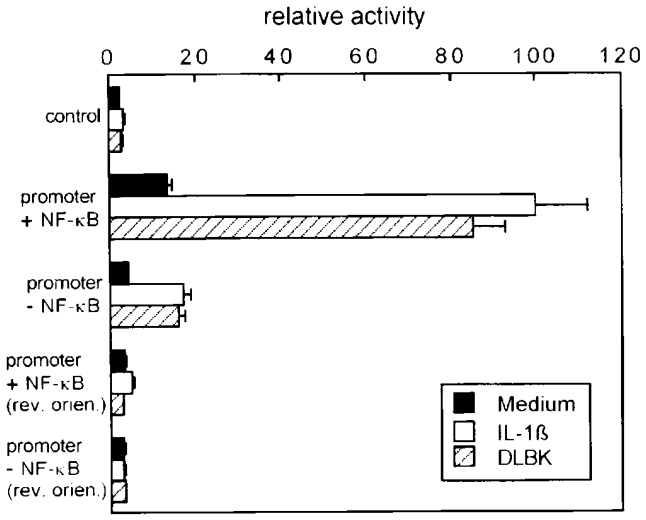

Figure 8. Gene-reporter assays with the human B_1-receptor promoter. IMR 90 cells were transiently transfected using pGL3-Basic with the following B_1-receptor promoter elements cloned upstream from the luciferase gene: the human B_1-receptor promoter with and without the NF-κB–like sequence in the normal orientation (promoter +/− NF-κB) and in the reverse orientation (promoter +/− NF-κB [*rev. orien.*]). Then cells were simulated during 2 h with medium only, IL-1β (200 pM) and DLBK (0.1 μM) followed by analysis of luciferase activities in crude cell extracts. Differences in transfection efficiencies were corrected by assaying the *Renilla* luciferase activities of plasmid pRL-CMV which was cotransfected with the constructs. The results are shown as the percentage of the maximum luciferase activity observed with the IL-1β–induced promoter + NF-κB construct. Each value is the mean±SEM (n = 3).

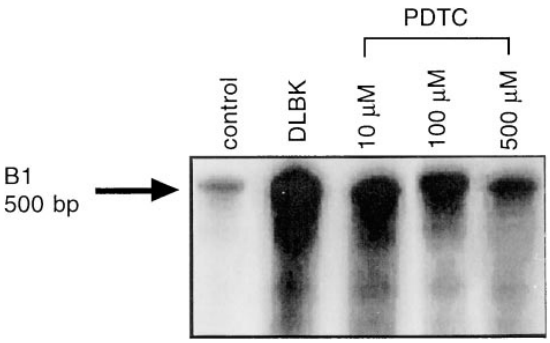

Figure 9. Inhibition of DLBK-induced B_1-receptor mRNA upregulation by antioxidant PDTC. IMR 90 cells were preincubated (30 min) with PDTC at three different concentrations before DLBK (0.1 μM) stimulation for 6 h. *Control*, untreated cells; *DLBK*, cells stimulated with DLBK; *PDTC*, cells pretreated with different concentrations (indicated) of PDTC before stimulation with DLBK. The autoradiograph of one representative experiment is shown.

cells and measure reporter gene-expression after stimulation (Fig. 8). Whereas the control plasmid (pGL3-Basic) showed only low level luciferase expression and showed no response to IL-1β (200 pM) or DLBK (0.1 μM) treatment (2 h), the construct with the B_1-receptor promoter induced significant luciferase expression upon stimulation with both IL-1β and DLBK. However, the IL-1β- and DLBK-induced luciferase expression of the construct in which we deleted the NF-κB like sequence from the B_1-receptor promoter was significantly lower, but still about fivefold higher than the value obtained with pGL3-Basic alone. Cloning of the B_1-receptor promoter in the reverse orientation did not significantly drive luciferase expression. These results suggest that IL-1β and DLBK-induced NF-κB activation participates in B_1-receptor expression via the NF-κB–like sequence located in the promoter region. Further support for this relationship was obtained by the use of the antioxidant PDTC, which is a known inhibitor of transcription factor NF-κB activation (39). A PDTC dose-dependent inhibition on DLBK-induced B_1-receptor upregulation was observed when cells were pretreated 30 min before DLBK stimulation (Fig. 9). PDTC at a concentration of 500 μM decreased strongly B_1-receptor upregulation induced by IL-1β and DLBK at the levels of mRNA and protein expression (Fig. 10, *A* and *B*). Pretreatment with PDTC resulted in a decreased activation of transcription factor NF-κB by both DLBK and IL-1β (Fig. 10 *C*). These results suggest that transcription factor NF-κB participates in B_1-receptor upregulation induced by DLBK and IL-1β.

Effect of bacterial toxins and involvement of PKC on DLBK-induced B_1-receptor expression and NF-κB activation. Cloning studies showed that B_1-receptors are 7-transmembrane G protein–coupled receptors (3). To determine which type of G_α protein is involved in DLBK-induced B_1-receptor upregulation, we treated IMR 90 cells with either PTX (100 nM) or CHX (1 μM) for 4 h before stimulation with DLBK (0.1 μM) for 4 h. RNase protection analysis of B_1-receptor mRNA did not show any differences in bacterial toxin-treated cells compared with nontreated control cells (Fig. 11 *A*). This was also observed on DLBK-induced NF-κB activation (Fig. 11 *B*). 12-h pretreatment with CHX and PTX was also without effect (not shown). However, pretreatment with PKC inhibitor GF 109203X (30 min, 1 μM) inhibited DLBK-induced B_1-receptor mRNA expression (Fig. 11 *C*). Direct stimulation of PKC by phorbol ester PMA (100 nM, 4 h) resulted in upregulation of B_1-receptor message that was also clearly inhibited by GF 109203X, indicating the specificity of the inhibitor. PKC inhibitor GF 109203X also significantly inhibited DLBK-induced NF-κB activation (Fig. 11 *D*).

Discussion

An increasing number of observations show an upregulation of the bradykinin B_1-receptor under chronic inflammatory conditions that suggests a role for this receptor in these situations. However, it is still unclear why and how this receptor is induced (12). For this reason, we have studied the molecular mechanism of B_1-receptor upregulation under in vitro inflammatory conditions (IL-1β stimulation) and during prolonged

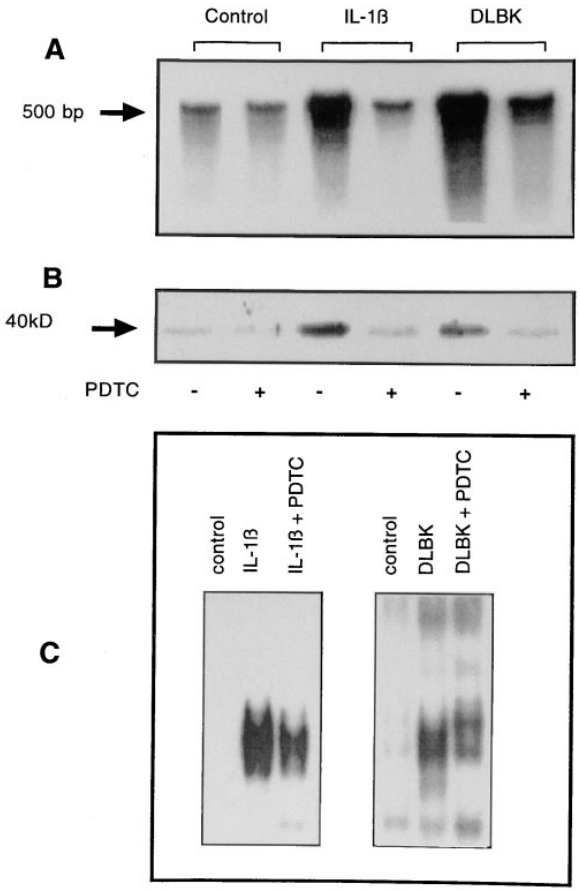

Figure 10. The effect of PDTC on B_1-receptor upregulation. IMR 90 cells were stimulated with IL-1β (200 pM) and DLBK (0.1 μM) for 6 h for Western blot (20 μg per lane) and RNase protection analysis, and 1 h 45 min for EMSA analysis. PDTC (500 μM) was added 30 min before drug treatment. (*A*) RNase protection analysis of B_1-receptor mRNA expression in the presence or absence of PDTC. (*B*) Western blot analysis of B_1-receptor protein expression in the presence or absence of PDTC. *PDTC*, treatment with (+) or without (−) PDTC. (*C*) EMSA-analysis of NF-κB activation in the presence or absence of PDTC. *Control*, untreated cells; *IL-1β*, cells treated with IL-1β; *DLBK*, cells treated with DLBK; *IL-1β + PDTC*, treatment with IL-1β and PDTC; *DLBK + PDTC*, treatment with DLBK and PDTC.

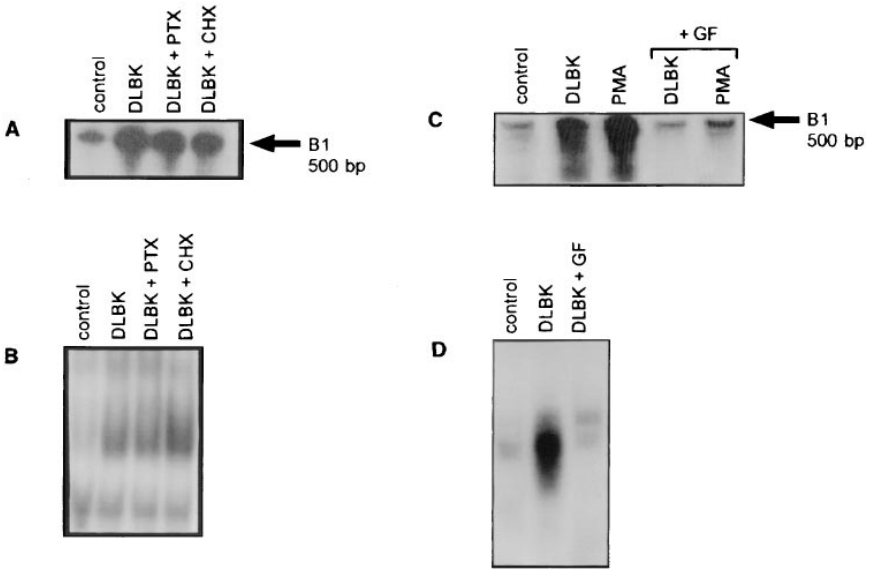

Figure 11. Absence of effects of pretreatment with PTX (100 nM) and CHX (1 μM) for 4 h on DLBK-induced (0.1 μM, 4 h) B_1-receptor mRNA expression (*A*) and NF-κB activation (*B*). Inhibition of B_1-receptor mRNA expression (*C*) and NF-κB activation (*D*) by 30-min pretreatment with 1 μM GF 109203X (+GF) before stimulation with DLBK (0.1 μM) or PMA (100 nM) for 4 h. *Control*, medium only. The autoradiographs of one representative experiment are shown.

agonist stimulation in cultured human lung fibroblasts (IMR 90 cells). The results presented here show that B_1-receptor up-regulation by IL-1β is regulated at the transcriptional level involving activation of transcription factor NF-κB and provide the first evidence that the B_1-receptor is up-regulated by its own agonist. Both IL-1β and the B_1-agonist activate transcription factor NF-κB, a transcription factor now recognized as playing a key role in immune and inflammatory responses (22, 40).

The observation of local production of IL-1β during inflammation accompanied by B_1-receptor up-regulation in several tissues has resulted in the hypothesis that this cytokine is directly involved in B_1-receptor upregulation (12). Furthermore, binding studies have shown a sevenfold increase in B_1-receptor binding sites after treatment of IMR 90 cells with IL-1β (3). For these reasons we have chosen IL-1β as an in vitro stimulus to study B_1-receptor up-regulation in cultured human lung fibroblasts. B_1-receptor protein expression was examined with immunoblotting using antipeptide antibodies. This relatively new strategy to obtain antibodies when no purified protein is available uses small synthetic peptides derived from cDNA sequences. All our tests showed that the antipeptide antibodies were B_1-receptor specific and thus were a good analytical tool to study the human B_1-receptor expression. Recently, antipeptide antibodies directed against the human B_2-receptor have been successfully used to study the involvement of extracellular loops in B_2-receptor ligand binding (41, 42).

Our data confirm, at the mRNA and protein levels, that IL-1β upregulates B_1-receptor expression. Furthermore, the functional response to the B_1-agonist DLBK as reflected by increased calcium mobilization, was enhanced following IL-1β treatment. To our knowledge, this is the first clear demonstration of B_1-receptor upregulation by IL-1β studied at different levels. This observed upregulation is the consequence of transcription activation as shown by the results of both B_1-receptor mRNA half-life and nuclear run-on experiments. Electrophoretic mobility shift assays were used with nuclear extracts from stimulated IMR 90 cells to examine the transcription mechanism for IL-1β–induced B_1-receptor expression. A DNA-binding activity specific for transcription factor NF-κB was observed after IL-1β treatment, which was strongly correlated to B_1-receptor upregulation since both receptor expression and NF-κB were inhibited by antioxidant PDTC, a known NF-κB inhibitor. This IL-1β–induced DNA-binding activity could also bind to the NF-κB–like sequence located in the promoter region of the B_1-receptor. Furthermore, gene-reporter experiments showed that this NF-κB–like sequence could contribute to IL-1β–induced B_1-receptor mRNA expression.

We report here the novel observation that the bradykinin B_1-receptor is upregulated by its own agonist DLBK. Usually, the activation of receptors by their agonists induces downregulation of the receptor. However, a few reports have shown the upregulation of G protein–coupled receptors, including the β3-adrenergic (43), the dopamine D2 (44), and the angiotensin II (45) receptors. Multiple mechanisms seem to be involved, like an increased efficiency of translation, modulation of cAMP production, stabilization of mRNA or stabilization of the receptor by ligands, depending upon the cells or receptor which is studied. The upregulation of the human B_1-receptor was identified by an increase in both B_1-receptor mRNA and protein, as well as the B_1-agonist–induced increase in free cytosolic calcium. Since this effect was fully prevented by the B_1-antagonist [des-Arg^{10}-Leu^{9}]-kallidin, the B_1-receptor upregulation resulted from the stimulation of the preexistent population of cell-surface B_1-receptors. Furthermore, as observed for IL-1β, B_1-receptor upregulation by DLBK is the consequence of transcription activation. In IMR 90 cells, the B_1-receptor was coupled to PTX and CHX insensitive G proteins. However, our results suggest that the transduction pathway activated by the B_1-agonist involves PKC activation. These observations indicate that the B_1-receptor might be coupled to the Gαq/11 subfamily which is insensitive to PTX but couples to the known β-isoforms of phospholipase C (46–49). Indeed, recombinant stable expression of the B_1-receptor in Chinese hamster ovary cells has recently demonstrated that the human B_1-receptor can be coupled to the Gαq/11 subfamily (23).

As observed in IL-1β treatment, activation of transcription factor NF-κB was observed in nuclear extracts of DLBK-

treated cells which was well correlated with B_1-receptor upregulation. Moreover, EMSAs with the NF-κB–like sequence, the effect of PDTC, and B_1-receptor promoter gene-reporter studies with DLBK showed a clear correlation between activation of transcription factor NF-κB, the NF-κB–like sequence, and B_1-receptor transcription activation. Transcription factor NF-κB is activated by phosphorylation and subsequent degradation of IκB which is an inhibitory protein that retains NF-κB in its inactive form in the cytosol. Upon dissociation of IκB, NF-κB translocates to the nucleus (36, 37, 40). The signaling pathways of NF-κB activation by IL-1β have been studied in some detail now (38), but very little is known about the transduction pathways of G protein–coupled receptor-simulated NF-κB activation. Our results indicate the involvement of PKC in NF-κB activation by DLBK, a kinase that is able to activate NF-κB (50). The precise mechanism of DLBK-induced NF-κB activation remains to be investigated, however. The B_1-receptor is not the only protein upregulated by NF-κB, many inflammatory genes are activated by NF-κB. The NF-κB activating proinflammatory cytokines IL-1β and TNFα are upregulated by NF-κB itself which may lead to amplification and perpetuation (through feedforward loops) of the inflammatory process (22).

From a pathophysiological point of view, the observation that the B_1-receptor is upregulated by its own agonist, should be kept in mind to better understand the role of the B_1-receptor in the pathological situations where the B_1-receptor is likely to be induced, such as chronic inflammation (12), hyperalgesia (24), and septic shock (51). Other pathologies may also be concerned by this B_1-upregulation mechanism because it has been reported that, in cultured cells, the B_1-agonist stimulates cell proliferation (32, 52) but also collagen production (52). Such effects have to be related to pathologies where fibrotic processes are observed. Furthermore, the accumulation of the B_1-agonist can occur in situations where the activity of the ACE is either decreased, or inhibited by the use of ACE inhibitors. Indeed, treatment with ACE inhibitors favors the conversion of B_2-agonists into B_1-agonists by arginine carboxypeptidase M and N (12). In this respect a decrease in ACE activity has been reported in animal models of fibrogenic lung injury (53, 54). Our results also agree with Nwartor and Whalley's study (55) which reported that treating rabbits with ACE inhibitors made these animals in vivo responsive to the B_1-agonist [des-Arg^9]-BK. Consistent with our results, it is likely that potentiation of [des-Arg^9]-BK formation upregulates the B_1-receptor and thereby explains the increased response to the B_1-agonist after ACE inhibitor treatment in their studies.

In summary, we present here the first analysis of the regulation of the expression of the human kinin B_1-receptor. The evidence of a homologous upregulation appears as an important new feature of this receptor. This observation introduces a new difference between the mechanisms of expression regulation of B_1- and B_2-receptors since the B_2-receptor is downregulated by prolonged exposure to its agonist (56), which is most likely linked to receptor internalization (23). Under inflammatory conditions B_1-receptors may take the relay of B_2-receptor function (12) and recently it was shown that BK, the high affinity B_2-receptor agonist, activates both NF-κB and IL-1β production (21). Prolonged B_2-receptor stimulation under inflammatory conditions in vivo thus could lead to the induction of the B_1-receptor, an effect that is likely to be amplified by the B_1-agonist DLBK. Under these pathological conditions the use of mixed B_1- and B_2-antagonists (57) will be appropriate to counteract all kinin related effects.

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