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Role of Nuclear Factor-κB and Protein Kinase C Signaling in the Expression of the Kinin B_1 Receptor in Human Vascular Smooth Muscle Cells

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ABSTRACT

Kinin B_1 receptor expression was characterized in human umbilical artery smooth muscle cells to further elucidate the function and specificity of three previously proposed pathways [nuclear factor-κB (NF-κB), protein kinase C, and agonist autoregulation] that regulate this inducible G protein-coupled receptor. Radioligand binding assays, real-time reverser transcription/polymerase chain reaction with an optional actinomycin D treatment period, and NF-κB immunofluorescence were primarily employed in these primary cell cultures. Various stimulatory compounds that increase receptor mRNA stability only (human and bovine sera, cycloheximide) or that stimulate NF-κB nuclear translocation and both mRNA concentration and stability [interleukin (IL)-1β, phorbol 12-myristate 13-acetate (PMA)] all increased the density of binding sites for the tritiated B_1 receptor agonist [^{3}H]Lys-des-Arg^9-bradykinin (without change in receptor affinity) in cell-based assays. Small interfering RNA assays indicated that NF-κB p65 is necessary for the effective expression of the cell surface B_1 receptor under basal or IL-1β, fetal bovine serum (FBS), or PMA stimulation conditions. Dexamethasone cotreatment reproduced these effects. IL-1β-, FBS-, or PMA-induced stabilization of B_1 receptor mRNA was inhibited by the addition of the protein kinase C inhibitor 3-[1-[3-(dimethylamino)propyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride (GF-109203x), which also diminished the B_{max} under FBS or PMA treatment. Lys-des-Arg^9-bradykinin had little effect on NF-κB activation, the B_{max}, or receptor mRNA abundance or stability. Both NF-κB and protein kinase C signaling are required for the effective expression of the kinin B_1 receptor in human umbilical artery smooth muscle cells.

Kinins (bradykinin-related peptides) are regulatory plasma-derived peptides that affect smooth muscle tone, microvascular permeability, and pain responses. Kinins bind to the G protein-coupled receptors B_1 and B_2. The B_2 receptor is preformed in endothelial and other cell types, and the B_1 type has been shown to be inducible in some tissue injury models (Leeb-Lundberg et al., 2005; Moreau et al., 2005b). The human B_1 receptor exhibits a particular pharmacological profile, with preference for both kallidin (Lys-bradykinin)-derived ligands and kinin metabolites without the C-terminal Arg residue; thus, lysyl-des-arginine^9-bradykinin (Lys-des-Arg^9-BK) is the optimal natural agonist of this receptor (Leeb-Lundberg et al., 2005).

Interleukin (IL)-1 and other cytokines and transcription factors, such as nuclear factor (NF)-κB are involved in the transcriptional regulation of the B_1 receptor (Schanstra et al., 1998; Medeiros et al., 2004; Leeb-Lundberg et al., 2005), and a role for tyrosine and MAP kinases in B_1 receptor expression has been proposed (Larrivée et al., 1998; Phagoo et al., 2001). Stabilization of B_1 receptor mRNA is involved with its up-regulation in IMR-90 cells, notably after IL-1 treatment or protein synthesis inhibition (Zhou et al., 1998).

Autoregulation of B_1 receptor expression is defined as kinin-induced B_1 receptor up-regulation (Phagoo et al., 1999). Although B_1 receptor autoregulation in human embryonic

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ABBREVIATIONS: BK, bradykinin; siRNA, small interfering RNA; IL, interleukin; NF-κB, nuclear factor-κB; HUA, human umbilical artery; SMC, smooth muscle cell; FBS, fetal bovine serum; PCR, polymerase chain reaction; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; DMSO, dimethyl sulfoxide; GF109203x, 3-[1-[3-(dimethylamino)propyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2,5-dione monohydrochloride; CHX, cycloheximide.

fibroblasts IMR-90 has been reported (Schanstra et al., 1998; Phagoo et al., 1999, 2001), this may not be the typical scenario, given that receptor expression in this cell line is dependent on G protein-coupled receptor-mediated secretion of IL-1β [including CXCR2 (Bastian et al., 1998)]. Autoregulation was not seen in rabbit smooth muscle cells (SMCs) stimulated with either B_1 or B_2 receptor agonists or live rabbits treated with dextran sulfate to activate the contact system [with ensuing massive production of kinins and depletion of kininogen (Sabourin et al., 2001)].

De novo synthesis of B_1 receptors causes isolated blood vessels, such as rabbit aorta, human umbilical vein, and rat portal vein, to contract in presence of kinins (Bouthillier et al., 1987; Gobeil et al., 1996; Sabourin et al., 2002b; Medeiros et al., 2004). In freshly isolated human umbilical arteries (HUA), stimulation of vascular smooth muscle B_1 receptors mediates contraction (Pelorosso et al., 2005). In cultured SMCs, B_1 receptor inhibits mitosis and migration (Agata et al., 2000; Dixon et al., 2002). HUA SMC responses to B_1 receptor agonist stimulation include phosphorylation of extracellular signal-regulated kinase 1/2 (Fortin et al., 2003b) and inhibition of cell migration in a wound closure assay (Morissette et al., 2006). The extent of migration inhibition mediated by the B_1 receptors in human SMCs correlated with the B_{max} of the receptor population (the B_{max} was varied by introducing or omitting IL-1β in the FBS-containing medium; Morissette et al., 2006). Accordingly, a reverse transcription (RT)-polymerase chain reaction (PCR) technique used previously suggested that the baseline B_1 receptor mRNA expression was up-regulated by IL-1 treatment in the HUA SMCs (Angers et al., 2000).

The present work aimed to verify and characterize several putative pathways implicated in the regulation of B_1 receptor levels in human SMCs. We compared the effects of IL-1, human and bovine serum, and phorbol 12-myristate 13-acetate (PMA) on NF-κB stimulation, B_1 receptor mRNA concentration and stability and the abundance and affinity of cell surface receptor expression. The results functionally illustrate the diversity of the B_1 receptor regulation mechanisms in a valuable primary culture system of human origin.

Materials and Methods

Cells. The institutional research ethics board approved the anonymous use of human umbilical cord segments obtained after normal deliveries. All culture surfaces were coated with gelatin. Primary cultures of SMCs were obtained from explants (1–2-mm cubes) of de-endothelialized HUAs maintained in medium 199 supplemented with antibiotics and 20% FBS [cell culture media and reagents from Invitrogen (Burlington, ON, Canada) and FBS from BioMedia Canada (Drummondville, QC, Canada)]. Cells sprouting from the explants invaded the culture surface in approximately 3 weeks and were passaged at confluence with a brief trypsin-EDTA treatment and used between passages 3 and 6. SMCs were identified by their narrow, pointed morphology as seen in phase-contrast microscopy and α-actin expression as seen in immunofluorescence (1A4 monoclonal antibody; Sigma-Aldrich, St. Louis, MO).

Drugs. Lys-des-Arg^9-BK (des-Arg^{10}-kallidin) was purchased from Bachem Bioscience Inc. (King of Prussia, PA), IL-1β from R&D Systems (Minneapolis, MN), and the remaining drugs from Sigma-Aldrich. DMSO was used as the primary solvent for dexamethasone, GF109203x, and PMA, and the final solvent concentration never exceeded 0.1%. In experiments using these drugs, the control cells were treated with DMSO vehicle in parallel.

B_1 Receptor Expression in Human Umbilical Artery SMCs. To evaluate the effect of several cell treatments on B_1 receptor expression, intact HUA SMCs were incubated with [^{3}H]Lys-des-Arg^9-BK ([^{3}H]des-Arg^{10}-kallidin; 64 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA) in 12-well plates according to published methods (Sabourin et al., 2002b). Each well (3.8 cm^2) contained an average of $224 \pm 16 \times 10^3$ SMCs ($n = 20$, estimated from four primary cell lines) or 11.5 ± 0.8 μg ($n = 6$) of membrane proteins, prepared as described previously (Houle et al., 2000). At near confluence, the FBS-containing medium was replaced by serum-free medium 199 for 48 h to reduce the basal B_1 receptor expression level, after which various stimulant or inhibitory drugs were added for 4 h (this included the restoration of the serum for some experimental groups). Finally, the wells were washed twice with the binding medium [medium 199 supplemented with 0.1% bovine serum albumin, 3 μM amastatin, 1 μM captopril, 1 μM phosphoramidon, and 0.02% (w/v) sodium azide] and filled with 1.0 ml of prewarmed (37°C) binding medium. The B_1 receptor ligand (0.125–4 nM) and unlabeled competing peptide (1 μM Lys-des-Arg^9-BK for the determination of nonspecific binding) were added to the wells. After 60 min of incubation at 37°C, each well was washed three times with 2 ml of ice-cold phosphate-buffered saline, pH 7.4, then 1 ml of 0.1 N NaOH was added to dissolve the cells. Radioactivity in the resulting suspension was determined by liquid scintillation counting (5–10 min per vial). Ancillary binding experiments aimed at studying the effect of temperature on both the association and dissociation reactions for the agonist radioligand in relation to the wild-type human B_1 receptors; very slow association and dissociation have been shown at 0°C with rabbit wild-type or recombinant receptors in intact cells (Levesque et al., 1995; Sabourin et al., 2002a).

Assays for the Nuclear Quantification of NF-κB. The translocation of NF-κB p65 subunit from the cytosol to the nucleus was studied in serum-starved (48 h) HUA SMCs using immunofluorescence as described previously (Sabourin et al., 2002b), except that incubation times with stimulants or drug combinations were limited to 1 h. The p65 staining intensity for each manually outlined nucleus in the photographic record was quantified as the median pixel intensity (0–255 scale; Photoshop v. 6; Adobe Systems, Mountain View, CA). These numerical values were averaged in each microscopic field, and results from several fields were compared using nonparametric statistical tests.

RT and Real-Time PCR. Total RNA was extracted from treated cells according to the method of Chomczynski and Sacchi (1987), then 1 μg of total RNA was transcribed into cDNA using 10 U of Transcriptor reverse transcriptase (Roche Diagnostics, Mannheim, Germany) and 5 μM oligo(dT)16 primer. The RNA was quantified by real-time PCR using the LightCycler 2.0 apparatus (Roche Diagnostics). Two microliters of cDNA were brought to a final volume of 20 μl containing 2 mM $MgCl_2$, 2 μl of LightCycler-FastStart DNA SYBRgreen I Mix (Roche Diagnostics), and 0.7 μM primers (Table 1) in water. DNA polymerase was activated at 95°C for 10 min. The amplification conditions were as follows: 47 cycles consisting of denaturation at 95°C for 15 s, annealing for 5 s at 60°C (62°C for GAPDH and B_1 receptor), respectively, and extension at 72°C. The extension times were calculated from the amplicon size (base pairs/25). Fluorescence was measured at the end of each extension phase. After amplification, a melting curve analysis from 65 to 98°C with a heating rate of 0.1°C/s with a continuous fluorescence acquisition was made. A mathematical model was used to calculate level of the target gene relative to the reference gene.

Silencing of p65 (Rel A). Lipofectamine-facilitated transfection of siRNAs has previously been applied to cultured human SMCs (Gravel and Servant, 2005). siRNA reagents were purchased from Upstate/Dharmacon RNA Technologies (Lake Placid, NY/Lafayette, CO) and used as directed. In brief, the "Smart Pool" mixture of four siRNA duplexes with selectivity for human NF-κB p65 (Rel A) was used, as well as a nonspecific control pool (nontargeting negative

control). Immunoblotting to control protein expression was done using 6-cm SMC Petri dishes and radioligand binding experiments in 12-well plates; 400 pmol of siRNA pools were added to Petri dishes, and the adherent SMCs were grown to 70% confluence [siRNA premixed with 10 or 15 μl of Lipofectamine 2000 (Invitrogen) in 1 ml of Opti-MEM, incubated 20 min at room temperature and added to 4 ml of serum- and antibiotic-free medium 199 in each dish]. After 24-h incubation at 37°C in humid air containing 5% CO_2, the medium was removed from the dish and replaced with regular culture medium (medium 199 with 20% FBS and antibiotics). The cells were extracted at 96 h for immunoblots of p65 [based on the same monoclonal antibody as immunofluorescence; dilution 1:250 (BD Transduction Laboratories, Lexington, KY); general procedures as in Houle et al. (2000)]. Cells used for binding experiments were transfected in a similar manner (reagent quantities used proportionally to the cell culture surface in 12-well plates), but starved in FBS in the 48- to 96-h interval after transfection. At that time point, 4-h treatments with stimuli were performed before the binding assay was applied, as described above.

Statistical Analysis. The Kruskal-Wallis test followed by the Mann-Whitney test were done using the InStat 3.05 computer program (GraphPad Software, San Diego, CA). The parameters of Scatchard plots (binding data treatment) were obtained using a computer program (Tallarida and Murray, 1987).

Results

Effects of Stimulants on Surface B_1 Receptor Expression. HUA SMCs maintained for 48 h in the absence of FBS showed low levels of saturable binding sites for the B_1 receptor ligand [^{3}H]Lys-des-Arg^9-BK (0.125–4 nM; Fig. 1A). The Scatchard regression analysis of the binding data (Fig. 1B) was compatible with a single class of sites with a K_D of 0.15 nM [95% confidence limits (CL), 0.09 to 0.40 nM] and a B_{max} of 1.69 ± 0.11 fmol/well. As in the rabbit SMCs (Galizzi et al., 1994; Sabourin et al., 2002b), IL-1β treatment (4 h) considerably increased the B_{max} (4.4-fold; 7.46 ± 0.36 fmol/well) in human SMCs without significant affinity change (K_D, 0.27 nM; 95% CL, 0.20 to 0.42). Separate tests showed that the IL-1β concentration in the range 2 to 10 ng/ml produced a similar effect, and that 5 ng/ml is maximal (data not shown). Serum restoration (to 20%, either human or FBS) or temporary protein synthesis blockade with cyclohex-

TABLE 1

Oligonucleotides for real-time PCR

Sequences derived from GenBank accession numbers NM_002046 (GAPDH) and NM_000710 (B_1 receptor). F and R indicate forward and reverse primers, respectively; numbers indicate the sequence position. Final concentration refers to forward and reverse primers.

mRNA Targets	Nucleotide Sequences (5′ → 3′)	ProductSize	Final Concentration
		bp	*μM*
GAPDH-GAPDH gene			
F479	TGAACCATGAGAAGTATGACAACA	156	0.7
R634	TCTTCTGGGTGGCAGTG		
B_1 receptor-BDKRB1 gene			
F731	CTGCGATCCATCCAAGC	158	0.7
R888	GCCAGGATGTGGTAGTTGA		

bp, base pair(s).

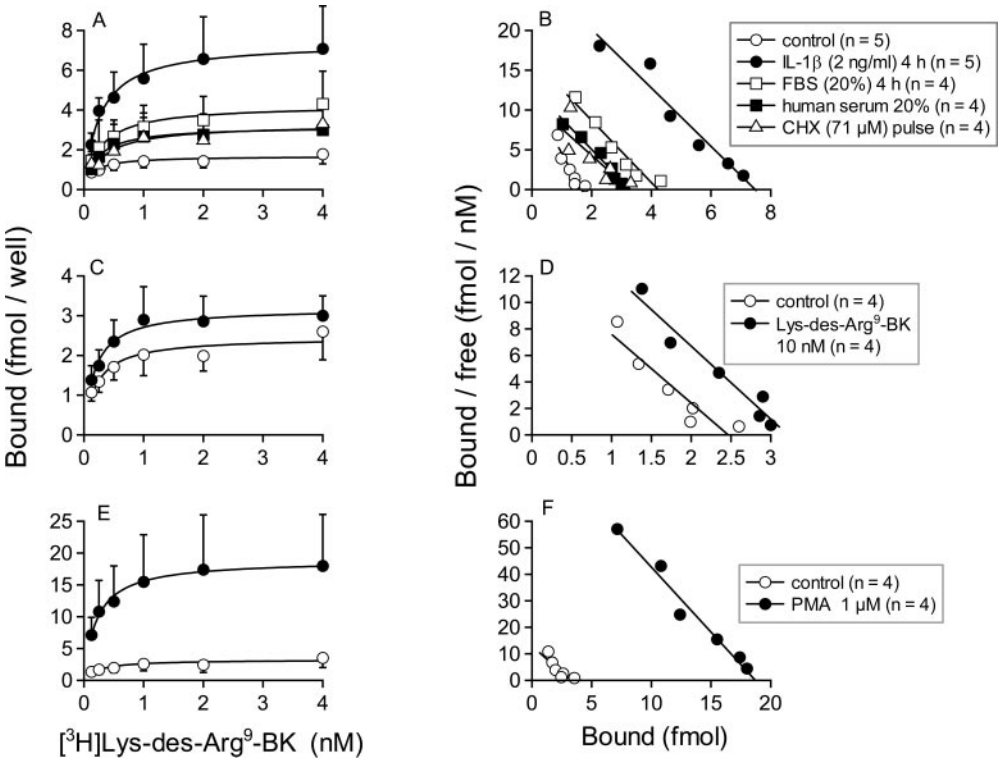

Fig. 1. Specific binding of [^{3}H]Lys-des-Arg^9-BK to serum-starved human umbilical artery SMCs further treated for 4 h with the indicated stimuli before the binding assays. Curves were fitted to the equation $B_{max} \times X/(X + K_D)$ using a least-squares method (software KyPlot 2.15 for Windows; Yoshioka, 2002). Values are means ± S.E.M. of the indicated number of determinations (n), each composed of duplicate observations. Scatchard plots (graphs on right) were derived from averaged values of the saturation curves (left). See text for further description.

imide also stimulated B_1 receptor expression in human SMCs [B_{max} of 3.28 ± 0.12, 4.20 ± 0.21, and 3.28 ± 0.48 fmol/well; K_D values of 0.25 (CL, 0.20–0.35), 0.26 (CL, 0.19–0.40), and 0.28 (CL, 0.14–25.8) nM, respectively; Fig. 1]. In subsequent experiments based on many different primary cell lines, the relative increase of B_{max} varied from one stimulus to the other, but the stimulants had consistent effects.

Serum-starved cells were incubated for 4 h in the presence of the agonist Lys-des-Arg^9-BK (10 nM, a saturating concentration) to determine whether presence of the agonist would determine an increased B_1 receptor level, thus testing for autoregulation of the receptor. It was assumed that the added agonist used during preincubation would be progressively degraded by the cells and thus would not interfere with the binding assay (based on the tritiated form of the agonist). This assumption was based on reports of the high level of activity of at least one inactivating kininase, aminopeptidase N, seen in these cells (Pelorosso et al., 2005; Gera et al., 2006). A 1.3-fold stimulation of the baseline levels of binding sites was observed in agonist-pretreated cells (Fig. 1, C and D). Measured K_D values indeed showed no unlabeled agonist leftover from the stimulation period (0.19 nM in controls, 0.18 nM in agonist-pretreated cells).

The phorbol ester PMA increased the B_{max} of the B_1 receptor in the human cell line IMR-90 (Zhou et al., 1998), although the mechanism of this effect remains unknown. In primary SMC cultures, this effect was remarkable for 1 μM PMA (4 h), with a 5.7-fold increase in B_{max} relative to that of serum-starved cells (Fig. 1, E and F). Again, the apparent affinities of the sites remained constant and compatible with those from previous experiments (0.23 and 0.21 nM in control and PMA-treated cells, respectively).

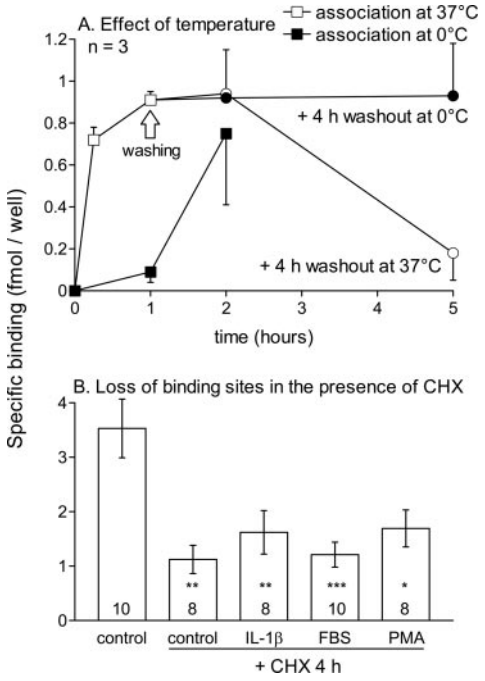

Fig. 2. Ancillary radioligand binding experiments involving serum-starved smooth muscle cells. A, association kinetics of 1 nM [^{3}H]Lys-des-Arg^9-BK to cells at 0 or 37°C. The association reaction at 37°C was followed by washing at time 60 min and further incubation for 4 h at either 0 or 37°C. Values are means ± S.E.M. of three separate experiments composed of duplicate determinations. B, before the regular binding assay (1 nM agonist radioligand, 37°C), other cells were incubated with CHX (71 μM) with or without a B_{max} stimulant [IL-1β, 5 ng/ml; FBS 20% (v/v); PMA, 1 μM]. Values are means ± S.E.M. of the indicated number of determinations drawn from four separate experiments. The nonparametric analysis of variance (Kruskall-Wallis test) indicated that values were statistically heterogeneous ($P < 0.01$). The Mann-Whitney test was applied to assess the effect of treatments relative to the controls (*, $P < 0.05$; **, $P < 0.01$; **, $P < 0.001$).

The results of ancillary radioligand binding experiments are presented in Fig. 2. The kinetics of [^{3}H]Lys-des-Arg^9-BK (1 nM) association at 0 or 37°C to serum-starved wells containing SMCs expressing the baseline B_1 receptor population is presented in Fig. 2A. The radioligand binding was rapid (complete after 60 min) at 37°C, but was slow at 0° (incomplete after 2 h), which was similar to binding to rabbit wild-type or recombinant B_1 receptors expressed by adherent and intact cells (Levesque et al., 1995; Sabourin et al., 2002a). After ligand association at 37°C, some wells were washed three times with phosphate-buffered saline at 60 min and further incubated in the azide-free binding medium for 4 h at 37 or 0°C. Most of the specifically bound radioligand was released at 37°C during the washout period, but the cells retained practically all the radioligand if the cells were incubated at 0°C (Fig. 2A). Other serum-starved SMCs were incubated at 37°C for 4 h with cycloheximide (71 μM), optionally combined with IL-1β, FBS, or PMA before the binding assay (1 nM radioligand; Fig. 2B). Blocking protein synthesis was associated with a 67% loss of surface binding that was not recovered by adding stimulants. These observations support the concept that the agonist-independent rapid clearance of surface B_1 receptors previously proposed for rabbit natural or recombinant B_1 receptors (Fortin et al., 2003a) applies to the human system and that B_{max} stimulation by IL-1β, FBS, or PMA acts on receptor synthesis but does not affect the receptor half-life.

NF-κB Translocation in Response to Stimulation. HUA SMCs were distinguished by their morphology and presence of α-actin (Fig. 3, top). In most serum-starved SMCs, p65 resided in the cytosol and not the nucleus (Fig. 3). IL-1β treatment (1 h) resulted in p65 translocation to the nuclei in most cells (Fig. 3, numerical analysis of digitized images in Fig. 4A). Neither serum restoration (to 20% with

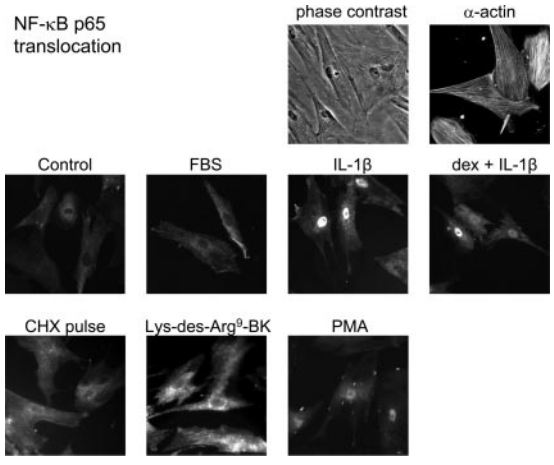

Fig. 3. Effect of drugs or drug combinations (1 h) on the subcellular localization of NF-κB subunits in serum-starved SMCs: sample photographic record of the immunofluorescence of p65 (original magnification, 400×). The morphology of the cells and their expression of α-actin are also shown (top). Agent concentrations: IL-1β, 5 ng/ml; FBS, 20% (v/v); Lys-des-Arg^9-BK, 100 nM; CHX, 71 μM; PMA, 1 μM; dexamethasone (dex), 100 nM.

human serum or FBS for 1 h) nor presence of Lys-des-Arg^9-BK (100 nM) or cycloheximide (71 μM) significantly induced p65 nuclear translocation (Figs. 3 and 4A). Dexamethasone cotreatment significantly but only partially reduced the effect of IL-1β on p65 translocation (Figs. 3 and 4A). The DMSO vehicle of dexamethasone had no effect (data not shown). An enzyme-linked immunosorbent assay to detect the p50 subunit of NF-κB in nuclear extracts confirmed that IL-1β was the only significant activator and that FBS, human serum, cycloheximide (CHX), or the B_1 receptor agonist were not active in this assay (data not shown; Mercury Transfactor NF-κB p50 kit; Clontech, Mountain View, CA). Figure 4B presents the effects of treatments related to protein kinase C (PKC) in the photographic p65 translocation assay. PMA (1 μM, 1 h) was a significant stimulant of this translocation, but was less active than IL-1β. The effect of PMA on p65 nuclear translocation was significantly inhibited by GF109203x, a broad PKC inhibitor that also significantly potentiated the effect of IL-1β (Fig. 4B).

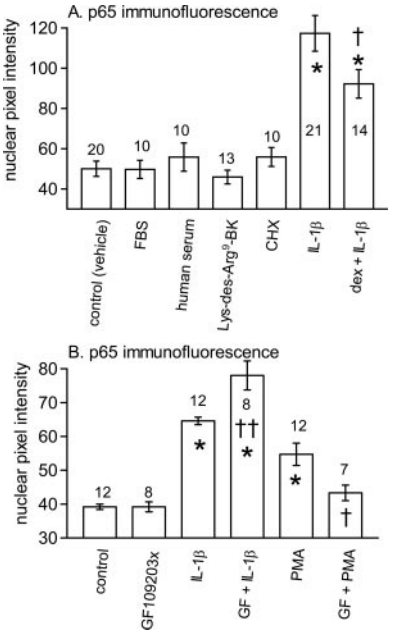

Fig. 4. NF-κB regulation of B_1 receptor expression in human SMCs. A, image analysis (see *Materials and Methods*) was used to assess the relative abundance of p65 in the nucleus of SMCs. Photographic record from 4 different days of experiments based on four different cell lines were exploited. Values are mean ± S.E.M. of the averaged median pixel intensity for nuclei from the indicated number of microscopic fields. The nonparametric analysis of variance (Kruskall-Wallis test) indicated that values were statistically heterogeneous ($P < 0.001$). The Mann-Whitney test was applied to assess the effect of treatments relatively to the controls (**, $P < 0.001$) or the effect of the concomitant treatment added to IL-1β relative to IL-1β alone (†, $P < 0.05$; ††, $P < 0.01$). B, relative abundance of p65 in the nucleus of SMCs based on the photographic techniques (as in A) for treatments related to protein kinase C (PMA, 1 μM; GF109293x, 5 μM; IL-1β, 5 ng/ml). Statistics as in A (*, $P < 0.001$ relative to control; †, $P < 0.05$; ††, $P < 0.01$ relative to same stimulus).

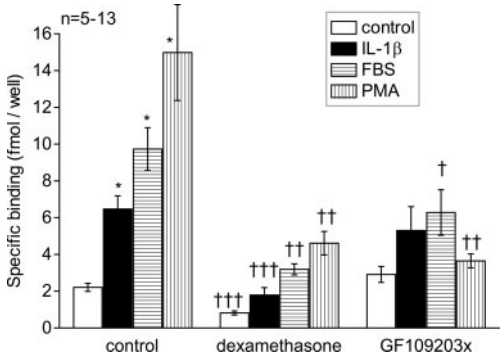

Fig. 5. Effect of inhibitory drugs on the stimulated cell surface B_1 receptor density in SMCs estimated using the specific binding of [^{3}H]Lys-des-Arg^9-BK (1 nM). HUA SMC lines were initially serum-starved then treated for a further 4-h period with the indicated drugs and stimuli before the binding assays (concentrations as in Fig. 4; control wells contained the DMSO vehicle of dexamethasone or GF 109230x). Values are the means ± S.E.M. of 5 to 13 determinations, each composed of duplicate observations in different cell lines. The Mann-Whitney test was applied to assess the effect of stimuli relatively to the control group in cells exposed to the drug vehicle (*, $P < 0.01$; *, $P < 0.001$) or the effect of the inhibitory drug relative to the control group subjected to the same stimulation (†, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.001$).

Effect of Drugs and p65 Silencing on the Cell Surface Density of B_1 Receptors. A saturating concentration of the agonist radioligand (1 nM) has been used to assess the effect of dexamethasone and of GF109203x on the cell surface B_1 receptor density; some of the cells were cotreated with IL-1β, FBS, or PMA (Fig. 5). Dexamethasone significantly diminished the baseline receptor expression in 4 h, under both basal and stimulated conditions. The inhibitory effect of GF109203x reached significance only in cells costimulated with FBS or PMA.

Four days of transfection with relevant siRNAs silenced p65 expression [immunoblot in total SMC extract (Fig. 6, top); two siRNA-lipofectamine ratios were tested and the second applied in the following experiments], but the nontargeting siRNA had no effect on p65 levels. NF-κB silencing reduced the abundance of cell surface binding sites for [^{3}H]Lys-des-Arg^9-BK under basal conditions and inhibited the stimulatory effect of IL-1β, FBS, and PMA (stimulation

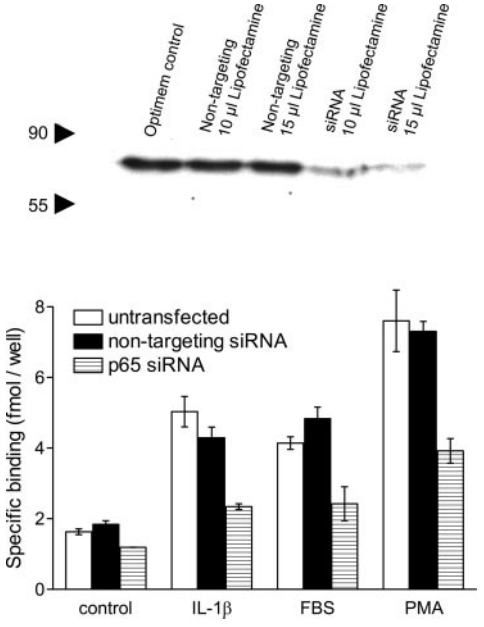

Fig. 6. Silencing of NF-κB p65 in human SMCs. Top, immunoblot of p65 in the total cell extract performed 4 days after siRNA transfection. Two siRNA/lipofectamine ratios were tested, and the second one was applied in the subsequent experiments. Bottom, effect of p65 silencing (4 days after transfection) on the cell surface expression of B_1 receptors (radioligand binding assay as in Fig. 5) as modified by stimulants (applied for the last 4 h of culture, concentration as in Fig. 4). Values are the means ± S.E.M. of two experiments composed of duplicate determinations.

during the last 4 h in serum-starved cells; Fig. 6, bottom). Pretreatment with the nontargeting siRNA did not affect B_1 receptor expression.

RT-PCR Analysis of Kinin B_1 Receptor mRNA in Cultured Human SMCs. Control B_1 receptor mRNA concentration in SMCs was measured in confluent, FBS-starved cells (48 h) and expressed in a ratio with the concentration of GAPDH mRNA (a stable molecule, Fig. 7). Of the stimulants tested, only IL-1β and PMA increased the cellular concentration of B_1 receptor mRNA. An additional 3-h incubation with the transcription inhibitor actinomycin D sharply reduced the B_1 receptor/GAPDH mRNA ratio in control cells, which indicated that the B_1 receptor mRNA was short-lived. CHX, FBS, or human serum mainly affected mRNA inactivation, in that actinomycin D treatment did not reduce the mRNA concentration in these cells (Fig. 7). This effect was also seen with IL-1β and PMA, with a nonsignificant concentration decrease with actinomycin D treatment. The B_1 receptor agonist Lys-des-Arg^9-BK had no significant effect on B_1 receptor mRNA concentration (with or without actinomycin D); compound 11 (100 nM, data not shown), a high affinity nonpeptide antagonist of the B_1 receptor (Morissette et al., 2004), had no effect as well.

Inhibitory drugs were combined with stimulants of B_1 receptor expression in SMCs to isolate effects on the acute rise of mRNA concentration or on the stability of the RNA (Fig. 8). Treatments with IL-1β or PMA acutely increased the B_1 receptor mRNA in control cells; the effect of FBS did not reach significance, but, as observed previously, all 3 stimulants prevented the drop of mRNA concentration caused by actinomycin D treatment. Dexamethasone did not significantly influence the acute effect of any of the three stimulants but did significantly reduce the effect of IL-1β and PMA seen after actinomycin D treatment (Fig. 8); the combined inhibition did not reach statistical significance for FBS. GF109203x cotreatment inhibited the acute stimulatory effect of IL-1β and PMA and all three conditions of stimulation in the presence of actinomycin D. These results indicated the possibility of a special role for PKC in B_1 receptor mRNA stabilization.

Discussion

Despite inherent genetic and environmental variability, we elected to use primary cultures of human SMCs to characterize the kinin B_1 receptor expression and identify physiological mechanisms that may not be intact in transformed or permanent cell lines. The culture procedures themselves, particularly the serum supplements (which are often not controlled), have important effects on the results. The regulation of B_1 receptor expression in the present cellular system has several distinct features relative to other documented systems. The B_1 receptor population in the present cellular model and the rabbit aortic SMCs was up-regulated in response to cycloheximide pulse or FBS treatment but in an NF-κB-dependent manner only in rabbit cells (Sabourin et al., 2002b). Kinin-induced B_1 receptor up-regulation was observed mainly in IMR-90 cells (see Introduction), and Lys-des-Arg^9-BK had a synergistic effect with IL-1β in that system (Phagoo et al., 2001); however, this regulatory pathway was not detected in primary cultures of human or rabbit SMCs (Sabourin et al., 2001; Marceau et al., 2002; present results). PKC regulation of B_1 receptor expression was first identified in IMR-90 cells (Zhou et al., 1998), and IL-1-induced enrichment of B_1 receptor mRNA underlies the ratio-

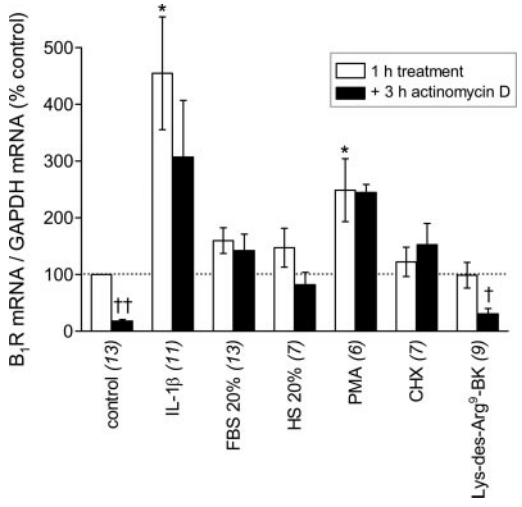

Fig. 7. Effect of actinomycin D (2 μM, 3 h) on the B_1 receptor mRNA/GAPDH mRNA ratio determined by real-time RT-PCR in HUA SMCs prestimulated with IL-1β (5 ng/ml, 1 h), FBS or human serum (HS) (20%, 1 h), PMA (1 μM, 1 h), CHX (71 μM, 1 h), or Lys-des-Arg^9-BK (100 nM, 1 h). Results are the means of the number of determinations indicated between parentheses and derived from different cell lines. Kruskal-Wallis test showed that the groups were not statistically homogeneous ($P < 0.001$). The effect of each 1-h treatment was further compared with the control group using Mann-Whitney test (*, $P < 0.001$). The 1-h treatments were also compared with the effect of the same treatment with the addition of actinomycin D (solid bar next to each open bar; Mann-Whitney; †, $P < 0.01$; ††, $P < 0.001$). Results are normalized within each cell line and day of experiment (100% = control mRNA ratio at 1 h).

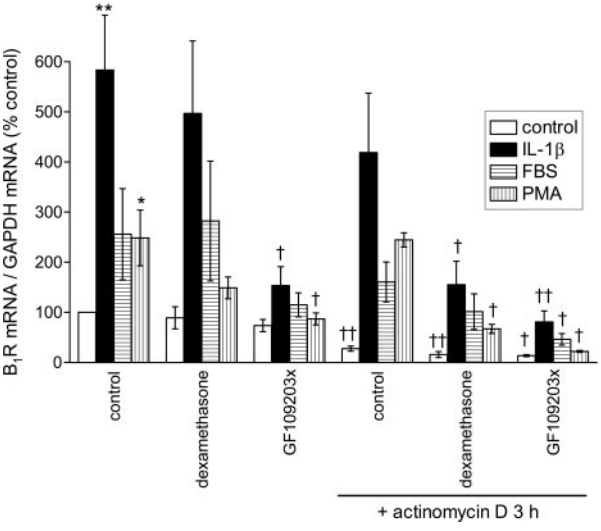

Fig. 8. Effect of inhibitory drugs on B_1 receptor mRNA regulated by IL-1β (5 ng/ml), FBS (20%), or PMA (1 μM) in SMCs. B_1 receptor mRNA was measured after 1 h of treatment and an optional 3-h period of treatment with actinomycin. Presentation as in Fig. 7 (n = 6–11). Kruskal-Wallis test showed that the groups were not statistically homogeneous ($P < 0.001$). In control cells treated with vehicle of inhibitory drugs, the effect of the stimulants was compared with the control group using the Mann-Whitney test (*, $P < 0.001$; **, $P < 0.0001$). The effect of inhibitory drugs added to stimulants (dexamethasone, 100 nM; GF109203, 5 μM; actinomycin D, 2 μM alone or combined) was also compared with the effect of the same stimulant without drug (leftmost set of histograms; Mann-Whitney; †, $P < 0.01$; ††, $P < 0.001$). Results are normalized within each cell line and day of experiment (100% = control mRNA ratio at 1 h).

nale of expression cloning of this gene in these cells (Menke et al., 1994).

The radioligand binding experiments reveal that the B_1 receptor population is tightly regulated in HUA SMCs; only the cell surface abundance (B_{max}) is variable, not affinity (K_D) (Fig. 1), an observation that has been reported in other cell-based systems (Galizzi et al., 1994; Bastian et al., 1998; Sabourin et al., 2002b). However, the baseline population of B_1 receptors in the human SMCs was functional and supported the phosphorylation of extracellular signal-regulated kinase 1/2 (data not shown) and specific effects on cell motility (Morissette et al., 2006). Evidence based on the wild-type rabbit B_1 receptors or its recombinant conjugate suggested that, like other inflammatory signaling molecules, the cell surface protein is short-lived (Fortin et al., 2003a); the same is true for the natural human receptor given that cycloheximide treatment diminished the radioligand B_{max} (Fig. 2B). IL-1, human serum, FBS, cycloheximide pulse, and PMA all altered the receptor B_{max} by increasing synthesis of the B_1 receptor during the standardized 4-h incubation period, based on specific effects on the corresponding mRNA and the absence of stimulation in the presence of cycloheximide.

The present siRNA results support for the first time the oft-proposed role of NF-κB signaling in B_1 receptor expression (see Introduction and Fig. 6). The p65 subunit, a component of the classic NF-κB heterodimer, is necessary for full stimulation of B_1 receptor B_{max} by IL-1, FBS, and PMA, and it is noteworthy that treatment with the siRNA somewhat decreased the baseline expression in control cells. The results of this approach support the idea that B_1 receptor expression is dependent on NF-κB, although the effects of the individual stimulants on NF-κB activation varied widely under the tested conditions. Whereas human serum, FBS, and cycloheximide did not activate NF-κB nuclear translocation (Figs. 3 and 4), IL-1β and PMA showed strong and intermediate tendencies, respectively, to induce p65 nuclear translocation. As expected, the effect of PMA on NF-κB nuclear translocation was mediated by a protein kinase C (shown by inhibition by GF109203x), although unexplained interference of this drug with IL-1β signaling increased the p65 translocation (Fig. 4). Therefore, certain stimulants could rely on the basal level of NF-κB activation in the cell-based model (SMCs subjected to 48-h FBS starvation) to increase the effective expression of a basal B_1 receptor gene transcription rate.

The real-time PCR measurement of the B_1 receptor-coding mRNA provided insight into possible NF-κB-independent stimulatory mechanisms. Although the capacity to stimulate NF-κB predicted the acute (1 h) effect of the stimuli of the mRNA cellular concentration (significant only for IL-1β and PMA; Fig. 7), an additional actinomycin D treatment period identified a second class of stimulants selectively stabilizing the mRNA concentration (the human and bovine sera and cycloheximide). However, PMA and IL-1β also completely stabilized the mRNA, as previously noted for the cytokine in another system (Zhou et al., 1998). PMA activates protein kinase C; thus, its effect on receptor mRNA stabilization was completely inhibited by the kinase inhibitor GF109203x (Fig. 8). This mediation has been generalized to the more physiological stimuli of stabilization (IL-1β, FBS) using the same drug, which reduced the total cell B_1 receptor mRNA after 1-h treatment with IL-1 and after the additional RNA synthesis blockade period for FBS (Fig. 8) and the binding B_{max} in FBS-treated cells (Fig. 5). These results warrant further investigation to identify relevant protein kinase C isoform(s) and signaling pathways. Whether protein kinase C-mediated stabilizing influence works in concert with a proposed destabilizing RNA sequence (AUUAAA) that overlaps the stop codon (Zhou et al., 1999) remains to be studied. The possibility that additional mechanisms of regulation are recruited by specific stimulants, such as FBS, which often had a large effect on receptor B_{max}, cannot be excluded. A role for activator protein 1 has been proposed in B_1 receptor expression (Yang et al., 2001). Such regulatory diversity has a possible interest to explain situations in which the B_1 receptor up-regulation occurs without frank systemic inflammation. We have recently observed that pigs undergoing long-term treatment with the angiotensin I-converting enzyme inhibitor enalapril exhibit a generalized B_1 receptor up-regulation (mRNA in several organs and endothelial immunoreactivity in tissue sections). This protracted response occurred without changes in the white blood cell counts or increases in the phase reactant C-reactive protein (Moreau et al., 2005a). On the other hand, pharmacological inhibition did not support important roles for tyrosine or MAP kinases in the basal or stimulated expression of B_1 receptors in the present cellular model (data not shown), in contrast to other in vitro systems (Larrivée et al., 1998; Phagoo et al., 2001).

The antiinflammatory glucocorticoid dexamethasone has been known for a long time as a negative regulator of B_1 receptor up-regulation (deBlois et al., 1988; Leeb-Lundberg et al., 2005). As discussed elsewhere, dexamethasone is an indirect inhibitor of NF-κB-induced gene transcription (Sabourin et al., 2002b); it only slightly diminished the IL-1β-induced nuclear translocation of NF-κB p65 (Figs. 3 and 4). The activated glucocorticoid receptor may inhibit NF-κB translocation through the stimulation of the de novo synthesis of IκB (an endogenous inhibitor of translocation) but is mainly inhibitory through a protein-protein interaction with activated NF-κB in the nucleus (Rhen and Cidlowski, 2005). Thus, the functional inhibition of NF-κB by dexamethasone may be more profound than that expected from a translocation assay, and this has been observed under all tested stimulation conditions using the B_1 receptor binding assay (Fig. 5), reproducing the effect of the siRNA. It is noteworthy that

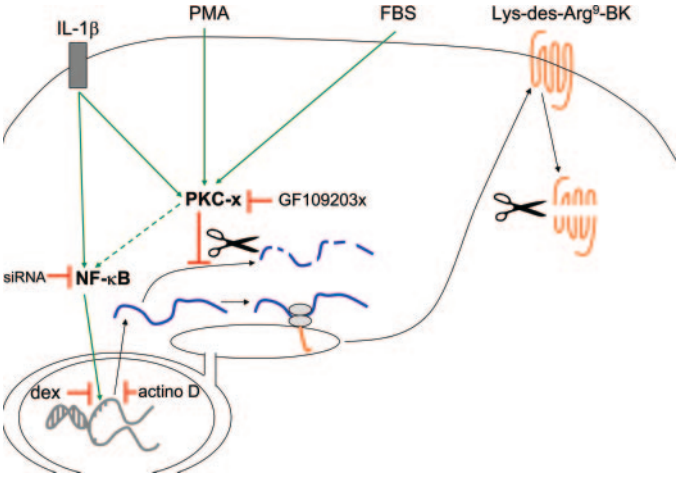

Fig. 9. Schematic representation of the regulation of B_1 receptor expression in SMCs. Green, stimulatory effects; red, inhibitory effects; blue, mRNA; orange, protein; dex: dexamethasone; actino D, actinomycin D.

the effect of dexamethasone on the mRNA concentration was nonsignificant after 1 h of stimulation but clearer in the control cells and in those treated with the NF-κB stimulants IL-1 and PMA after RNA synthesis inhibition (Fig. 8). The measurements of receptor mRNA in the total cell extract as applied in the present experiments seem to be more sensitive to stabilizing influences than to the transcriptional rate.

Lys-des-Arg^9-BK stimulated neither NF-κB translocation nor stabilization of B_1 receptor mRNA. The Lys-des-Arg^9-BK–induced effect on B_1 receptor B_{max} was comparatively small (1.3-fold) and does not support the idea that autoregulation (kinin-induced up-regulation of kinin B_1 receptors, see Introduction) is generally applicable.

In conclusion, two types of stimuli were identified for kinin B_1 receptor up-regulation in HUA SMCs (Fig. 9): those that increased mRNA stability only (sera, cycloheximide) and others that also stimulated NF-κB (IL-1β, PMA). Protein kinase C is a likely mediator of the mRNA stabilization by physiological stimuli. The siRNA approach indicated that NF-κB is indispensable for the effective expression of this important signaling molecule (control of vascular tone, cell migration etc.) at the SMC cell surface.

Acknowledgments

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