Expression of kinin B₁ receptor in fresh or cultured rabbit aortic smooth muscle: role of NF-κB

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THE KININS [peptides related to bradykinin (BK)] are known to activate two types of G protein-coupled receptors, termed B₁ receptors (B₁R) and B₂ receptors (B₂R) (27). Lys-des-Arg⁹-BK (des-Arg¹⁰-kallidin) is the optimal agonist sequence of human and rabbit B₁Rs, and des-Arg⁹-BK is also a selective agonist, but of lower affinity in these species. The initial pharmacological definition of the B₁R was based on the analysis of the contractile effects of kinins on the isolated rabbit aorta (39). The B₁R is now widely recognized as an inducible gene, as certain forms of tissue injury trigger its de novo synthesis in tissues. For instance, bacterial lipopolysaccharide (LPS) injection in several animal species induces B₁R expression at the vascular cell level (14, 27, 32). The analysis of isolated rabbit blood vessel contractility has provided early insight into the inducible behavior, as the preparations, initially insensitive to kinins, specifically developed increasing maximal response (Eₘₐₓ) in response to B₁R agonists in a time-, temperature-, and metabolism-dependent manner (7, 40). This phenomenon, coined the postisolation induction paradigm (27), was prevented by treatment with inhibitors of RNA or protein synthesis and was potentiated by certain cytokines such as epidermal growth factor (EGF), interleukin-1 (IL-1), and oncostatin M, but only modestly by LPS applied in vitro. Glucocorticoids and some mitogen-activated protein kinase inhibitors could reduce specifically the spontaneous or cytokine-mediated upregulation of the contractile response to des-Arg⁹-kinins without acutely interfering with kinin-induced contraction (11–13, 20). Similar reasoning has been applied to the postisolation increase in responsiveness to a B₁R agonist in the isolated human umbilical vein (42). These observations suggested a complex regulatory control of B₁R expression, but the demonstration of receptor upregulation was limited to the description of specific alterations of the contractile response.

The molecular analysis of the expression of the B₁R gene has been mostly dependent on human or animal cultured cells capable of expressing B₁Rs, but these systems may not be satisfactory in all respects. For instance, cultured smooth muscle cells (SMCs) derived from the rabbit arteries express a population of B₁R that mediates such effects as increased inositol phosphate turnover, intracellular calcium increase, prosta-cylin secretion, and DNA synthesis; radioligand-binding techniques show that there is a functional baseline receptor population that is variably increased by cytokine treatment (16, 24–26, 30, 44). Furthermore, studies of various human cell lines have shown that the baseline B₁R mRNA concentration is increased partly sensitive to kinins, specifically developed increasing maximal response (Eₘₐₓ) in response to B₁R agonists in a time-, temperature-, and metabolism-dependent manner (7, 40). This phenomenon, coined the postisolation induction paradigm (27), was prevented by treatment with inhibitors of RNA or protein synthesis and was potentiated by certain cytokines such as epidermal growth factor (EGF), interleukin-1 (IL-1), and oncostatin M, but only modestly by LPS applied in vitro. Glucocorticoids and some mitogen-activated protein kinase inhibitors could reduce specifically the spontaneous or cytokine-mediated upregulation of the contractile response to des-Arg⁹-kinins without acutely interfering with kinin-induced contraction (11–13, 20). Similar reasoning has been applied to the postisolation increase in responsiveness to a B₁R agonist in the isolated human umbilical vein (42). These observations suggested a complex regulatory control of B₁R expression, but the demonstration of receptor upregulation was limited to the description of specific alterations of the contractile response.

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tion is a powerful upregulator of BR mRNA in human cell lines (37, 51) via the inhibition of mRNA degradation (51). These findings may be related to the stimulation of the postisolation induction of BRs by temporary inhibition of protein synthesis in the rabbit aorta (13). The present experiments aimed at comparing the regulation of BR expression in rabbit freshly isolated aortic tissue (postisolation induction) and primary SMCs. Because nuclear factor (NF)-κB is a transcription factor controlling the expression of numerous genes associated with immunity and inflammation (3) and previous experimental results support a role of NF-κB in BR induction (8, 34, 43), its implication in the upregulation of the kinin BR was studied in the present systems.

MATERIALS AND METHODS

Drugs. Des-Arg⁸-BK was purchased from Bachem Bioscience (King of Prussia, PA), MG-132 (Cbz-Leu-Leu-leucin- nal) and BAY 1-7082 were purchased from BioMol Research Laboratories (Plymouth Meeting, PA). Human recombinant EGF was from Calbiochem (San Diego, CA), IL-1β was purchased from R&D Systems (Minneapolis, MN), and actinomycin D (actinomycin) was obtained from Merck (West Point, PA). The other drugs used were purchased from Sigma (St. Louis, MO).

Treatment of animals used as sources of tissues. Groups of male New Zealand White, pathogen-free rabbits (Charles River Canada) weighing 1.5–2.2 kg were used as a source of tissues for all experiments. Most animals were not treated and directly euthanized as outlined below to aseptically remove the thoracic aorta to study the postisolation induction of the BR (see below). Two additional groups of four rabbits each were treated before euthanization: the acute treatments consisted of the intravenous injection of LPS (50 μg/kg, extracted from Escherichia coli serotype O111:B4, Difco;Detroit, MI) or its saline vehicle (500 μl/kg). Five hours after the injection, all treated rabbits were consecutively euthanized by CO₂/O₂ asphyxiation, and the thoracic aorta was quickly removed, frozen in liquid N₂, and kept at −80°C until RNA isolation (methods described previously) (28).

Postisolation treatment of rabbit aorta segments. This series of experiments was designed to mimic the procedures applied in previous contractility studies based on the rabbit aorta but in a manner compatible with mRNA harvesting. Aseptically removed thoracic aortas were placed in serum-free medium 199 (Life Technologies), further cleaned, deendothelialized, and cut into five pieces. Some pieces were immediately frozen in liquid N₂ (nunincubated controls). The other pieces were distributed in 12-well plates containing sterile medium 199 (2 ml) and, in some wells, a drug documented to influence the upregulation of BR. After 3 h of incubation (37°C, 5% CO₂), the tissue pieces were frozen and the RNA was later extracted as outlined above.

Contractility experiments. The contractile effect of the BR agonist des-Arg⁸-BK and of the α-adrenoceptor agonist phenylephrine (PE) were recorded precisely as described (20) to test the effect of some treatments that inhibit the function of NF-κB on the postisolation induction of BR and the specificity of the effects, respectively. Briefly, all tissues were submitted to the construction of five full cumulative concentration-effect curves. The ones for the BR agonist des-Arg⁸-BK were conducted after 1, 3, and 6 h of incubation. The concentration-effect curves for PE were established at 1.5 and 7.5 h as more stable contractile responses. Tissues were amply washed with fresh Krebs solution between stimulations. The selected inhibitory drugs, postulated to exert no direct myotropic effect and no overt toxicity on the contractile mechanisms, were introduced in the bathing fluid of some tissues (continuous application) to analyze the mechanism of the postisolation BR induction. Contractions are expressed as the percentage of the maximum PE-induced contraction recorded at a time of 1.5 h, an internal standard for each tissue. Sigmoidal concentration-effect curves are characterized by the half-maximal effective concentration (EC₅₀) and the maximum relative contraction maximum (Eₘₐₓ; percentage of the internal standard).

BR expression in cultured rabbit aortic SMCs. Rabbit aortic SMCs, cultured and characterized as previously described (24), were the alternate model to study BRs regulation in vitro. Cells were used at passages 3–6, at a stage where the BR basal expression is relatively low and its hormonal induction (EGF treatment) is high (44). Separate protocols dealt with the effect of drugs or fetal bovine serum (FBS) on BR expression; both mRNA and radioligand were assessed in these experiments, which were based on cells cultured in 6- or 12-well plates, respectively. To reduce the baseline BR expression level, the FBS-containing medium was replaced by serum-free medium 199 for 24 h; various stimulant or inhibitory drugs were then added to the serum-free medium and the total RNA was extracted after 3 h according to Chomczynski and Sacchi (10), or the binding was determined as outlined above after 4 h of incubation. When inhibitory drugs were tested against stimulatory drugs, the first type of compounds was introduced 30 min before the second (at a time of −4.5 h relative to the binding assay). The binding assay was conducted as described (24) except for the identity of the ligand, which was [³H]Lys-des-Arg⁸-BK (³H)des-Arg⁸-kallidin, NEN Biosciences, 64 Ci/mmol). Briefly, cells were seeded at a density of 1.5 × 10⁵ cells/well in 12-well plates coated with gelatin. After 24 h, the wells were washed twice with the binding medium (consisting of medium 199 supplemented with 0.1% bovine serum albumin, 3 μM amastatin, 1 μM captopril, 1 μM phosphoramidon (Sigma), and 0.02% (wt/vol) sodium azide) and filled with 1.0 ml of prewarmed (37°C) binding medium. The BR ligand (0.125–8 nM) and cold competing peptides (1 μM Lys-des-Arg⁸-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 ice-cold PBS (pH 7.4). One milliliter of 0.1 N NaOH was finally added to dissolve the cells. Radioactivity in the resulting suspension was determined by scintillation counting (5–10 min/vial).

To detect stimulus effect on mRNA stability, a variation of the mRNA extraction protocol was applied to cells submitted to short stimulations (from 30 min to 2 h) and then treated for up to 4 h with actinomycin D to inhibit transcription.

Semi-quantitative duplex RT-PCR. The RT-PCR experiments were applied to RNA extracted from fresh or incubated aortic tissue or from cultured aortic SMCs and were conducted using Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech) as indicated by the manufacturer. The general conditions (primers used, PCR conditions, and Southern blot analysis of the RT-PCR) were as previously reported (28). Briefly, 2 μg of total RNA sample, 250 ng of the sense and antisense primers for a specific fragment of the rabbit BR, 25 ng of the sense and antisense primers for a specific fragment of rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 250 ng of an oligo (dT)₁₁₅, and water to a final volume of 50 μl were added to each tube of Ready-To-Go RT-PCR Beads. The tubes were incubated for 30 min at 42°C for the RT reaction. The samples were then submitted to a
PCR followed by a Southern blot analysis as described previously (28).

**Nuclear runon.** This protocol was modified from Chacko et al. (9). Three days before the experiment, 30 75-cm² flasks were plated with 10⁶ rabbit aortic SMCs each and grown up to confluence in medium 199 containing 10% serum and 1% penicillin-streptomycin. Cells were serum starved 24 h before the experimentation. Cell flasks were treated with saline, IL-1 (5 ng/ml), EGF (100 ng/ml), cycloheximide (CHX; 71 μM), or FBS (10%) 2 h before SMCs were collected in their serum-free medium. The lysates were spun for 5 min at 2,500 g at 4°C. The pellets were washed in cold PBS and centrifuged once more. The pellets were resuspended in 2.5 ml lysis buffer (10 mM Tris-HCl, 5 mM MgCl₂, 0.1 mM EDTA, and 40% glycerol), and frozen in liquid N₂.

Nuclear aliquots (10⁷ nuclei) were thawed on ice and spun for 5 min at 300 g. The pellets were resuspended in 50 μl transcription buffer 1× (25 mM Tris-HCl, 1 mM dithiothreitol, 3 mM spermidine, 2.5 mM MgCl₂, and 500 mM NaCl containing 5 μl ATP, 3 mM GTP and CTP, and 10 μl α-32P[UTP (100 μCi)] and incubated 30 min at 37°C. The mixes were digested with DNase I (Amersham Pharmacia, 100 U/ml, 37°C, 30 min) and then digested with proteinase K (USB, 200 μg/ml, 37°C, 1 h). RNA was then obtained with two successive phenol-chloroform-isooamylic alcohol extractions, precipitated with ethanol, and resuspended in 50 μl of 10 mM Tris-HCl (pH 8)-1 mM EDTA. To perform the dot blots, a nylon membrane layer was created on a 3 M Whatman filter, both prewet in 1.4 M NH₄OAc, was inserted in the dot blot apparatus. Ten micrograms of plasmidic DNA (rabbit wild-type B₁R coding sequence in pcDNA3) (21) were denatured, fragmented by a 15-min boiling treatment in 0.3 M NaOH, and neutralized with NH₄OAc to a 1.4 M final concentration before being loaded in a slot of the dot blot apparatus. The slot was then washed with 1.4 M NH₄OAc, and the nylon membrane was rinsed, dried, and exposed to ultraviolet light for 5 min. Each slot was cut, inserted in separate tubes, and prehybridized (50% formamide, 50 mM phosphate buffer, 5× saline-sodium citrate (SSC), 1% SDS, 5× Denhardt, and 100 μg/ml ssDNA) for 3 h at 42°C. Prehybridization buffer was then replaced by 0.5 ml hybridization buffer (50% formamide, 50 mM phosphate buffer, 5× SSC, 1% SDS, 1× Denhardt, 100 μg/ml ssDNA, and 50 μl radiolaabeled RNA). Membranes were incubated 72 h at 42°C before being washed three times for 30 min in SSPE-SDS solutions. Membranes were exposed for 24 h, and the results were analyzed (PhosphorImager) and quantified (Imagequant software).

**Drug effect on the translocation of NF-κB p65 from the cytosol to the nucleus.** The effect of stimulants and inhibitors of B₁R expression was tested in an immunofluorescence assay of subcellular localization of the NF-κB p65 subunit p65, as this protein is translocated from the cytosol to the nucleus upon activation of NF-κB (3). Subconfluent rabbit aortic SMCs were transferred in a serum-free medium for 24 h and then treated with a drug for 3 h or with a drug combination for 3.5 h (a drug tested for inhibition was applied first, and then IL-1β was applied 30 min later). After incubation at 37°C, the cells were fixed (1% paraformaldehyde), permeabilized (0.5% Triton X-100), and stained with monoclonal antibodies to p65 (Transduction Laboratories, 1:100 dilution) or to α-actin (a smooth muscle marker, Sigma, dilution 1:200). The monoclonal antibody staining was revealed using Alexa fluor 594-conjugated anti-mouse IgG (Molecular Probes; Eugene, OR).

**Statistical analysis.** Statistical analysis was performed using the Kruskal-Wallis test followed by the Mann-Whitney test using the InStat 2.0 computer program (GraphPad Software; San Diego, CA). The parameters of Scatchard plots (binding data treatment) were obtained using a computer program (45).

**RESULTS**

**Multiplex RT-PCR analysis of kinin B₁R mRNA in fresh aortic tissue.** Deendothelialized aortic tissue freshly isolated from healthy rabbits and frozen as quickly as possible contains a low measurable concentration of B₁R mRNA according to the RT-PCR techniques applied (Fig. 1, nonincubated). A 3-h incubation
at 37°C was associated with a nearly fourfold stronger signal (Fig. 1, incubated control). This experimental situation mimics the postisolation induction of B1R in the contractility assays based on the rabbit aorta. The latter system is known to be influenced by several drugs introduced in the tissue bathing fluid, and some of these treatments were assessed for an effect on tissue mRNA (Fig. 1, postisolation induction). Incubation of aortic tissue with IL-1β or FBS (10%) significantly increased the B1R mRNA concentration above the level of incubated tissues, whereas the effects of EGF or CHX coincubation did not reach statistical significance. Actinomycin D or dexamethasone treatments significantly reduced the B1R mRNA concentration, but the DMSO vehicle of the latter drug had no significant effect.

Separate pieces of aortic tissue were derived from saline-treated rabbits (500 μl/kg iv 5 h before death) or LPS-treated animals (50 μg/kg iv 5 h before death). These tissues were not further incubated ex vivo. The RT and PCR reactions as well as the subsequent Southern blots were performed simultaneously on extracts from tissues treated ex vivo or obtained from rabbits treated in vivo to provide comparative data expressed on the same scale. The mRNA values from the tissues removed from saline-treated rabbits did not differ from the ones derived from nonincubated tissues from untreated rabbits (Fig. 1). However, LPS treatment applied before death increased mRNA concentration relative to the saline-treated control group.

Effect of treatments intended to inhibit NF-κB function on fresh rabbit aortas. Control aortic rings isolated from normal rabbits exhibited the well-characterized transition from a null response to a time-dependent increase in the maximal response to the B1R agonist des-Arg⁹-BK (Fig. 2, control curves). In this graphic representation, Fig. 2, C and D, represents the responses recorded after 3 or 6 h of incubation, respectively. The maximal level of response to the kinin recorded at 1 h was always close to zero (0.4 ± 0.1% of the maximal PE-induced contraction recorded at 1.5 h in the control group, not significantly influenced by drugs). BAY 11-7082 (used here at 5 μM) is an inhibitor of inhibitory (IκB phosphorylation indirectly inhibiting NF-κB function (38). Proteasome inhibitors also indirectly inhibit NF-κB by stabilizing ubiquitinated IκB in the cytosol (47); we used MG-132 (1 μM) as a representative proteasome inhibitor (22). The two drugs were remarkably effective to prevent the time-related increase of responsiveness to the B1R agonist (Fig. 2; both drugs significantly reduce 6-h \( E_{\text{max}} \) and BAY 11-7082 reduces the 3-h \( E_{\text{max}} \), Mann-Whitney test). However, the two drugs also significantly depressed the late response to the \( \alpha \)-adrenoceptor agonist PE, an outcome that was never observed with treatments based on the other drugs shown in Fig. 1 or additional ones (21).

Multiplex RT-PCR analysis of kinin B₁R mRNA in cultured aortic SMCs. The control B₁R mRNA concentration in rabbit aortic SMCs was measured in confluent cultured aortic SMCs. The control B₁R mRNA concentration in rabbit aortic SMCs was measured in confluent cultured aortic SMCs.

![Fig. 2.](http://ajpheart.physiology.org/) Cumulative concentration-effect of des-Arg⁹-bradykinin (BK) and phenylephrine as modified by time and treatment with BAY 11-7082 or MG-132 (5 or 3 μM, respectively; continuous application). Values are means ± SE of 7 determination for BAY 11-7082-treated tissues, 13 for MG-132-treated tissues, and 25 for controls (DMSO vehicle of the drug). See text for analysis.
ent cells starved of FBS for 24 h (Fig. 3). The set of drug treatments applied to fresh tissues incubated ex vivo was applied to cultured cells (Fig. 3A). IL-1β, CHX, EGF, or FBS treatment (that is, restoring the FBS concentration to 10% after serum starving) were statistically significant stimulants, whereas actinomycin D reduced the B1R-to-GAPDH mRNA concentration ratio. Dexamethasone or the NF-κB inhibitors MG-132 or BAY 11-7082 or their DMSO vehicle exerted no significant effect (Fig. 3A) as well as diclofenac sodium (500 nM). The latter drug, a cyclooxygenase inhibitor that prevents the synthesis of prostaglandins, was tested because it prevented interference from autocrine prostaglandins in an assay based on these cells (B1R-mediated DNA synthesis) (26).

Fig. 3. Concentration of B1R mRNA in rabbit aortic smooth muscle cells (6-well plates). A: serum-starved cells were submitted to 3-h treatments (drug concentrations identical to those in Fig. 1 with, in addition, 500 nM diclofenac, 5 μM BAY 11-7082, or 1 μM MG-132). Results (B1R-to-GAPDH ratio) are means ± SE of 8 determinations except for actinomycin D, diclofenac, dexamethasone, BAY 11-7082 (n = 4), or DMSO (n = 16). A Kruskall-Wallist test showed that the groups were not statistically homogenous (P = 0.0004). The effect of each drug treatment was further compared with the control group using the Mann-Whitney test (*P < 0.05; **P < 0.01; ***P < 0.001).

B: effect of MG-132 or BAY 11-7082 pretreatments (30 min) on the concentration of B1R in cells treated for 3 h with cytokines, FBS, or CHX. Drug concentrations and presentation are as in A (n = 12 for pooled vehicle with or without stimulants, 8 for MG-132-treated cells, and 6 for BAY 11-7082-treated cells). For each stimulation condition, values obtained in the presence of MG-132 or BAY 11-7082 were compared with those observed with the DMSO vehicle using the Mann-Whitney test (**P < 0.05).

Pretreatment with the proteasome inhibitor MG-132 (1 μM) prevented the increase of B1R mRNA induced by IL-1β or FBS (Fig. 3B). The alternate NF-κB inhibitor BAY 11-7082 (5 μM) prevented the effect of EGF and FBS on B1R mRNA concentration. Other inhibitory interactions did not reached statistical significance (Fig. 3B).

The multiplex RT-PCR assay for B1R mRNA was applied to cells stimulated for short periods with IL-1β, EGF, FBS, or CHX (2 h except for CHX, which was 30 min) and then treated with actinomycin D for up to 4 h to inhibit transcription (Fig. 4A). Both the basal and the cytokine- or FBS-stimulated mRNA concentrations decreased in cells over the 4-h period; more precisely, the B1R mRNA decreased faster than that coding for GAPDH during transcription inhibition by actinomycin, producing the negative slopes shown in Fig. 4A. The effect of CHX was opposite: the positive slope indicates that GAPDH mRNA decreased faster than...
that coding for B1R, evidencing a major effect of CHX on B1R mRNA stability.

Nuclear runon. The nuclear runon experiments support that IL-1β, EGF, FBS, and CHX are all transcriptional activators of the B1R gene (Fig. 4B). The effect of CHX was particularly strong.

Effect of treatments on the subcellular localization of NF-κB p65. The marker α-actin was expressed as stress fibers in aortic SMCs, whereas p65 was distributed mainly as a smooth labeling of the cytosol in FBS-starved cells (Fig. 5, top microphotographs and control-saline frame). The nuclear staining was markedly reinforced in cells treated with IL-1β, EGF, FBS, or CHX, but weak in cells treated with dexamethasone, MG-132, BAY 11-7082, or their DMSO vehicle (continuous 3-h application; Fig. 5). The inhibitory drugs or their DMSO vehicle were combined with two of the strong stimulants of nuclear translocation, IL-1β or

Fig. 5. Effect of drugs or drug combinations on the subcellular localization of the nuclear factor (NF)-κB p65 subunit. Rabbit aortic smooth muscle cells were fixed, permeabilized, and stained with no primary antibody or anti-α-actin (top microphotographs). The p65 nuclear staining was taken as a measure of NF-κB activation after cell treatments (all other microphotographs). Magnification: ×400.
FBS. Dexamethasone, MG-132, or BAY 11-7082 reduced p65 translocation to the cell nucleus induced by these stimuli; dexamethasone being relatively less effective.

Effect of treatments on the binding of [3H]Lys-des-Arg9-BK to cultured SMCs. In initial studies, the specific binding of [3H]Lys-des-Arg9-BK to FBS-starved SMCs was found to be saturable (Fig. 6; Scatchard regression at bottom). The calculated regression parameters were a dissociation constant ($K_d$) of 0.13 nM and a maximal receptor binding ($B_{max}$) of 1.39 ± 0.14 fmol/well for this particular cell line. Treating the cells continuously with the protein synthesis inhibitor CHX (71 μM) for 4 h before the binding assay slightly reduced the $B_{max}$ (0.88 ± 0.10 fmol/well). However, a pulsed application of the drug (a period of 3.5–4 h before the binding assay, followed by rinsing with the serum-free cultured medium prewarmed at 37°C) variably upregulated B1R in these cells (Fig. 6; $B_{max}$ of 1.86 ± 0.29 fmol/well; $K_d$ of 0.18 nM in this example), as reported for IMR-90 cells (48). This system is reminiscent of the contractility assay in the fresh aorta for which either inhibition or stimulation of $E_{max}$ was observed depending whether CHX was applied continuously or temporarily (13). A sharp stimulatory effect of FBS restoration was also observed ($B_{max}$ increased to 5.09 ± 0.23 fmol/well; $K_d$ constant at 0.14 nM; Fig. 6).

IL-1 and EGF have been previously documented to upregulate the B1R $B_{max}$ essentially without modifying the $K_d$ in rabbit cultured vascular SMCs (16, 25, 44). This also applies to pulsed CHX and FBS restoration in the culture medium (Fig. 6). This set of four stimuli (4-h treatments) was combined with inhibitory drugs (applied 30 min before treatments were initiated) in serum-starved cells; the B1R abundance was assessed by measuring the specific binding of 4 nM [3H]Lys-des-Arg9-BK to these cells (Fig. 7), as this concentration level equilibrates with a receptor proportion close to $B_{max}$ (Fig. 6). Figure 7 represents experiments based on different primary cultures of SMCs and the relative effect of the four stimulatory treatments varied from one line to the other but was always clear relative to controls. Diclofenac cotreatment had no important effect of the radioligand binding, whereas actinomycin D cotreatment inhibited the binding as stimulated with IL-1β, EGF, or FBS only, not the basal value or the one increased by pulsed CHX treatment (Fig. 7A). Dexamethasone did not modify basal binding but prevented a variable fraction of the stimulatory effect of IL-1β, EGF, FBS, or pulsed CHX (Fig. 7B). Similarly, cotreatment with dexamethasone partially inhibited the stimulation of B1R-mediated contractility in rabbit aortas treated with EGF, IL-1, or pulsed CHX (11, 12). The drugs inhibiting the function of NF-κB, MG-132 and BAY 11-7082, prevented the stimulatory effect of IL-1β, EGF, or FBS but were not effective against pulsed CHX on binding (Fig. 7, C and D).

DISCUSSION

Several in vivo systems have evidenced the induction of B1Rs from a null level by inflammatory stimuli (27, 32); in a fraction of them, Northern blot or RT-PCR techniques have supported the functional data by showing that B1R mRNA, expressed at very low levels in normal tissues, is sharply upregulated. These systems include LPS injection in the rabbit (28, 29, 41) and the rat (32), ischemia and reperfusion in the rabbit heart (31), and inflammatory models, systemic heat shock, myocardial infarction, and ischemia-induced angiogenesis in the rat (5, 6, 15, 19, 46).

In previous studies of postisolation induction of the B1R, the maximal contractile effect mediated by agonists of this receptor was evaluated after 6 h of incubation in organ baths containing Krebs medium. Most of the drugs shown in Fig. 1 were used at the same concentration as in the present RT-PCR experiments and exerted a statistically significant effect on the in vitro development of this contractile effect without affecting the effect of an α-adrenoceptor agonist (see original publications for statistical analysis). Inhibitory drugs were actinomycin D (unpublished data) and dexamethasone (11). The stimulant treatments were human recombinant IL-1β (13, 20), recombinant human EGF (20), and FBS (5%, D. deBlois, PhD Thesis,

Fig. 6. Binding of [3H]Lys-des-Arg9-BK to a representative rabbit aortic smooth muscle cell line starved of serum for 24 h and then treated for a further 4-h period with the indicated stimuli before the binding assays. See text for analysis.
DMSO (0.1% vol/vol) has a slightly stimulatory effect. CHX can be either inhibitory if applied continuously to aortic tissue (2, 7, 12) or stimulatory if applied during the first hour of in vitro incubation and then washed out (13). Nonincubated aortic tissue or tissue derived from saline-treated animals essentially do not functionally respond to B1R agonists in the first hour of ex vivo incubation (7, 41). In contrast, tissue derived from LPS-treated rabbits exhibits an immediate and strong response to a B1R agonist when tested during the first hour after isolation (41). The effect of stimuli of various nature (long in vitro incubation, cytokines, FBS, pulsed CHX, LPS given before animal death) and of some inhibitors (actinomycin, dexamethasone) on B1R mRNA concentration in the fresh rabbit aorta is well correlated to the contractile response to a B1R agonist, thus validating older functional work on B1R regulation based on contractility (see Introduction).

The same sets of stimulants (cytokines, FBS, pulsed CHX) and inhibitors (actinomycin D, dexamethasone) generally retain their effect on B1R expression (mRNA, binding site abundance) in SMC derived from the rabbit aorta (Figs. 3 and 6). Thus the cultured cell model is relevant to the tissue reactions and allows a fuller mechanistic investigation of the induction mechanism. Inhibition of NF-κB with the drugs MG-132 or BAY 11-7082 reduced the stimulatory effect of isolation in the fresh aorta (Fig. 2) and of at least IL-1, EGF, and FBS in the cultured cells (mRNA, Fig. 3B; binding sites, Fig. 7). The inhibitory drug effect may be better appreciated in the binding assays (Fig. 7) than in the RT-PCR (Fig. 3B), as the receptor protein population perhaps integrates the effect of the cytokines and the drugs over a longer time period and represents a less fragmented temporal image. However, some drug effects seem of questionable selectivity (nonspecific depression of contractility in the fresh aorta, Fig. 2; a certain stimulatory effect of MG-132 that seemed to counteract the expected inhibitions of cytokine-induced increase of B1R mRNA, Fig. 3B; the theoretical accumulation of p53 when proteasome is blocked, a situation that may be significant as p53 inhibits B1R gene promoter activity, Ref. 49). NF-κB is antiapoptotic in many experimental system (3) and its functional inhibition may result in accelerated cell death in isolated tissues, thus explaining the nonspecific depression of contractility. Although BAY 11-7082 and MG-132 did not produce apoptosis at the concentrations employed in the present experiments in rabbit aortic SMCs, the alternate proteasome inhibitor TPCK produced apoptosis in 6 h at 50 μM (DNA Ladder Isolation kit, Oncogene; Boston, MA; data not shown), supporting that cell survival may be affected by such drugs. However, the p65 nuclear translocation assay supports that MG-132, BAY 11-7082, and dexamethasone are capable of reducing the activation of NF-κB by IL-1β or FBS (Fig. 5), which was the primary effect desired.

Dexamethasone inhibition of postisolation induction of B1R in fresh aortic tissue (mRNA, function, Fig. 1) and of the upregulation of B1Rs in cultured cells by all tested stimuli (Fig. 7) is consistent with an effect of NF-κB. Glucocorticoids may inhibit this transcription factor by inducing the synthesis of IκBα, by direct protein-protein interaction between the activated glucocorticoid receptor and the p65 subunit of NF-κB, and by competition for coactivators common to the glucocorticoid receptor and NF-κB (47). Because dexamethasone inhibits at least a part of p65 translocation to the cell nucleus (Fig. 5), induction of IκBα is likely to have a primary role in the present SMC model. However, glucocorticoids also affect other systems of transcrip-
tion factors and their action may exceed that of more selective NF-κB inhibitors, as suggested by the partial inhibition of CHX-induced increased radioligand Bmax by dexamethasone not seen with more selective inhibitors (Fig. 7B).

As reported in other systems, CHX is a powerful agent to upregulate B1R mRNA and, if applied as a pulse to allow translation, the receptor protein itself in rabbit aortic SMCs (Figs. 3, 6, and 7). The drug may stimulate NF-κB activation in a powerful manner, as other cytokine regulators of B1R expression (Fig. 5), and indeed the nuclear runon experiments support that CHX is a transcriptional activator of the B1R gene (Fig. 4B). NF-κB activation by CHX has been previously observed and analyzed in other systems (33). However, experiments involving the measurement of the B1R mRNA-to-GAPDH mRNA ratio in the presence of actinomycin D suggest that CHX treatment increases the half-life of B1R mRNA over that of GAPDH (itself greater or equal to 24 h) (18, 23), a finding not observed with the other stimuli (Fig. 4A). It is striking that actinomycin D did not decrease the stimulatory effect of pulsed CHX on the production of new B1R proteins (Fig. 7A), as though the high mRNA stability achieved in the absence of transcription was sufficient for efficient translation. Actinomycin D decreased the basal B1R mRNA concentration in cells as well as those increased by cytokines or FBS (Figs. 3A and 4A), implying that transcription stimulation is probably a major effect of cytokines and FBS. Another important difference between CHX and the other stimuli was the sensitivity to treatments that inhibit NF-κB: MG 132 or BAY 11-7082 failed to reduce the stimulatory effect of CHX on binding (Fig. 7) and the drugs did not exert a consistent effect on the corresponding mRNA (Fig. 3B). Thus a model of B1R expression in cultured aortic SMCs could include a transcriptional effect of cytokines (including those present in FBS) or CHX mediated by NF-κB, a basal expression not clearly dependent on NF-κB activity in serum-starved cells and a profound stabilization of B1R mRNA after CHX treatment, suggesting that a labile factor regulates B1R mRNA stability, as previously discussed (48).

The site of interaction of activated NF-κB within the B1R gene is presently rather controversial. Fragments of the human B1R gene promoter supported reporter gene expression in several cell lines, but cytokine-regulated behavior only in one (rat SMCs; attributed to a NF-κB-binding sequence proximal to the transcription initiation site) (34). Other investigators (1, 49) have characterized upstream elements in the core promoter of the gene, such as a possible activator protein-1-binding site, but those did not confer significant responsiveness to LPS, tumor necrosis factor-α, or phorbol ester. Perhaps in line with these negative findings, extensive protein-DNA interaction was observed at the core promoter region of the B1R gene but was not influenced by LPS or IL-1 treatments in three types of cultured human cells, including SMCs derived from the human umbilical artery (footprinting established in living cells using ligation-mediated PCR) (1). Recent evidence based on a reporter gene coupled to large parts of the B1R gene, including introns and exons 1 and 2, suggests that the inducibility resides in motifs exclusive of the core promoter and stress the importance of c-Jun (50). The present data support that NF-κB is obligatory for B1R gene expression induced by cytokines but also reveal a B1R gene basal transcription rate independent of NF-κB in cultured SMCs. We have also isolated a important NF-κB mediated effect of serum on B1R expression in the cellular model (FBS is one of the most potent stimulus in the sampled cell lines shown in Figs. 6 and 7), a factor not systematically accounted for in previous studies. The precise factor(s) responsible for FBS activity remain to be identified.

Human embryonic lung fibroblasts (IMR-90, WI-38, and HEL 299 lines) have assumed a particular importance in the study of the regulation of B1Rs, perhaps because they are so permissive in this respect (17, 35–37, 51). At least in the IMR 90 cells, there is autocrine secretion of IL-1β which indirectly makes the B1R regulation reactive to a wide range of stimuli, including the stimulation of some G protein-coupled receptors (B1R, B2R, and IL-8 receptor) (4, 36, 43). However, recent investigations based on the rabbit (in vivo kallikrein-kinin system activation or in vitro treatment of aortic SMCs with B1R or B2R stimulants) suggest that the stimulation of kinin receptors is not generally followed by B1R expression in several tissues (41). Specifically, rabbit vascular SMCs appear to be a less permissive system for the study of B1R regulation, relative to IMR 90 cells, as kinin receptor stimulation in these cells does not upregulate B1R expression (mRNA, binding) (41).

In summary, the B1R mRNA concentration in the fresh rabbit aorta is highly correlated to pharmacological response in the fresh rabbit aorta; cultured SMCs derived from this tissue respond to the same set of stimulants as the fresh tissue by an increased expression of B1Rs. In this case, the cytokine-related stimulations are dependent on NF-κB, but the action of CHX is more complex.

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