Further Pharmacological Characterization of Bradykinin B₁ Receptor Up-Regulation in Human Umbilical Vein

SERGIO PABLO SARÌ, FEDERICO MANUEL DARAY, ANDREA EMILSE ERRASTI, FACUNDO GERMAN PELOROSSO, VIRGINIA ANDREA PUJOL-LEREIS, VERONICA REY-ARES, MARÌA PÌA ROGINES-VELO, and RODOLFO PEDRO ROTHLIN

Departamento de Farmacología, Facultad de Medicina, Universidad de Buenos Aires, Buenos Aires, Argentina

Accepted for publication May 12, 1999

This paper is available online at http://www.jpet.org

ABSTRACT

Previous reports have provided evidence to support the view that the de novo synthesis of bradykinin (BK) B₁ receptor is involved in the induction of vascular responses in human umbilical vein (HUV). In the present study, we evaluated different pharmacological tools to further analyze this up-regulation process in HUV. Concentration-response curves to des-Arg⁹-BK, a selective BK B₁ receptor agonist, were performed after 5 h of incubation. Tumor necrosis factor-α (TNF-α) and is selectively inhibited by pyrrolidine dithiocarbamate, suggesting that BK B₁ receptor up-regulation in HUV involves nuclear factor-κB activation. The effects of actinomycin D and tunicamycin provide evidence that the de novo synthesis of a transmembrane glycoprotein has an obligatory role in the BK B₁ up-regulation. The reversion of the cycloheximide effect on BK B₁ receptor indicates that the time necessary for synthesis, trafficking, and functional membrane expression of this receptor would be less than 1 h.

In 1977, two mammalian bradykinin (BK) receptor subtypes were proposed, B₁ and B₂ receptors (Regoli et al., 1977). Whereas BK and kallidin (Lys-BK) are BK B₂ receptor agonists, BK B₁ receptors are activated by their carboxy terminal-truncated metabolites, des-Arg⁹-BK and des-Arg¹⁰-Lys-BK. Molecular cloning has revealed the primary structures of BK B₁ (Menke et al., 1994) and BK B₂ (McEachern et al., 1991) receptors, and identified them as members of the G protein-coupled receptor family characterized by seven membrane-spanning α-helices.

The BK B₂ receptors are constitutively expressed in a variety of tissues and mediate most of the in vivo effects to kinins (Bathon and Proud, 1991). On the other hand, the BK B₁ receptors are not present in any significant amount in normal tissues and their expression is often inducible rather than constitutive. BK B₁ receptor-mediated responses are up-regulated in a time- and protein synthesis-dependent process (Regoli et al., 1978; Bouthillier et al., 1987). It has been proposed that the up-regulated responses to des-Arg-kinins result from inflammation, or from trauma during tissue isolation and incubation (Marceau et al., 1983).

Vascular BK B₁ receptors were first described in isolated rabbit anterior mesenteric vein by Regoli et al. (1978) after a long in vitro incubation. These authors have postulated the de novo formation of BK B₁ receptors to account for this phenomenon. Thereafter, induction of BK B₁ receptor-mediated responses was documented in different isolated tissue preparations (Marceau et al., 1998). The presence of BK B₁ receptors has been demonstrated previously in the isolated human umbilical vein (HUV) using des-Arg⁹-BK and des-Arg⁹-[Leu⁸]-BK, selective BK B₁ receptor agonist and antagonist, respectively. In this tissue, these receptors mediate contraction and exhibit a sensitization phenomenon during in vitro incubation (Sardi et al., 1997). Furthermore, it has been observed that continuous exposure to cycloheximide, a protein synthesis inhibitor, or brefeldin A, a protein-trafficking inhibitor across the Golgi apparatus, decreases the BK B₁ receptor-sensitized responses but does not modify the maxi-
polymeric response to an unrelated agonist (Sardi et al., 1998). In different tissues, it has been well established that BK B

receptor sensitization is completely prevented either by protein synthesis inhibitors, such as cycloheximide or anisomycin, or by transcription inhibitors, such as actinomycin D (Marceau et al., 1998). On the other hand, in the rabbit aorta preparation, when cycloheximide was transiently applied to a complete reversion of its inhibitory effect was obtained (DeBlois et al., 1991).

BK B receptor is a transmembrane glycoprotein that is likely to be processed through the endoplasmic reticulum-Golgi pathway after its ribosomal synthesis. Moreover, three potential sites of N-linked glycosylation have been proposed (Menke et al., 1994). In isolated rabbit aorta, permanent exposure to the protein glycosylation inhibitor, tunicamycin, has partially inhibited the development of des-Arg^9-BK-mediated responses (Audet et al., 1994).

In vitro and in vivo studies have demonstrated a link between inflammatory mediators and the expression of BK B receptors (Marceau et al., 1998). In rabbit isolated tissues and in the HUV, it has been reported that lipopolysaccharide or interleukin (IL)-1β treatment potentiates BK B receptor-mediated responses (Bouthillier et al., 1987; DeBlois et al., 1991; Sardi et al., 1998). Ni et al. (1998) have observed the increase in BK B receptor mRNA levels in rat aorta smooth muscle cells treated with IL-1β or tumor necrosis factor (TNF)-α. These cytokines have been linked to activation of the nuclear factor (NF)-κB pathway (Baladin, 1996). NF-κB is an ubiquitous cytoplasmic pleiotropic transcription factor that has been described as an activator of immediate early genes involved in inflammatory and immune response (Baladin, 1996). Pyrrolidine dithiocarbamate (PDTC), an antioxidant and NF-κB activation inhibitor (Schreck et al., 1992), has been reported to inhibit BK B receptor mRNA expression in rat vascular smooth muscle cells (Ni et al., 1998).

In the present study, we evaluated different pharmacological tools to further analyze the BK B receptor up-regulation process in HUV. Therefore, it was considered interesting to examine the effects of TNF-α or PDTC on concentration-response curves to des-Arg^9-BK in HUV. Furthermore, the process of BK B receptor induction was addressed by incubating tissues in the presence of the transcription inhibitor, actinomycin D. On the other hand, we have analyzed the possible reversibility of the inhibitory cycloheximide treatment on the BK B receptor sensitization phenomenon. Finally, the requirement of protein glycosylation for the expression of functional BK B receptor-mediated responses in HUV was assessed using tunicamycin.

### Materials and Methods

#### Preparation of Tissues for Tension Measurements

Human umbilical cords excised midway between the placenta and infant were obtained from normal full-term deliveries. Immediately, cords were placed in modified Krebs’ solution at 4°C of the following composition: 119 mM NaCl, 4.7 mM KCl, 25 mM NaHCO_3, 1.2 mM KH_2PO_4, 2.5 mM CaCl_2, 1.0 mM MgSO_4, 0.004 mM EDTA, and 11 mM D-glucose. The veins were carefully dissected out of the cords and cut into rings of approximately 3-mm width. The preparations were suspended in 10-ml organ baths and stretched with an initial tension of 3 to 5 g as described previously (Elgoyhen et al., 1993). Changes in tension were measured with Grass isometric transducers (FT-03C, Grass Instrument Co., Quincy, MA) and displayed on Grass polygraphs (model 7D). The time from delivery until the tissue was set up in the organ baths was approximately 3 h.

During the incubation period, Krebs’ solution was maintained at 37°C and at pH 7.4 by constant bubbling with 95% O_2/5% CO_2. Bath solution was replaced every 15 min. After 70 min of equilibration, each preparation was contracted with 40 mM KCl to test their functional state. Tissues were incubated with captopril (1 μM) 30 min before the BK receptor stimulation to avoid peptide degradation by kininase II (angiotensin-converting enzyme).

**BK-Receptor Stimulation.** Cumulative concentration-response curves were obtained for the BK B receptor-selective agonist, des-Arg^9-BK, after a 5-h incubation period. Only one agonist concentration-response curve was performed on a single ring.

Some HUV rings were continuously exposed to PDTC (0.1 and 1 mM), actinomycin D (4 μM), cycloheximide (70 μM), or tunicamycin (5 μg/ml) for the 5-h equilibration period, before cumulative addition of the agonist. Furthermore, other HUV rings were incubated in the presence of cycloheximide (70 μM) for the initial 4 h, then cumulative concentration-response curves were obtained at 5 h.

To evaluate possible toxic effects of these metabolic inhibitors, concentration-response curves for the unrelated agonist serotonin (5-HT) were constructed on HUV rings. Thus, PDTC (0.1 and 1 mM), actinomycin D (4 μM), cycloheximide (70 μM), or tunicamycin (5 μg/ml) were evaluated in tissues continuously exposed to each inhibitor.

In other series of experiments, some HUV rings were treated with human recombinant TNF-α (10 ng/ml) for the initial 75 min. Then, cumulative concentration-effect curves were constructed for the BK B receptor-selective agonist at 5 h.

Control trials for actinomycin D- and tunicamycin-treated tissues were performed in the presence of the corresponding concentration of ethanol (<1%).

Experiments were performed in parallel in rings from the same umbilical vein. At the end of each experiment, the BK B receptor agonist, BK (0.1 μM) was applied to determine the tissue maximal response.

**Chemicals and Solutions.** Actinomycin D, BK, captopril, cycloheximide, PDTC, and TNF-α were purchased from Sigma Chemical Co. (St. Louis, MO). Tunicamycin was a mixture of homologous in the fatty acid moiety from Biomol Research Laboratories (Plymouth Meeting, PA) and des-Arg^9-BK was obtained from Bachem Biochemicals, Inc. (Torrance, CA). 5-HT creatinine sulfate complex was purchased from Research Biochemicals Inc. (Natick, MA).

All concentrations of drugs are expressed as final concentrations in the organ bath. Actinomycin D and tunicamycin stock solutions were made in ethanol (99.5%), stored at −20°C in aliquots and used daily. Stock solution of TNF-α was made in Krebs’ solution, stored frozen in aliquots, and thawed daily. Stock solutions of peptides, 5-HT, and captopril were made in distilled water, stored frozen in aliquots, and thawed and diluted daily. PDTC and cycloheximide were made up fresh in distilled water or Krebs’ solution, respectively, on the day of use.

**Expression of Results and Statistical Analysis.** All data are presented as mean ± S.E.M. Responses are expressed as grams of developed contraction. The pEC_{50} values, negative logarithms of the agonist concentration that produces 50% of the maximum, were determined using ALLFIT, a nonlinear curve-fitting computer program (De Lean et al., 1978). Statistical analysis was performed by means of paired Student’s t test. p values lower than .05 were taken to indicate significant differences between means.

### Results

**Effect of Recombinant Human TNF-α on BK B Receptor Sensitization in HUV.** Some tissues were exposed to recombinant human TNF-α during the initial 75 min to evaluate the possible potentiating effect of this proinflamma-
tory cytokine on the BK B1 receptor sensitization process. When HUV rings were exposed to TNF-α (10 ng/ml), concentration-response curve to des-Arg9-BK at 5 h was significantly potentiated (Fig. 1, pEC50, control: 7.19 ± 0.13, treated: 7.67 ± 0.10, p < .05, n = 5). However, maximal response to the BK B1 receptor-selective agonist was unaffected by TNF-α treatment (control: 13.0 ± 1.8 g, treated: 15.2 ± 2.2 g, n = 5).

Effect of PDTC on BK B1 Receptor Sensitization in HUV. Some HUV rings were exposed to PDTC for 5 h to examine the possible effect of this antioxidant and inhibitor of NF-κB activation on the BK B1 receptor sensitization process. PDTC (0.1 mM) produced a significant rightward shift of the concentration-response curve to des-Arg9-BK without affecting the maximal response (Fig. 2A, pEC50, control: 7.42 ± 0.06, treated: 6.94 ± 0.09, p < .05; max. resp., control: 13.0 ± 1.8 g, treated: 11.9 ± 2.2 g, n = 5).

When tissues were treated with PDTC (1 mM), contractile response to the BK B1 receptor agonist was significantly reduced (Fig. 2B, max. resp., control: 13.5 ± 1.3 g, treated: 7.3 ± 2.1 g, p < .05, n = 8). However, maximal response to BK (0.1 μM) at the end of each experiment was not modified by this treatment (control: 15.8 ± 1.2 g, treated: 15.1 ± 1.2 g, n = 8).

To determine possible toxic effects of PDTC on the contractile response in this tissue, 5-HT concentration-response curves were performed. Incubation for 5 h with PDTC (0.1 and 1 mM) produced a rightward shift in the concentration-response curves to 5-HT (Fig. 2C, pEC50 control: 8.48 ± 0.04; PDTC 0.1 mM: 8.23 ± 0.04; PDTC 1 mM: 8.18 ± 0.04, n = 10) without affecting the maximal response (Fig. 2C, control: 12.6 ± 0.4 g; treated: 12.8 ± 0.6 g, n = 8).

Effect of Actinomycin D on BK B1 Receptor Sensitization in HUV. Continuous exposure to the transcription inhibitor, actinomycin D (4 μM), significantly inhibited the BK B1 receptor sensitization in isolated HUV rings. The maximal response to des-Arg9-BK was diminished in 5-h-treated tissues (Fig. 3A, control: 13.5 ± 1.3 g, treated: 7.3 ± 2.1 g, p < .05, n = 8). In addition, maximal response to BK (0.1 μM) at the end of each experiment was not modified by this treatment (control: 15.8 ± 1.2 g, treated: 15.1 ± 1.2 g, n = 8).

To evaluate any change resulting from possible toxic effects, 5-HT concentration-response curves were constructed on HUV rings exposed for 5 h to actinomycin D (4 μM). This transcription inhibitor produced a rightward shift in the concentration-response curve (Fig. 3B, pEC50 control: 8.51 ± 0.04; treated: 8.17 ± 0.08, n = 5, p < .05) with no decrease in the maximal response (Fig. 3B, control: 12.6 ± 0.4 g; treated: 12.8 ± 0.6 g, n = 5).
Effect of Cycloheximide on BK B<sub>1</sub> Receptor Sensitization in HUV. BK B<sub>1</sub> receptor sensitization was profoundly inhibited by continuous exposure to the protein translation inhibitor cycloheximide (70 μM) in isolated HUV rings. The maximal response to des-Arg<sup>9</sup>-BK was significantly diminished in 5-h-treated tissues (Fig. 4A, control: 11.7 ± 1.1 g, treated: 3.4 ± 1.0 g, p < .05, n = 13). Additionally, maximal response to BK (0.1 μM) at the end of each experiment was not modified by this treatment (control: 13.8 ± 1.1 g, treated: 12.8 ± 1.5 g, n = 13).

When HUV rings were exposed to cycloheximide (70 μM) for the initial 4 h of incubation, the concentration-response curve for the BK B<sub>1</sub>-selective agonist at 5 h was not modified (Fig. 4B, pEC<sub>50</sub>, control: 7.31 ± 0.06, treated: 7.28 ± 0.09; max. resp., control: 13.1 ± 0.9 g, treated: 12.4 ± 1.9 g, n = 6).

In other HUV rings, 5-HT concentration-response curves were constructed at 5 h after permanent exposure to cycloheximide (70 μM) to evaluate possible toxic effects of this agent. This treatment produced a rightward shift in the concentration-response curve (Fig. 4C, pEC<sub>50</sub>, control: 8.52 ± 0.02, treated: 8.21 ± 0.06, n = 8; p < .05) without affecting the maximal response (Fig. 4C, control: 14.2 ± 8.6 g; treated: 16.6 ± 1.5 g, n = 8).

Effect of Tunicamycin on BK B<sub>1</sub> Receptor Sensitization in HUV. Continuous exposure to the N-linked glycosylation inhibitor, tunicamycin (5 μg/ml), significantly inhibited the BK B<sub>1</sub> receptor sensitization in isolated HUV rings. The maximal response to des-Arg<sup>9</sup>-BK was diminished in 5-h-treated tissues (Fig. 5, control: 14.4 ± 1.4 g, treated: 4.1 ± 1.7 g, p < .05, n = 7). In addition, maximal response to BK
as a function of in vitro incubation time (Bouthillier et al., 1987). In the rabbit aorta rings, this sensitization process has also been demonstrated to be selective to BK B1 agonists when it was evaluated against other unrelated agonists, such as phenylephrine (Audet et al., 1994). In the isolated HUV, we have demonstrated previously that contractile response to des-Arg9-BK increased as a function of incubation time (Sardi et al., 1997). This temporal sequence was evaluated against a vasoconstrictor agonist, 5-HT, that does not show a sensitization process. The approximated maximal response to BK B1 receptor-selective agonist was 10% of the maximal response to 5-HT at 15 min, 55% at 120 min, and 80% at 300 min (Sardi et al., 1997).

In HUV, the BK B1 sensitization process is sensitive to cycloheximide, a protein synthesis inhibitor, and it is dependent on protein trafficking across the Golgi apparatus, as it is blocked by brefeldin A (Sardi et al., 1998). Furthermore, in this tissue, it has been shown that this phenomenon is inhibited by the glucocorticoid dexamethasone, and potentiated either by lipopolysaccharide or by human recombinant IL-1β (Sardi et al., 1998).

Studies in different tissues provide evidence that several proinflammatory cytokines are involved in BK B1 receptor induction (Marceau et al., 1998). Epidermal growth factor, IL-1, and IL-2 have been shown to increase BK B1 receptor-mediated responses in rabbit aorta (DeBlois et al., 1988). Ni et al. (1998) have observed the increase in BK B1 receptor mRNA levels in rat aorta smooth muscle cells treated with IL-1β or TNF-α. Furthermore, it has been reported that these two cytokines increase BK B1 receptor-mediated rat paw edema (Campos et al., 1998). In the present study, TNF-α treatment also potentiated the BK B1 receptor responses, showing a leftward shift of the concentration-response curve to des-Arg9-BK in HUV at 5 h without modifying the maximal response.

Ni et al. (1998) have demonstrated, in transfected cultured cells, that transcription factor NF-κB is involved in the inducible expression of the human BK B1 receptor gene during inflammatory processes. The NF-κB pathway is activated by exposure to lipopolysaccharide or inflammatory cytokines, such as TNF-α or IL-1β, phagocytic viruses and bacteria, and by other physiological and nonphysiological stimuli (Baldwin, 1996). NF-κB is an heterodimeric transcription factor that is held inactive by an inhibitory subunit IκB in the cytoplasm of unstimulated cells. Activation of NF-κB involves removal of IκB. Six IκB proteins have been suggested to control the activity of NF-κB dimers. Only the IκB-α, -β, and -ε are stimulus-dependent regulators (Baue, 1998). Whereas IκB-α is targeted by a signaling pathway initiated by lipopolysaccharide, IL-1, and TNF-α, IκB-β is targeted only by pathways initiated by lipopolysaccharide or IL-1 (Baldwin, 1996). It has been shown that IκB is subject to both phosphorylation and proteolysis in the process of NF-κB activation (Baldwin, 1996). Traenckner et al. (1994) have reported that PDTC, an antioxidant inhibitor of NF-κB, prevents de novo phosphorylation of IκB-α as well as its subsequent degradation. Therefore, we consider it interesting to examine the possible effects of PDTC on the concentration-response curve to des-Arg9-BK in HUV. In this tissue, contractile responses to the selective BK B1 receptor agonist were inhibited by continuous incubation with PDTC in a concentration-dependent manner. These results suggest that...
NF-κB activation is necessary for the BK B1 receptor up-regulation in this tissue. Furthermore, the previously reported BK B1 receptor potentiation with both lipopolysaccharide and IL-1β (Sardi et al., 1998) and the present results with TNF-α also support the involvement of NF-κB activation in the BK B1 receptor sensitization in HUV.

The antibiotic actinomycin D, produced by Streptomyces species, binds to double-helical DNA blocking the RNA polymerase movement, thus preventing RNA synthesis (Chabner et al., 1996). In HUV rings, BK B1 receptor-mediated sensitized responses were inhibited by continuous exposure to actinomycin D. This result supports the hypothesis of the de novo synthesis of BK B1 receptors to account for the up-regulation phenomenon in this tissue. In addition, in different tissues, it has been well established that BK B1 receptor responses are abolished by transcription inhibitors (Marceau et al., 1998).

Cycloheximide is a protein synthesis inhibitor that blocks the translocation reaction on ribosomes (Alberts et al., 1994). As mentioned above, continuous exposure of the HUV to cycloheximide has been shown to inhibit the BK B1 receptor sensitization (Sardi et al., 1998). This inhibitory action was described previously in rabbit vascular strips (Regoli et al., 1978). On the other hand, in this tissue, when cycloheximide was transiently applied, a complete reversal of its inhibitory effect was obtained (DeBlois et al., 1991). Furthermore, in human lung fibroblasts, Zhou et al. (1998) have shown the reversal of the inhibitory effect of cycloheximide on BK B1 receptor binding within 120 min after removal of this protein synthesis inhibitor. In the present study, this phenomenon was also evaluated in the HUV by exposing rings to cycloheximide only for the first 4 h. In these experimental conditions, a complete recovery of BK B1 receptor-mediated responses was observed. The curves to des-Arg9-BK at 5 h were not affected by this transitory treatment, neither pEC50 nor maximal response were modified. This result suggests that in this tissue the temporal sequence of the process involving BK B1 receptor synthesis from its mRNA, trafficking, and functional membrane expression is relatively rapid, less than 1 h.

According to Menke et al. (1994), the human BK B1 receptor contains three potential N-linked glycosylation sites. These sites may confer varying functional roles depending on the receptor. For example, they could be important for efficient G protein coupling, ligand binding, or membrane expression (Rands et al., 1990; Walsh et al., 1998). The nucleoside antibiotic tunicamycin, produced by Streptomyces lyosuperificus, is a highly selective direct inhibitor of N-linked glycosylation of various proteins. Apparently, its mechanism of action is blocking the enzymatic transfer of N-acetylglucosamine 1-phosphate to dolichol monophosphate (Tkacz and Lampen, 1975; Elbein, 1987). Moreover, it has little or no effect on de novo protein synthesis (Struck and Lenzar, 1977). Thus, in this study, experiments were undertaken to investigate the possible loss of BK B1 receptor function in HUV because of N-glycosylation inhibition. In our experimental conditions, tunicamycin almost completely abolished BK B1 receptor-mediated contractions. Furthermore, this action was apparently selective as the maximal response to BK, a B2 receptor agonist, was not modified. Therefore, in HUV, N-linked glycosylation of the BK B1 receptor seems to be functionally relevant for the expression of contractile responses.

To determine possible toxic effects of these metabolic inhibitors on the HUV, concentration-response curves to an unrelated agonist as 5-HT were constructed. The 5-HT maximal response in tissues continuously exposed to each inhibitor was not modified. These results indicate the lack of toxic effects of PDTC, actinomycin D, cycloheximide, or tunicamycin on HUV rings contractility. On the other hand, the depression on the 5-HT sensitivity promoted by these agents suggests the presence of a turnover process on the 5-HT receptor during the 5-h incubation time in this tissue. Nevertheless, further experiments should be performed to confirm this hypothesis.

Apparently, in the HUV there is a proportion of spare BK B1 receptors in the BK B1 receptor population after 5 h of incubation (Sardi et al., 1998). The results obtained in the present study with TNF-α and PDTC (0.1 mM), promoting respectively a leftward or a rightward shift of the concentration-response curve to des-Arg9-BK without affecting the maximal response, provide additional evidence to support the presence of BK B1 spare receptors in the HUV in control conditions after a 5-h incubation period.

In summary, this study proves that in the HUV:

1) PDTC selectively inhibits the BK B1 sensitization process, suggesting that BK B1 receptor up-regulation in this tissue involves NF-κB activation. To our knowledge, this is the first report showing a pharmacological activity of PDTC on BK B1 receptor-mediated responses;

2) the BK B1 sensitization process is potentiated by TNF-α, providing additional pharmacological evidence that NF-κB activation would be required in this phenomenon;

3) actinomycin D produces an inhibitory effect on des-Arg9-BK, BK-sensitized responses supporting the view that the de novo synthesis of BK B1 receptors is involved;

4) tunicamycin treatment decreases the development of the BK B1 receptor sensitization, suggesting that glycosylation of these newly synthesized receptors is required for their functional expression; and

5) the cycloheximide inhibitory effect on BK B1 sensitization process is reversible. Moreover, in these experimental conditions the time for synthesis, trafficking, and functional membrane expression of this receptor would be less than 1 h.

Acknowledgments

We thank the Instituto Médico de Obstetricia (Buenos Aires) for their efforts in providing umbilical tissues.

References


De Lean A, Munson PJ and Rodbard D (1978) Simultaneous analysis of families of


Send reprint requests to: Prof. Rodolfo Pedro Rothlin, Departamento de Farmacología, Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, Piso 15, C.P. 1121, Buenos Aires, Argentina. E-mail: farmaco3@fmed.uba.ar