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Several cytokines and LPS regulate the population of the B₁ receptors (B₁Rs) for kinins; these are responsive to des-Arg⁹-bradykinin (BK) and Lys-des-Arg⁹-BK. B₁R activation contributes to inflammatory vascular changes and pain. Aortic rings isolated from normal rabbits and incubated in vitro in Krebs physiological medium were used as a model of tissue injury. From a null level of response, these rings exhibit a time- and protein synthesis-dependent increase in the maximal contractile response to des-Arg⁹-BK. Exposure to exogenous IL-1β or epidermal growth factor (EGF) considerably increases the process of sensitization to the kinins. Freshly isolated control aortic rings showed high mitogen-activated protein (MAP) kinase activities (persistent activation of p38, but less prolonged for extracellular signal-regulated kinase and c-Jun-N-terminal kinase/stress-activated protein kinase pathways) relatively to the basal activities found in various types of cultured cells. IL-1β or EGF further increased the activities of the extracellular signal-regulated kinase and c-Jun-N-terminal kinase/stress-activated protein kinase MAP kinases. The inhibitor of the p38 MAP kinase, SB 203580 (10 μM), massively (~75%) and selectively inhibited the spontaneous sensitization to des-Arg⁹-BK over 6 h. SB 203580 also significantly reduced the development of the response to des-Arg⁹-BK as stimulated by IL-1 or EGF. Both spontaneous and IL-1β-stimulated up-regulation of responsiveness to des-Arg⁹-BK were significantly inhibited by the MAP kinase extracellular signal-regulated kinase 1 inhibitor PD 98059 (~40%). The protein kinase inhibitors failed to inhibit protein synthesis and to acutely inhibit the contractile effect of des-Arg⁹-BK, suggesting that they do not influence B₁ receptor transduction mechanisms. In cultured aortic smooth muscle cells stimulated with EGF, MAP kinase activation preceded B₁R mRNA induction. Protein kinase inhibitors reveal the role of cell injury-controlled MAP kinase pathways, and singularly of the p38 pathway, in the induction of B₁R. The Journal of Immunology, 1998, 160: 1419–1426.

Bradykinin (BK)³ and sequence-related peptides (hereafter termed kinins) are blood-derived peptides that are suspected to be inflammatory mediators. The current classification of receptors for kinins distinguishes two types of receptors, namely B₁ and B₂, and is now supported by molecular biology findings. Both kinin receptors belong to the superfamily of G protein-coupled seven-transmembrane domain receptors (1). The B₂ type of receptor is optimally stimulated by the full sequence of BK or Lys-BK; the discovery of the prototype antagonist for the B₂ receptors, [D-Phe⁹]-BK, was followed by the development of many effective and specific antagonists (2). B₂ receptors mediate most, if not all, the in vivo effects usually assigned to kinins in normal rodents, rabbits, and humans: vasodilation, pain, increased vascular permeability, and increased production of eicosanoids and nitric oxide (3).

The B₁ type receptors are selectively sensitive to fragments of kinins without the C-terminal arginine (e.g., des-Arg⁹-BK and Lys-des-Arg⁹-BK, also called des-Arg¹⁰-kallidin); the prototype selective antagonist is [Leu⁷]des-Arg⁹-BK (3, 4). B₁ receptors (B₁Rs) have a special interest due to their strong up-regulation following some types of tissue injury. A large body of evidence indicates that the B₁Rs are generally absent from normal tissues and animals, but are rapidly induced following some types of injuries (4). A time- and protein synthesis-dependent up-regulation of B₁R-mediated mechanical responses from a null initial level has been described in the rabbit aorta and several other smooth muscle preparations (4), including some of human origin (5, 6), following isolation and in vitro incubation in simple physiologic solutions. The postulated up-regulation of B₁Rs by tissue injury may explain several observations of in vivo enhanced functional responses to the corresponding agonists in systems pertaining to hemodynamics, smooth muscle contractility, pain perception, and leukocyte recruitment (4, 7–9). For instance, bacterial products sensitize the whole cardiovascular system of rabbits, rats, or pigs to the B₁ agonist, des-Arg¹⁰-BK (10–12). Accordingly, the maximal binding capacity (B max ) of the B₁R population is increased in rabbit vascular smooth muscle cells by treatment with LPS (13), and transcription of the B₁R gene has been shown in hearts from rabbits pretreated with endotoxin, but not in organs from control animals (14). Thus, unlike the constitutively expressed BK B₂ receptor, the

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3 Abbreviations used in this paper: BK, bradykinin; B₁,R, B₁ receptor; B max , maximal binding capacity; EGF, epidermal growth factor; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK/SAPK, c-Jun-N-terminal kinase/stress-activated protein kinase MAP kinases; EGF, epidermal growth factor; MAP, mitogen-activated protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase MEK1, MAP kinase extracellular signal-regulated kinase kinase 1.

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B1R appears to be dynamically regulated. The sequence analysis of the 5′-flanking region of the human B1R gene revealed the presence of a consensus TATA box and numerous candidate transcription factor binding sequences, including some associated with cytokine-induced gene expression, such as AP-1 and nuclear factor-xB (15), which is consistent with a highly regulated gene. While the B1R promoter is currently being dissected to explain its inducibility, a parallel immunologic and pharmacologic approach was followed to study the mechanism of B1R induction. The role of inflammatory cytokines, particularly IL-1, in the vascular up-regulation of B1 receptors, has been suspected for some time (4). Recombinant IL-1 can reproduce the B1R up-regulation in vivo in the cardiovascular system and in a hyperalgesia model (7, 16) and accelerate the in vitro sensitization of the rabbit aorta to B1R agonists (17) or increase the B1R Bmax in binding assays in cultured cells (1, 13, 18). However, several other cytokines and growth factors are active in this respect in one or several of these experimental systems: epidermal growth factor (EGF), oncostatin M, nerve growth factor, IL-2, and IL-8 (7, 10, 17, 19–21). While some of these factors may recruit autocrine or paracrine IL-1 (7) and others may exert postreceptor potentiating effects in functional assays (20, 22), the redundancy and multiple signaling pathways recruited by these factors prompted us to probe the role of downstream intracellular biochemical pathways that can be common to several stress situations. We investigated the roles of different protein kinase pathways, namely tyrosine kinases and the multiple mitogen activated-protein (MAP) kinases ERK, SAPK/JNK, and several stress situations. We investigated the roles of different pro-

Contractility studies pursued several objectives. Firstly, the selected inhibitory drugs for protein kinase pathways, 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 spontaneous (induction-induced) sensitization to the B1 receptor agonist, des-Arg9-BK. As some exogenous cytokines and growth factors, such as IL-1 and EGF, respectively, can up-regulate B1 receptors in functional (10, 17) and binding assays based on rabbit arterial smooth muscle (13, 18, 19), other tissues were exposed to human rIL-1β (5 ng/ml) or human rEGF (100 ng/ml) for the first 3 h of incubation as described previously (17, 20). These treatments do not interfere with the construction of the concentration-effect curves to des-Arg9-BK or Pe, with those described above. The incubation with the cytokine treatment in some tissues to analyze the pathways used by these substances to potentiate B1 receptor regulation. The late response to Pe in these tissues may also provide an insight into another independent functional response induced by IL-1 in blood vessels, i.e., the induction of iNOS (revealed as a nonspecific depression of the contractility), and inhibitory drugs may also reveal the mechanism of this gene activation. Vascular rings from each animal were assigned randomly to each of the treatments and to the control group, so that the statistical weight of each animal is equal in each group.

Constrictions are expressed as the percentage of the maximum Pe-induced contraction recorded at 1.5 h, an internal standard for each tissue. This contractility value is equivalent to 4.47 ± 0.17 g of weight in the isolated organs (n = 30) and 50% inhibition of contractile treatments (not shown). Sigmoidal concentration-effect curves are characterized by the half-maximal effective concentration (EC50) and the maximal absolute contraction amplitude (Emax, percentage of internal standard). Statistical analysis was performed by Kruskal-Wallis test followed by Mann-Whitney test or Student’s t test, using the InStat 2.0 computer program (GraphPad Software, San Diego, CA).

MAP kinase assays in aortic tissue

Aortic rings were prepared from normal rabbits as described above (ring weight, 40–50 mg). The rings were incubated for 15 min in 5% CO2 at 37°C in a humidified Krebs buffer containing, or not MAP kinase inhibi-

The thoracic aorta was isolated from New Zealand White rabbits of either sex (20) (1.5–2 kg). The vessels were cut into rings (~4 mm in diameter, 3–4 mm in length) and were suspended between a metal hook and a thread loop under a tension of 2 g in 5-mL bath tubes containing oxygenated (95% O2/5% CO2) Krebs solution. The composition of Krebs was 117.5 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.18 mM MgSO4, 2.5 mM CaCl2, 25.0 mM NaHCO3, and 5.5 mM d-glucose (20). Isometric changes in vascular tone were measured by force transducers (model 52-9545, Harvard Apparatus, South Natick, MA) coupled to LKB chart recorders (model 2210 or REC 102, LKB, Rockville, MD).

Constrictions and Methods


test, using the InStat 2.0 computer pro-

Contractility studies were always based on the construction of five full cumulative concentration-effect curves. The ones for the B1 receptor agonist, des-Arg9-BK, were constructed after 1, 3, and 6 h of incubation. The repeated stimulations with des-Arg9-BK were performed to monitor the progressive increase in the responsiveness of aortic strips to the kinin metabolite, thus revealing the up-regulation of receptors by monitoring their functional effects. The concentration-effect curves for the α-adrenoceptor agonist phenylephrine (Pe) were established at 1.5 and 7.5 h as more stable contractile responses (20); in addition, the late response to Pe was depressed in tissues exposed to IL-1 (IL-1), and there is ample functional evidence that nitric oxide mediates this nonspecific decrease in contractility in this system (23, 24), thus presumably representing the cytokine up-regulation of inducible nitric oxide synthase (iNOS). Tissues were amply washed with fresh Krebs buffer between stimulations.
The phosphorylated GST/c-Jun was boiled in SDS sample buffer to stop the reaction. The activity of the various kinases was quantified by measuring the incorporation of radioactivity onto the specific substrate after SDS-PAGE.

\[^{1}H\]leucine incorporation by rabbit aortic tissue

As continuous exposure to protein synthesis inhibitors prevents the sensitization to kinins in the rabbit aortic preparation (10, 16), we further validated the specificity of other drugs that inhibit this phenomenon using a \[^{1}H\]leucine incorporation assay to exclude a global depression of protein synthesis. Rings of rabbit aorta (average wet weight, 20 mg) were prepared as described above and further divided into four or five pieces with fine scissors. The effect of each drug on the incorporation of \[^{1}H\]leucine was tested at the same concentration as that used in organ bath, and cycloheximide (71 \(\mu\)M) was used as a positive control. The tissue fragments from each drug were incubated together for 6 h at 37°C(95% air/5% CO\(_2\)) in 3 ml of Krebs buffer containing 0.5 \(\mu\)Ci/ml of \[^{1}H\]leucine (New England Nuclear Corp., Boston MA; sp. act., 144 Ci/mmol). Incubated fragments were rinsed three times in PBS, pH 7.4. Tissue fragments exposed to \[^{1}H\]leucine were homogenized in 2 N NaOH, and the associated radioactivity was determined.

**Assays based on cultured smooth muscle cells**

Rabbit aortic smooth muscle cells were isolated, cultured and characterized as described previously (29), and used at passage 5. Groups of five 75-cm\(^2\) flasks (~5 × 10\(^6\) cells) were used for Northern blot analysis of \(\beta_1\) receptor expression as affected by EGF (100 ng/ml). Seventy-five to eighty percent confluent cells, grown in medium 199 supplemented with 10% FBS, fresh L-glutamine, and antibiotics, were fed a reduced serum medium (0.4%) containing 2% formaldehyde. The gel was transferred to a nylon membrane, and the membrane was hybridized at 65°C with a 0.8-kb \(^32\)P-labeled probe corresponding to a PstI fragment of the coding sequence of the rabbit \(\beta_1\) receptor (31) (a gift from Dr. Fred Hess). The final wash was with 0.1 × SSC/0.1% SDS at 65°C. The membrane was then subjected to autoradiography. The filter was further washed with boiling 0.1% SDS and rehybridized with a probe corresponding to GAPDH, a housekeeping gene. Results were reported as the ratio of \(\beta_1R\) signal to the GAPDH one. Individual 75-cm\(^2\) flasks of rabbit aortic smooth muscle cells maintained in reduced serum (0.4%) medium for 24 h were also used for extraction and quantification of basal and EGF-stimulated MAP kinase activities (15-min stimulation; assayed as described above) to monitor whether increased activities of these enzymes precede the receptor up-regulation.

**Materials**

SB 203580 (4-(4-fluorophenyl)-2-(4-methylsulfanylphenyl)-5-(4-pyridyl)imidazole) and its less active structural analogue, SKF 106978, were gifts from Dr. John C. Lee, SmithKline Beecham Pharmaceuticals (King of Prussia, PA). SB 203580 is a specific inhibitor of the p38 MAP kinase (25), PD 98059 (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one) was purchased from Research Biochemicals International (Natick, MA). This compound is a specific inhibitor of the MAP kinase kinase (MEK1) (33). Genistein, a tyrosine kinase inhibitor (34), was obtained from Biomol (Plymouth Meeting, PA), and des-Arg\(^9\)-BK was obtained from Bachem (Torrance, CA). Recombiant IL-1\(\beta\) and 15-\(\beta\)SDS at 65°C. The membrane was then subjected to autoradiography. The filter was further washed with boiling 0.1% SDS and rehybridized with a probe corresponding to GAPDH, a housekeeping gene. Results were reported as the ratio of \(\beta_1R\) signal to the GAPDH one. Individual 75-cm\(^2\) flasks of rabbit aortic smooth muscle cells maintained in reduced serum (0.4%) medium for 24 h were also used for extraction and quantification of basal and EGF-stimulated MAP kinase activities (15-min stimulation; assayed as described above) to monitor whether increased activities of these enzymes precede the receptor up-regulation.

**Results**

**Kinase assays of rabbit isolated aortic rings**

Aortic rings isolated from normal rabbits were incubated in Krebs buffer in the presence of cytokines (5 ng/ml IL-1\(\beta\) or 100 ng/ml EGF) or saline vehicle. The kinase assays showed a high level of basal activity of all the MAP kinases tested (Fig. 1, insets), most notably of p38 MAP kinase (Fig. 1A), whose activity in control rings was about 10- to 20-fold higher than that in untreated cultured HUVECs (25), IMR-90, or CCL 39 cells (data not shown). Treatment of the rings with either cytokine did not significantly augment the observed activity of p38 (Fig. 1A) or that of the downstream MAPKAP kinase 2/3 in separate tests (Table I). Activation of the later kinase did not decline significantly after the 3-h incubation (Table I). The SAPK/JNK assay also revealed that the basal activity of this kinase was elevated and was further stimulated by the addition of cytokines to the medium (Fig. 1B, statistical analysis in Table I). Also, even though the ERK2 basal activity was high in the control rings, it was inducible in presence of IL-1\(\beta\) (~1.5-fold increase) and EGF (~2.5-fold increase; Fig. 1C and Table I). SAPK/JNK and ERK2 activities generally declined as a function of incubation time, as assessed at 3 h (Table I). The decrease in ERK2 activity was less pronounced in the EGF-treated tissues. These findings suggest that upon tissue isolation or animal death, the p38 MAP kinases are persistently activated to a high level, which is probably maximal, since the addition of cytokines did not affect it. The other MAP kinases are also activated, but
more transiently and to a lesser extent, as indicated by the induction of their activity by IL-1β or EGF.

**Effects of inhibitory drugs on the contractility and MAP kinase activities in rabbit aortic rings**

Control aortic rings isolated from normal rabbits exhibited the well-characterized passage from a null response to a time- and protein synthesis-dependent increase in the maximal response to the B₁ receptor agonist des-Arg⁹⁻BK (Figs. 2 and 3). In these graphic representations of the concentration-effect curves, C and D represent the responses recorded after 3 and 6 h of incubation, respectively. The maximal level of response to the kinin recorded at 1 h was always close to zero (see Table II, which also contains statistical analyses). The inhibitor of the p38 MAP kinase, SB

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**Table I. Relative MAP kinase activities in extracts of rabbit aortic rings as modified by time, cytokines, and drugs**

<table>
<thead>
<tr>
<th>Stimulation Time&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Drug</th>
<th>Control (no cytokine)</th>
<th>IL-1β (5 ng/ml)</th>
<th>EGF (100 ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MAPKAP kinase 2/3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 min</td>
<td>None (control)</td>
<td>1.0 ± 0.3</td>
<td>2.0 ± 1.0</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>SB 203580 (10 μM)</td>
<td>0.4 ± 0.2*</td>
<td>0.5 ± 0.2*</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>PD 98059 (25 μM)</td>
<td>1.1 ± 0.2</td>
<td>2.0 ± 0.8</td>
<td>2.1 ± 0.4</td>
</tr>
<tr>
<td>3 h</td>
<td>None</td>
<td>1.0 ± 0.3</td>
<td>2.0 ± 1.0</td>
<td>1.6 ± 0.6</td>
</tr>
</tbody>
</table>

**SAPK/JNK**

| 15 min                      | None (control)   | 1.0 ± 0.3             | 2.0 ± 1.0       | 1.6 ± 0.6       |
|                             | SB 203580 (10 μM)| 1.2 ± 0.04*           | 1.2 ± 0.05      | 1.7 ± 0.1       |
|                             | PD 98059 (25 μM)| 1.1 ± 0.2             | 1.7 ± 0.5       | 1.5 ± 0.3       |
| 3 h                         | None             | 0.5 ± 0.1***           | 0.4 ± 0.1**     | 0.5 ± 0.2*      |

**ERK2**

| 15 min                      | None (control)   | 1.0 ± 0.3             | 2.0 ± 1.0       | 1.6 ± 0.6       |
|                             | SB 203580 (10 μM)| 0.9 ± 0.3             | 1.0 ± 0.1       | 3.1 ± 0.3       |
|                             | PD 98059 (25 μM)| 0.8 ± 0.1             | 0.9 ± 0.1*      | 1.6 ± 0.1**     |
| 3 h                         | None             | 0.7 ± 0.1**            | 0.7 ± 0.2**     | 1.8 ± 0.5       |
The drug reduced the activity of the downstream kinase MAPKAP kinase 2/3 under all conditions, significantly for the control or IL-1β-treated tissues. SB 203580 exerted no overt effect on other MAP kinase activities, except for a marginal increase in SAPK/JNK in control tissues.

Both the spontaneous and the IL-1β-stimulated up-regulation of responsiveness to des-Arg<sup>9</sup>-BK were significantly inhibited (by about 40%) by PD 98059 (25 μM) at 6 h (Table II); however, the drug did not affect des-Arg<sup>9</sup>-BK-induced responses in tissues treated with EGF or the late depression of the response to Pe in tissues treated with IL-1β. An acute exposure to PD 98059 30 min before the 6-h recording of the concentration-effect curve of des-Arg<sup>9</sup>-BK failed to influence the responses (Table III). PD 98059 is an inhibitor of MEK1 MAP kinase (the upstream activator of ERK). Accordingly, the drug selectively and significantly inhibited ERK2 activity in cytokine-treated aortic rings, but the inhibition was not as profound as that obtained by SB 203580 against the p38 pathway (Table I).

Continuous exposure to the tyrosine kinase inhibitor, genistein (40 μM), markedly and selectively inhibited the response to the kinin des-Arg<sup>9</sup>-BK in a manner similar to that of SB 203580, except for the cytokine-induced depression of the 7.5 h response to Pe, which was not prevented by genistein. The acute exposure to genistein failed to significantly depress the 6 h response to des-Arg<sup>9</sup>-BK (Table III), as reported previously (22).

### Inhibition of protein synthesis by drugs

The drugs SB 203580, PD 98059, and genistein failed to inhibit [<sup>3</sup>H]leucine incorporation into fragments of rabbit aortic tissues incubated for 6 h in Krebs solution (Fig. 4). Cycloheximide was effective in this respect.
Experiments based on rabbit aortic cultured smooth muscle cells

Under culture conditions, rabbit aortic smooth muscle cells expressed a basal population of B1Rs that seems to be up-regulated more efficiently by exogenous EGF than by IL-1, as assessed by binding assays (18, 19). Northern blot analysis, performed on total RNA extracted 3 h after cytokine stimulation, showed that EGF treatment (100 ng/ml) determined a 1.7-fold increase in the concentration of B1R mRNA in cells, relative to the control value. The effect of EGF on receptor mRNA was preceded by an activation of all three tested MAP kinase pathways, as assessed at 15 min (2.1-fold for MAPKAP kinase 2/3, 3.5-fold for ERK2, and 1.4-fold for SAPK/JNK).

Table III. Acute effects of inhibitory drugs (applied from time 5.5 h to 6 h) on the parameters of the contractile response to des-Arg<sup>9</sup>-BK recorded at time 6 h of incubation

<table>
<thead>
<tr>
<th>Drug</th>
<th>Response in the Presence of Drug</th>
<th>Response in Control Tissues from the Same Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E&lt;sub:max&lt;/sub&gt;</td>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
</tr>
<tr>
<td>SB 203,580</td>
<td>25 ± 3 (10)</td>
<td>149 ± 27 (6)</td>
</tr>
<tr>
<td>(10 μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKF 106,978</td>
<td>34 ± 8 (4)</td>
<td>97 ± 14 (4)</td>
</tr>
<tr>
<td>(10 μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PD 98,059</td>
<td>41 ± 6 (6)</td>
<td>74 ± 8 (6)</td>
</tr>
<tr>
<td>(25 μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genistein</td>
<td>21 ± 4 (8)</td>
<td>97 ± 5 (8)</td>
</tr>
<tr>
<td>(40 μM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*E<sub:max</sub> values are expressed as the percent of the maximal Pe-induced contraction recorded at 1.5 h; EC<sub>50</sub> values are nM concentrations; both types of values are the means ± SEM of the number of replicates indicated between parentheses. No significant difference between values from drug treated tissues and those from control tissues was found using the Mann-Whitney test.
Discussion

It is becoming increasingly clear that the $B_1$R is up-regulated in several inflammatory situations in experimental animals, such as infection or treatment with bacterial materials (including LPS and Freund’s adjuvant) (4). These treatments amplify the tissue response to blood-derived kinins by conferring an active role on des-Arg$_2$-metabolites, which are always more abundant than native kinins in blood (36, 37) and are actively produced in inflammatory exudates from native kinins (38). Further, the cytokine regulation of the $B_1$R population and the important role of secondarily released eicosanoids in the in vivo pharmacology of the $B_1$Rs (7, 39) suggest that this pharmacological entity is at a crossroad of several types of inflammatory mediators.

The rabbit isolated aorta contractile response to kinins, mediated only by the $B_1$R, was instrumental in defining its pharmacologic profile (40). The contraction is independent of prostanoids, although they are produced and released under the effect of des-Arg$_2$-BK (20). The evidence for the de novo synthesis of $B_1$R in the rabbit aorta is based on the selective abolition of the up-regulation of responses to des-Arg$_2$-BK by protein synthesis inhibitors in isolated tissues (10, 16) and on the induction of both a pharmacologic responsiveness to des-Arg$_2$-BK (41) and of the $B_1$R mRNA (data not shown) by LPS pretreatment applied in vivo before sacrifice. The Northern blot approach notably shows that the basal level of $B_1$R mRNA is very low or null in the normal rabbit aorta (not shown), as recently shown in the normal rabbit heart (14). Aortic rings that have been isolated and incubated in vitro for some time are not a source of mRNA of sufficiently high quality for Northern blot analysis.

The present data suggest that specific protein kinases are recruited following tissue isolation to trigger the de novo synthesis of $B_1$Rs. In particular, p38 MAP kinase activity is very high in control aortic rings (~10- to 20-fold higher level of stimulation in the immunoprecipitated fractions) relative to that in various types of untreated cultured cells. The spontaneous up-regulation of $B_1$Rs is preceded by an activation of p38 catalytic activity, as also reflected by the high and persistent activation of its downstream substrate, MAPKAP kinase 2/3, in extracts of aortic rings (Fig. 1 and Table I). At a concentration that effectively inhibits MAPKAP kinase 2/3 activation, SB 203580 massively (~75%) inhibited the spontaneous up-regulation of responsiveness to the kinin, indicating that p38 MAP kinase is an important determinant of $B_1$R expression. The p38 MAP kinase pathway is one of three known homologous pathways that transduce the messages generated by stressing agents or cytokines and is notably activated by oxidants, LPS, IL-1, heat shock, and hyperosmolality in various cell types (25, 26). The p38 inhibitor, SB 203580, inhibits the production of inflammatory cytokines in response to LPS (42). The p38 kinase and the downstream MAPKAP kinase 2/3 activities were only moderately increased by cytokines (Fig. 1A and Table I), but SB 203580 could still effectively block the sensitization of cytokine-stimulated rings to des-Arg$_2$-BK (Figs. 2 and 3, and Table II); this further supports an important role for the p38 pathway in the expression of $B_1$R. Several transcription factors are known to be activated more or less selectively by this pathway (growth arrest and DNA damage inducible 153, cAMP response element binding protein, ATF-1, and ATF-2) (43), and this may represent a link between tissue injury and $B_1$R induction. On the other hand, the p38 pathway may not be recruited by $B_1$R agonists to contract the tissue when the receptors are formed, as SB 203580 failed to acutely influence the response to des-Arg$_2$-BK. SB 203580 exerts profound anti-inflammatory effects in several animal models of immunopathology (44), and repression of the expression of $B_1$R may represent one of the modes of action of this drug. Accordingly, a peptide $B_1$R antagonist clearly has an analgesic and anti-inflammatory potential in animal models (8, 45).

Other cytokine-regulated genes may contribute to the therapeutical effect of SB 203580, as suggested by the partial prevention of IL-1β-induced loss of Pe contractile efficacy at the late time of 7.5 h (Table II). From our previous work, we interpret this late effect of IL-1 on Pe-induced contractility as the induction of a NOS isoform (i-NOS) (23, 24). The nonselective, agonist- and endotoxin-independent depressant effect of NO on contractility in IL-1-treated tissues was indirectly identified by its complete reversal by nitro-l-arginine, a guanylyl cyclase inhibitor, or hemoglobin. The effect of IL-1 was dependent on protein synthesis and was suppressed by dexamethasone. Thus, the p38 pathway may be recruited by IL-1 to induce i-NOS in the rabbit aorta.

PD 98059 is a specific inhibitor of both tyrosyl and threonine protein phosphorylation directed by MEK1, thus stopping the downstream activation of the ERK MAP kinase pathway (33). The partial inhibition of $B_1$R induction of the contractile effect in rabbit aortic rings by this drug suggests a modulatory function of this pathway on receptor expression, possibly of a less critical nature than the role played by the p38 pathway. An incidental finding in our study is that ERK2 is strongly activated by EGF in fresh aortic tissue (Fig. 1C and Table I). EGF up-regulates the number of $B_1$R on rabbit vascular smooth muscle (19), but also acutely potentiates the effect of $B_1$R agonists (22), suggesting interactions at multiple levels between $B_1$R and EGF receptors.

A practical limitation of the use of the inhibitory drug is the partial nature of the inhibition obtained, as assessed by the MAP kinase assays, especially for PD 98059 (Table I). For instance, contractility tests showed that addition of exogenous EGF abrogated the inhibitory effect of PD 98059 (Table II), but ERK2 assays in aortic extracts suggests that the drug inhibition was partially surmountable by the growth factor action. Other cases of partial surmountability of drug inhibition by cytokines were observed (Table I) and may explain why SB 203580 and PD 98059 were proportionally less effective in cytokine-stimulated aortic rings to prevent the development of contractile responses to des-Arg$_2$-BK (Figs. 2 and 3, and Table II). In the fresh, compactly organized aortic tissue, the access of drugs to their molecular targets might have been less optimal than in cultured cells, where the same concentrations were proportionally more effective (25).

The drugs used in the present study do not cover specifically the c-Jun NH$_2$-terminal kinase pathway, which is, however, activated by the isolation procedure and IL-1 (Fig. 1B and Table I). We cannot exclude that this alternate MAP kinase pathway may also influence $B_1$R expression. Activation of the three families of serine and threonine kinases depends on both tyrosyl and threonyl phosphorylation of the MAP kinase proteins. Genistein, a tyrosine kinase inhibitor, was the least specific of the three inhibitors used in the present study. Its potent and selective inhibitory effect on the development of tissue response to des-Arg$_2$-BK is consistent with the role of MAP kinase pathways in the regulation of $B_1$R.

Cultured rabbit aortic smooth muscle cells express a relatively high basal population of $B_1$Rs relative to the inducible levels following cytokine treatments. The regulation of $B_1$Rs in these cells is conceivably influenced by the proliferative phenotype, the MAP kinase response to serum, and the alien environment (plastic vessels, culture medium, endothoxin traces, etc.). A 6-fold stimulation of $B_{max}$ by EGF was reported under experimental conditions that differed from ours (19). The $B_1$R mRNA level is null or very low in the aorta immediately isolated from normal rabbits (data not shown), but is measurable in cultured aortic smooth muscle cells and is increased 1.7-fold 3 h after EGF stimulation. This effect was
preceded by a stimulation of all measured MAP kinase in these cells, consistent with the role of MAP kinase pathways in the regulation of B1Rs.

Protein kinase inhibitors reveal the role of cell injury-controlled MAP kinase pathways, singularly of the p38 pathway, in the induction of B1R by tissue injury in rabbit aortic tissue. It is expected that specific inhibitory drugs will be useful in dissecting the pro-motor/enhancer function of the B1R gene.

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References


