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Autoregulation of Bradykinin Receptors: Agonists in the Presence of Interleukin-1β Shift the Repertoire of Receptor Subtypes from B2 to B1 in Human Lung Fibroblasts

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ABSTRACT

Elevated formation of bradykinin (BK) and Lys-BK or kallidin (KD) and their carboxypeptidase metabolites desArg^{9}BK and desArg^{10}KD is evident at sites of inflammation. Moreover, B2 receptors (B2R), which mediate the action of BK and KD, participates in the acute stage of the inflammatory and pain response, whereas B1 receptors (B1R), through which desArg^{9}BK and desArg^{10}KD act, partake in the chronic stage. We hypothesized that kinins autoregulate B2R and B1R expression in favor of B1R. Incubation of IMR-90 cells with BK (100 nM) led to a loss (89%) of B2R with a half-life ($T_{1/2}$) of 7.0 min. Concomitantly, BK increased B1R (2- to 3-fold) with a $T_{1/2}$ of 120 min. DesArg^{10}KD (100 nM) had no effect on B2R but increased B1R (3- to 4-fold) with the same rate as BK. Interleukin-1β (IL-1β; 500 pg/ml) also increased B1R (4- to 6-fold). Although both desArg^{10}KD and BK increased the level of IL-1β mRNA, IL-1β receptor antagonist inhibited the increase in B1R only in response to BK. DesArg^{10}KD and BK synergistically increased B1R (9-fold), which was further increased by inclusion of IL-1β (36-fold). Therefore, kinin metabolism and kinin-stimulated production of cytokines may play a pivotal role in shifting the repertoire of kinin receptor subtypes in favor of B1R during inflammation.

Tissue injury triggers the formation of kinins (Proud and Kaplan, 1988; Bhoola et al., 1992), and kinins elicit pain, inflammation, and hyperalgesia (Proud and Kaplan, 1988; Dray and Perkins, 1993). Consequently, kinins have been implicated in the inflammatory and pain response that occurs after injury (Dray and Perkins, 1993). Activation of tissue or plasma kallikreins results in formation of the first set of kinins, bradykinin (BK) and Lys-BK or kallidin (KD), from kininogen precursors (Bhoola et al., 1992). These products are subsequently degraded in part by carboxypeptidases to yield the second set of kinins, desArg^{9}BK and desArg^{10}KD, which remain biologically active. Receptors for kinins have been divided into two subtypes named B1 and B2 (Regoli and Barabe, 1980). The B2 receptor subtype mediates the action of BK and KD, whereas the B1 receptor subtype mediates the action of desArg^{9}BK and desArg^{10}KD. Both receptor subtypes are members of the superfamily of seven-transmembrane domain, G protein-coupled receptors (Hess et al., 1992; Menke et al., 1994) and couple to similar, if not identical, effector systems, including stimulation of phosphoinositide hydrolysis and intracellular Ca^{2+} mobilization (Tropea et al., 1993; Mathis et al., 1996). However, these receptors differ significantly in the short-term regulation to which they are subjected. The B2 receptor elicits a transient response that rapidly desensitizes (Mathis et al., 1996), and the receptor is rapidly internalized (Munoz and Leeb-Lundberg, 1992; Munoz et al., 1993). In contrast, the B1 receptor elicits a sustained response that is subject to very limited desensitization (Mathis et al., 1996), and the receptor is internalized only very slowly (Austin et al., 1997).

The B2 receptor is constitutively expressed in relative high numbers in many tissues and cultured cells, whereas the B1 receptor is expressed in very low numbers in few tissues and cells. Furthermore, tissues are generally unresponsive to desArg^{9}BK and desArg^{10}KD. These observations have led to the belief that the B2 receptor is responsible for most of the actions of kinins in vivo under nonpathological conditions (Dray, 1997). Indeed, animal models have directly implicated the B2 receptor in the acute phase (hours) of the inflammatory and pain response (Proud and Kaplan, 1988; Dray and Perkins, 1993). The same models suggest that the B1 receptor is involved in the chronic phase (days) of this response (Dray and Perkins, 1993). The time-dependent rise in the function of the B1 receptor in the inflammatory and pain response is consistent with the up-regulation of this receptor by various inflammatory stimuli. Originally observed in the

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ABBREVIATIONS: BK, bradykinin; KD, kallidin; IL, interleukin; RT, reverse transcription; TNF, tumor necrosis factor; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; IL-1ra, interleukin-1 receptor antagonist.

rabbit aorta as an induction in the responsiveness of the tissue to B1 agonists on prolonged in vitro incubation, a direct link with proinflammatory cytokines was established after the observation that B1 responsiveness could be induced in several vascular tissues by pyrogens such as bacterial lipopolysaccharide, muramyl-dipeptide, and interleukin (IL)-1 (Marceau, 1995). Even though several cytokines such as IL-1β, tumor necrosis factor (TNF)-α, interferon γ, and IL-2 and the chemokine IL-8 have been implicated in B1 receptor up-regulation, only IL-1β has been shown to do so both in vitro (Menke et al., 1994; Marceau, 1995; Phagoo et al., 1997; Zhou et al., 1998) and in vivo (Davis and Perkins, 1994; Perkins et al., 1995). In all, these observations have led to the belief that the B2 receptor serves in triggering the inflammatory and pain response, whereas the B1 receptor serves in maintaining the response (Dray and Perkins, 1993).

We hypothesized that kinins themselves are able to coordinate the activities of B1 and B2 receptors during inflammation. To study the regulation of the receptors by kinins, we used IMR-90 human fetal lung fibroblasts. These cells were chosen for two reasons. First, under basal conditions, these cells express both B2 and B1 receptors at levels that reflect reasonably well those observed in tissues in vivo. Second, kinin receptors have been proposed to play an important role in allergic inflammation of the airways (Polosa, 1992), and the airways of asthmatic subjects contain elevated levels of both kallikrein activity and kinins (Christiansen et al., 1992).

Experimental Procedures

Materials. [2,3-*prolyl*-3,4-^{3}H]Bradykinin (114 Ci/mmol), des-(Arg^{10})[3,4-*prolyl*-3,4-^{3}H]kallidin (82–91 Ci/mmol), and *myo*-[^{3}H]-inositol (22 Ci/mmol) were obtained from DuPont-NEN (Boston, MA). IMR-90 human fetal lung fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). Primers for reverse transcription-polymerase chain reaction (RT-PCR) were synthesized at the University of Texas Health Science Center at San Antonio. All cell culture reagents were obtained from Life Technologies (Gaithersburg, MD), except fetal bovine serum (FBS), which was obtained from Sigma Chemical Co. (St. Louis, MO). rRNasin was obtained from Promega (Madison, WI). DesArg^9[Leu^8]BK, HOE140, and desArg^{10}KD were obtained from Bachem (Torrance, CA). IL-1β was purchased from R&D Systems (Minneapolis, MN). IL-1 receptor antagonist (IL-1ra) and TNF-α were obtained from the National Institute for Biological Standards and Control (Potters Bar, UK). IL-6 and IL-8 neutralizing antibodies were purchased from Sigma Chemical Co. and Endogen (Woburn, MA), respectively. All other chemicals were obtained from Sigma. Six-well plates (Primaria) and tissue culture plastics were from Falcon.

Culture of IMR-90 Human Fetal Lung Fibroblasts. IMR-90 fibroblasts were cultured in complete growth media composed of Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 50 IU/ml penicillin, 50 μg/ml streptomycin, 4 mM L-glutamine, and 1% nonessential amino acids. The cells were maintained in a humidified atmosphere in 5% CO_2 at 37°C. Cells were subcultured by incubation with 0.05% trypsin-0.5 mM EDTA at a ratio of 1:2 or 1:3, twice weekly. For all experiments, cells were plated at a density of 150,000 cells/well in 6-well (3.5-cm-diameter) plates and used at confluency (4–5 days) between passages 15 and 25. Before experimentation, the IMR-90 cells were washed once with growth medium excluding FBS (hence referred to as DMEM) before being incubated in the absence and presence of receptor agonists and/or cytokines in 2 ml of DMEM as described in the figure legends.

Radioligand Binding. To determine B1 and B2 receptor-specific binding on cells that had been exposed to receptor agonists, a previously described acid-stripping technique was used that effectively removes bound ligand from cells by washing with low pH buffer (Munoz and Leeb-Lundberg, 1992; Munoz et al., 1993). As we have shown previously, this treatment was not deleterious to the cells and did not significantly affect receptor affinity or number. This procedure was performed at 4°C. In short, after exposure of the cells to receptor agonist, bound ligand was removed by first rinsing with PBS, followed by two incubations in 0.05 M glycine-HCl, pH 3.0, for 6 and 0.5 min and then two brief rinses in PBS. As determined by cell viability staining with Trypan blue, this acid-washing procedure was not detrimental to the IMR-90 cells and did not alter significantly [^{3}H]BK and [^{3}H]desArg^{10}KD binding.

Radioligand binding assays were performed at 4°C in duplicate 6-well dishes in a final volume of 1.25 ml. The variation between duplicate wells was $\leq$8%. For saturation studies, IMR-90 cells were incubated for 75 min in the presence of various concentrations of [^{3}H]BK or [^{3}H]desArg^{10}KD ranging from 0.125 to 5 nM in binding buffer that was composed of 20 mM HEPES, pH 7.4, 125 mM *N*-methyl D-glucamine, 5 mM KCl, 0.14 g/l bacitracin, 1 mM 1,10-phenanthroline, and 1 g/l BSA. All other experiments were performed using a radioligand concentration of 1 nM. Nonspecific binding was defined as the amount of radiolabeled ligand bound in the presence of 1 μM nonradioactive ligand. After incubation, the assay buffer was removed, and the cells were washed with 2 $\times$ 4 ml of ice-cold PBS. The cells were then lysed with 0.05% SDS. Specific binding was expressed in fmol/mg protein, and protein was determined according to the method of Bradford (1976) using a Bio-Rad (Hercules, CA) kit. Binding data were processed using ORIGIN (Microcal).

RT-PCR. Total RNA was extracted from cells using TRIZOL reagent as described by the manufacturer (Life Technologies, Gaithersburg, MD). Single-stranded cDNA was generated using Superscript II reverse transcriptase (100 U) in a 20-μl reaction mixture containing reaction buffer (50 mM Tris · HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol), 0.5 mM dNTP, 0.5 μg of oligo(dT)$_{12\text{-}18}$, 10 U of rRNasin, and 2 μg of total RNA. The reaction was carried out for 1 h at 42°C. Amplification of cDNA by PCR was performed using oligonucleotide primer pairs for IL-1β and β-actin as described by Jung et al. (1995). The reactions were carried out using a thermal cycler (M.J. Research, Watertown, MA) in a 30-μl reaction mixture containing reaction buffer (20 mM Tris · HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 mM dNTP, 1.5 U of *Taq* polymerase, and 2 μl of cDNA. Each primer was added at a final concentration of 0.1 μM. PCR was for 35 cycles with each cycle consisting of 1 min at 95°C and annealing/extension at 60°C for 2.5 min. Amplification of cDNA for the B1 receptor was performed using oligonucleotide primer pairs as described by Bachvarov et al. (1996). In this case, the above reaction mixture was modified to include 1.0 mM $MgCl_2$ and a primer concentration of 0.25 μM. PCR was for 30 cycles, each cycle consisting of 1 min at 95°C, annealing at 55°C for 1 min, and extension at 72°C for 1 min. PCR products were separated on 1% agarose gels containing 50 μg/ml ethidium bromide and visualized under UV light.

Cytokine/Chemokine Assays. Human recombinant cytokines/chemokines were handled according to the manufacturers' instructions (see *Experimental Procedures*). Samples of the media from stimulated cells were collected by centrifugation at 14,000*g* for 5 min at 4°C and then frozen on dry ice and stored at −70°C until assay (1–3 months). The supernatant was assayed for the presence of IL-6 and IL-8 using two-site enzyme-linked immunosorbent assays as described by Steffen and Ebersole (1996). Neutralizing antibodies were incubated with cells at 5 μg/ml for 1 h before the addition of agonists.

Phosphoinositide Hydrolysis. Total inositol phosphate production was assayed as described by Tropea et al. (1993) with a few modifications. Briefly, cells were labeled with 10 μCi/ml *myo*-[^{3}H] inositol for 18 h. They were then washed once and incubated for 6 h in the presence of various factors in 2 ml of DMEM. After stimula-

tion, the cells were washed with low pH buffer as described to remove any bound ligand and then allowed to equilibrate at room temperature for 5 min. At 30 min before the assay, the medium was changed to DMEM/50 mM LiCl at 37°C. The cells were stimulated with 1 μM desArg^{10}KD in 2 ml of DMEM for 20 min before termination of the reaction by aspirating off the medium and adding ice-cold 10% trichloroacetic acid. Total inositol phosphates were then assayed by anion exchange chromatography.

Data Analysis. Data are reported as the mean ± S.E. and were compared using Student's t test. Values of $p \leq .05$ were considered to be significant.

Results

Constitutive Expression of B1 and B2 BK Receptors in IMR-90 Cells. IMR-90 fibroblasts express both B1 and B2 BK receptor subtypes as determined by specific [^{3}H]desArg^{10}KD and [^{3}H]BK binding, respectively (Menke et al., 1994). When these cells are cultured under standard conditions in the presence of 10% serum, the B1 and B2 receptor subtypes are present in a ratio of approximately 1:14. Furthermore, B1 receptors in these cells are up-regulated in response to IL-1β (Menke et al., 1994; Zhou et al., 1998). To investigate the regulation of B1 and B2 receptor expression without interference by any regulatory factors that may be present in the serum, the current study incorporated the stimulation of the cells at 37°C in DMEM rather than in serum. After incubation of the cells in DMEM for 6 h at 37°C, the IMR-90 cells expressed B1 and B2 receptors in a ratio of approximately 1:85 (Table 1).

B2 Receptor Agonists Promote B2 Receptor Internalization and a Decrease in Available Cell Surface B2 Receptors. Figure 1A shows a typical saturation binding isotherm of [^{3}H]BK to cell surface B2 receptors on IMR-90 cells after incubation in DMEM for 6 h. Under these conditions, [^{3}H]BK identified a relatively large number of B2 receptors (B_{max} = 1,283 fmol/mg protein) with high affinity (K_D = 0.59 nM; Table 1). Exposure of the cells to 100 nM BK for 6 h at 37°C resulted in a dramatic loss in the number of B2 receptors available for [^{3}H]BK binding (B_{max} = 145 fmol/mg protein) without a significant change in the affinity of [^{3}H]BK for the receptors (K_D = 0.39 nM; Fig. 1A and Table 1). The BK-promoted response was rapid ($T_{1/2}$ = 7.0 ± 1.5 min) and reached a plateau at approximately 2 h that was maintained for ≥6 h in the continued presence of BK (Fig. 1B). The response was concentration dependent with an EC_{50} value for BK of 7.0 ± 1.5 nM (Fig. 1C). Furthermore, the response was inhibited by preincubation of the cells for 1 h with the B2 receptor-specific antagonist HOE140 (30 μM) but not with the B1 receptor-specific antagonist desArg^9[Leu^8]BK (100 μM; data not shown). These results are similar to those previously described elsewhere for B2 receptors in a variety of cell types and show that agonist binding to B2 receptors leads to a rapid internalization of the receptor. This response is specific for the B2 receptor as incubation of the cells with the B1 receptor agonists desArg^{10}KD (10 μM) or desArg^{9}BK (10 μM) for 6 h had minimal or no effect on the availability of B2 receptors (Fig. 1B).

B2 and B1 Receptor Agonists and IL-1β Promote an Increase in B1 receptor Gene Expression and an Increase in Available Cell Surface B1 Receptors. Figure 2A shows a typical saturation binding isotherm of [^{3}H]desArg^{10}KD to cell surface B1 receptors on IMR-90 cells after incubation in DMEM for 6 h. Under these conditions, [^{3}H]desArg^{10}KD identified a relatively small number of B1 receptors (B_{max} = 15.1 fmol/mg protein) with high affinity (K_D = 0.50 nM; Table 1). Exposure of the cells to 100 nM BK for 6 h at 37°C resulted in a 2- to 3-fold increase in the number of B1 receptors available for [^{3}H]desArg^{10}KD binding (B_{max} = 31 fmol/mg protein) without any significant effect on the affinity of [^{3}H]desArg^{10}KD for the receptors (K_D = 0.36 nM; Fig. 2A, Table 1). As shown in Fig. 2B, the BK-promoted response was transient with a maximum at 4 to 6 h. The increase was clearly apparent at 0.5 h, half-maximal at approximately 2 h, and had returned to near basal levels by 8 h. The response was concentration dependent with an EC_{50} value for BK of 11.3 ± 3.5 nM (Fig. 2C). To confirm that the BK-promoted increase in B1 receptor expression was mediated through the B2 receptor, the cells were preincubated for 1 h with receptor-selective antagonists. The B2-selective antagonist HOE140 (30 μM) inhibited the increase in the number of B1 receptors, whereas the B1-selective antagonist desArg^9[Leu^8]BK (100 μM) was unable to perturb the response (Fig. 2D). These results show that agonist binding to B2 receptors leads to an increase in the number of B1 receptors in IMR-90 cells.

B1 agonists also increased B1 receptor expression in IMR-90 cells (Fig. 2A). Exposure of the cells to 100 nM desArg^{10}KD for 6 h at 37°C resulted in a 3- to 4-fold increase in the number of B1 receptors available for [^{3}H]desArg^{10}KD binding (B_{max} = 36.7 fmol/mg protein) without any significant effect on the affinity of [^{3}H]desArg^{10}KD for the receptors (K_D = 0.23 nM; Fig. 2A, Table 1). An increase was also observed after exposure to 100 nM desArg^{9}BK (data not shown). As shown in Fig. 2B, the desArg^{10}KD response was similar to that for BK with a peak response at approximately 6 h that was half-maximal at approximately 2 h. In contrast to the BK response, the desArg^{10}KD response remained elevated at 8 h and had returned to near basal level by 20 h. The

TABLE 1

Binding constants of BK and desArg^{10}KD to IMR-90 cells before and after treatment with receptor agonists and IL-1β

Values for binding constants were calculated from saturation curves of radioligand binding as shown in Figs. 1A, 2A, 3A, and 6A. Values are averages of two independent experiments with each assay done in duplicate, and values differed by <10%.

Treatment	K_D		B_{max}		ΔB_{max}	
	BK	des Arg^{10}KD	BK	des Arg^{10}KD	BK	des Arg^{10}KD
	nM		*fmol/mg protein*		*% of basal*	
Basal	0.59	0.50	1,283	15.1	100	100
+ BK	0.39	0.36	145	31.0	11	205
+ desArg^{10}KD		0.23		36.7		243
+ IL-1β		0.26		56.5		370
+ desArg^{10}KD and IL-1β		0.24		346		2,290

response was concentration dependent with an EC_{50} value for desArg^{10}KD of 7.6 ± 2.5 nM (Fig. 2C). The B1-selective antagonist desArg^{9}[Leu^{8}]BK (100 μM) inhibited the desArg^{10}KD-promoted response, whereas the B2-selective antagonist HOE140 (30 μM) was without effect (Fig. 2D). These results show that B1 receptors on IMR-90 cells are autoregulated by B1 agonists (i.e., agonist binding to the B1 receptor leads to an increase in the expression of B1 receptors in these cells).

Previous studies in a number of cell types, including fibroblasts, show that B1 receptors are up-regulated by several cytokines including IL-1β (Menke et al., 1994; Phagoo et al., 1997; Zhou et al., 1998) and TNF-α (Phagoo et al., 1997). These responses are preceded by an increase in B1 receptor mRNA, indicating that the regulation occurs at the level of receptor gene expression (Phagoo et al., 1997; Zhou et al., 1998). As shown in Fig. 3A and Table 1, incubation of IMR-90 cells with 500 pg/ml IL-1β for 6 h resulted in a 4- to 6-fold

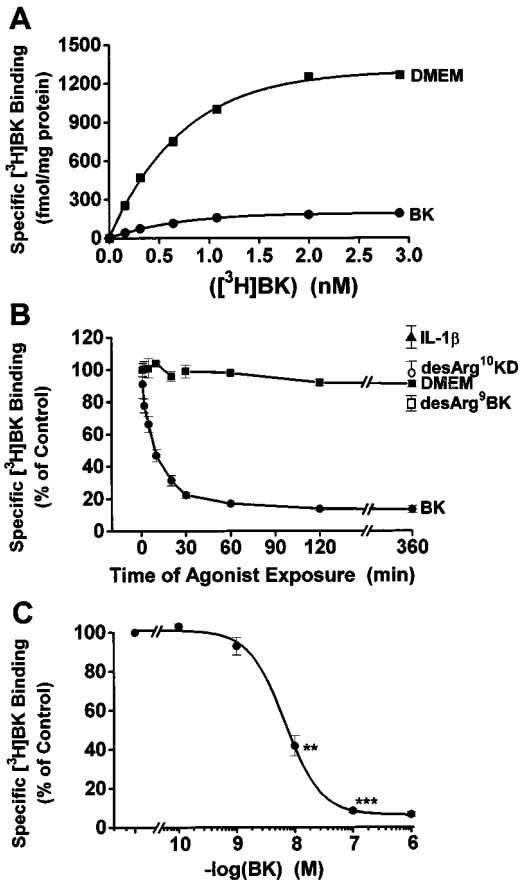

Fig. 1. The effect of kinins and IL-1β on the availability of cell surface B2 receptors. IMR-90 cells were treated at 37°C with DMEM (■), 100 nM BK (●), 10 μM desArg^{10}KD (○), 10 μM desArg^{9}BK (□), or 500 pg/ml IL-1β (▲), washed with low pH buffer, and then assayed for specific [^{3}H]BK binding at 4°C as described in *Experimental Procedures*. A, cells were treated as indicated for 6 h and then assayed for binding by saturation binding analysis. The result is a representative of two experiments. B, cells were treated for various times as indicated and then assayed for binding. The data shown are from three experiments. The results are presented as a percentage of control where 100% refers to the binding before any treatment. C, cells were treated for 6 h with various concentrations of BK and then assayed for binding. The data shown are from three experiments. The results are presented as a percentage of control where 100% refers to the response to DMEM treatment alone. Comparison with DMEM treatment for 6 h at 37°C: $^{**}p < .01$; $^{***}p < .001$.

increase in the density of B1 receptors available for [^{3}H]desArg^{10}KD binding (B_{max} = 56.5 fmol/mg protein) without any significant effect on the affinity of [^{3}H]desArg^{10}KD for the receptors (K_D = 0.26 nM). A similar increase in B1 receptor expression (4.8 ± 0.09-fold) was observed in response to TNF-α (10 ng/ml). In contrast, IL-1β had only a minimal effect (1.2 ± 0.07-fold increase) on the number of B2 receptors in these cells (Fig. 1B).

To investigate whether the agonist-promoted B1 receptor up-regulation occurs at the level of gene expression, we first determined whether protein synthesis was required for the up-regulation. To do so, IMR-90 cells were treated for 1 h with 10 μg/ml cycloheximide, a protein synthesis inhibitor, before exposing the cells to BK (100 nM) or desArg^{10}KD (100 nM) for 6 h. Cycloheximide treatment completely abolished the increase in B1 receptor expression observed in response to both BK and desArg^{10}KD (data not shown). These results show that agonist-promoted up-regulation of B1 receptor expression involves de novo receptor synthesis.

RT-PCR was used to determine whether the up-regulation of B1 receptors by agonists involves an increase in B1 receptor mRNA. As shown in Fig. 3B, treatment of IMR-90 cells for 6 h with either 100 nM BK or 100 nM desArg^{10}KD resulted in a significant increase in the amount of PCR product encoding for B1 receptor mRNA. As expected, an increase in receptor mRNA was also observed in response to 500 pg/ml IL-1β. In all, these results show that agonist stimulation of B1 and B2 receptors leads to an up-regulation of B1 receptor gene expression and a subsequent increase in the number of B1 receptors available for binding desArg^{10}KD on the cell surface.

IL-1β Mediates B2 Agonist-Promoted But Not B1 Agonist-Promoted Up-regulation of B1 Receptors. Considering that IL-1β is capable of up-regulating B1 receptors in IMR-90 cells, this cytokine represents one candidate mediator of the B2 and B1 agonist responses. If so, B2 and B1 agonists should be able to stimulate the expression of IL-1β in these cells. As demonstrated in Fig. 3B, exposure of cells to 100 nM BK or desArg^{10}KD for 6 h resulted in a significant increase in the level of the PCR product encoding for IL-1β mRNA. These results show that both B1 and B2 receptor agonists promote the transcriptional activation of the IL-1β gene and the formation of IL-1β mRNA. Treatment with 500 pg/ml IL-1β also up-regulated IL-1β gene expression (Fig. 3B), confirming that IL-1β gene expression is autoregulated by IL-1β (Dinarello et al., 1987).

IL-1ra was used to directly evaluate the involvement of IL-1β in agonist-promoted up-regulation of B1 receptor expression. This antagonist binds to cell surface IL-1 type I receptors and competitively inhibits the binding of both IL-1α and IL-1β. Figure 4A shows, as expected, that the increase in B1 receptors produced by a peak concentration of exogenously added IL-1β (500 pg/ml) was almost completely inhibited in the presence of a 400-fold excess of IL-1ra (200 ng/ml). IL-1ra also almost completely inhibited the BK-induced response. In contrast, IL-1ra did not significantly perturb the increase in B1 receptors promoted by desArg^{10}KD (Fig. 4A). These results clearly show that although both B1 and B2 receptor agonists are capable of increasing the level of IL-1β mRNA, only the B2 receptor-mediated up-regulation of B1 receptors involves the action of mature IL-1β protein. IL-1ra almost completely inhibited the BK- and desArg^{10}KD-

stimulated increase in IL-1β mRNA, confirming an autoregulatory mechanism of IL-1β gene expression in the action of these factors (Fig. 4B).

B2 and B1 Receptor Agonists and IL-1β Act Synergistically to Up-regulate B1 Receptor Expression. Because sites of inflammation contain elevated levels of both B2 and B1 agonists, it can be expected that these agonists act in concert. Figure 5 shows that at 0.5 and 2 h of exposure, the effect of adding BK and desArg^{10}KD in combination was more or less additive to the individual effects of the agonists at these times. On the other hand, at 6 h of exposure, a dramatic synergism was observed between these two agonists. Indeed, the increase observed in response to the two receptor agonists (9-fold) was significantly higher than that observed in response to IL-1β (4–6-fold) These results suggest that at early time points ($\leq$2 h), B2 and B1 agonists may act via the same mechanism to increase B1 receptor gene expression. On the other hand, the synergism observed between B2 and B1 agonists at later time points ($\geq$6 h) confirms earlier conclusions that these agonists act at least in part through distinct mechanisms.

Coincubation of 100 nM desArg^{10}KD and 500 pg/ml IL-1β for 6 h produced a 23-fold increase in the B_{max} value of [^{3}H]desArg^{10}KD binding without an effect on the K_D value of the binding (Fig. 6A and Table 1). This increase in B1 receptors was much larger than that observed with IL-1β alone, which was 4- to 6-fold, indicating that it is not simply due to an increased production of IL-1β (Fig. 6A and Table 1). Instead, these factors must synergize in their action to increase B1 receptor expression. IL-1β synergized considerably more with desArg^{10}KD than with BK (Fig. 6B). This is not surprising because the effect of BK appears to be mediated to a major extent by IL-1β. Receptor agonists also synergized in a similar way to IL-1β with a peak concentration of TNF-α (10 ng/ml; data not shown).

B1 and B2 Agonists Stimulate Release of IL-6 and IL-8. The cytokine IL-6 and chemokine IL-8 have been implicated in inflammation (Schindler et al., 1990; Cunha et al., 1991; Ferreira et al., 1993b; Davis and Perkins, 1994). To evaluate the involvement of these cytokines in the action of B2 and B1 agonists and, specifically, in the up-regulation of B1 receptors, we first quantified the levels of these cytokines in the media after agonist stimulation of IMR-90 cells. Incubation of cells with either 100 nM BK or desArg^{10}KD at 37°C

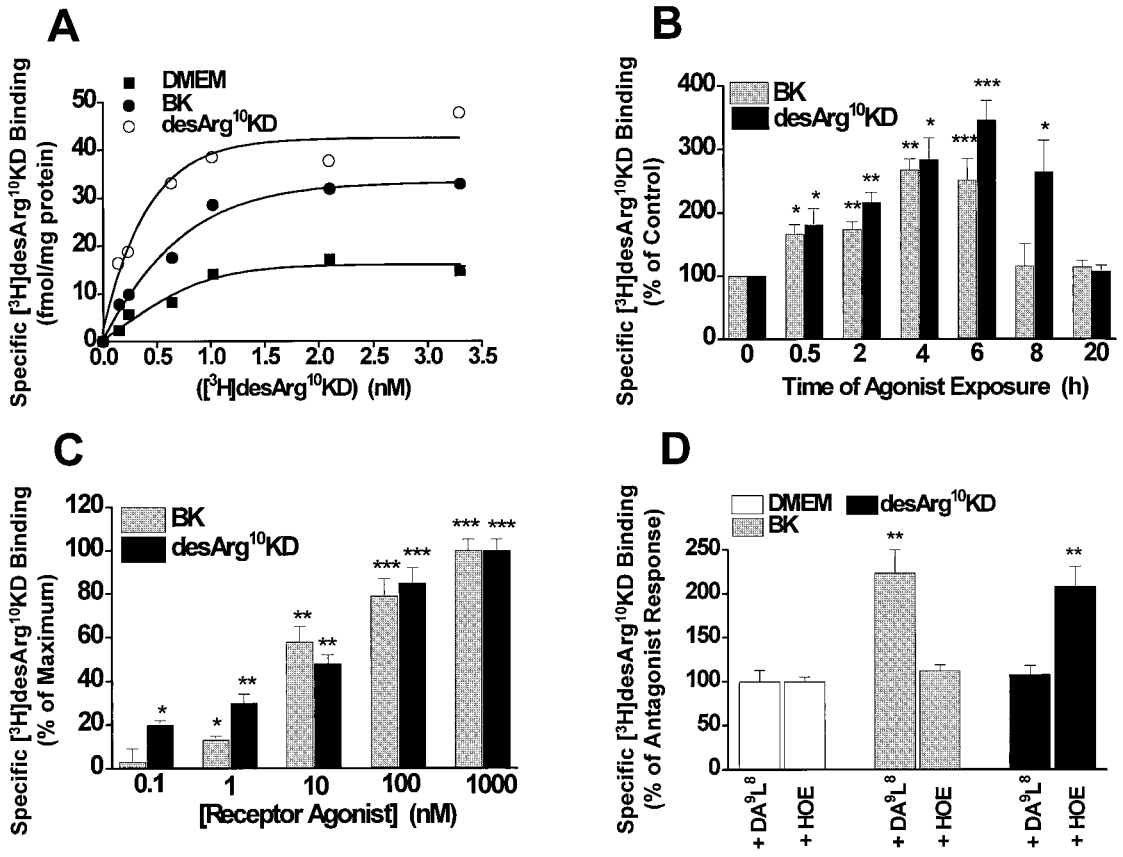

Fig. 2. The effect of kinins on the expression of cell surface B1 receptors. IMR-90 cells were treated at 37°C with DMEM, 100 nM BK, or 100 nM desArg^{10}KD, washed with low pH buffer, and then assayed for specific [^{3}H]desArg^{10}KD binding at 4°C as described in *Experimental Procedures*. A, cells were treated as indicated for 6 h and then assayed for binding by saturation binding analysis. The result is representative of two experiments. B, cells were treated for various times as indicated and then assayed for binding. The data shown are from at least five experiments. The results are presented as a percentage of control where 100% refers to the response to DMEM treatment alone. Comparison with DMEM treatment at each time point at 37°C: $^*p < .05$; $^{**}p < .01$; $^{***}p < .001$. C, cells were treated for 6 h with various concentrations of receptor agonists as indicated and then assayed for binding. The data shown are from at least five experiments. The results are presented as a percentage of maximum where 100% refers to the response to 1000 nM receptor agonist treatment alone. Comparison with DMEM treatment for 6 h at 37°C: $^*p < .05$; $^{**}p < .01$; $^{***}p < .001$. D, cells were pretreated for 1 h at 37°C with the B1 receptor antagonist desArg^9[Leu^8]BK (DA^{9}L^8; 100 μM) or the B2 receptor antagonist HOE140 (HOE; 30 μM) before treatment for 6 h at 37°C with receptor agonists as indicated and then assayed for binding. The data shown are from three experiments. The results are a presented as a percentage of antagonist response where 100% refers to the response to antagonist treatment alone. Comparison with agonist treatment for 6 h at 37°C: $^{**}p < .01$.

resulted in a time-dependent increase in the amount of both immunoreactive IL-6 (Fig. 7A) and IL-8 (Fig. 7B) in the media, with BK being the more efficacious agonist in stimulating the release of these cytokines.

To determine whether IL-6 and IL-8 are capable of increasing B1 receptor expression, cells were incubated for 6 h with various concentrations of IL-6 (1–30 ng/ml) and IL-8 (10–200 ng/ml). Neither IL-6 nor IL-8 had any significant effect on [^{3}H]desArg^{10}KD binding at these concentrations (data not shown). Consistent with the lack of any direct effect of these factors on [^{3}H]desArg^{10}KD binding, IL-6- and IL-8-neutralizing antibodies (5 μg/ml) were unable to inhibit the increase in B1 receptor expression in response to either B1 or B2 agonists (data not shown). These results indicate that both B1 and B2 receptors are capable of mediating an increase in the release of IL-6 and IL-8. However, neither factor, at the concentrations tested, affected the receptor-mediated up-regulation of B1 receptor expression.

Up-Regulation of B1 Receptors Leads to an Increase in B1 Receptor-Mediated Phosphoinositide Hydrolysis. To determine the functional significance of agonist-promoted B1 receptor up-regulation in IMR-90 cells, we analyzed B1 receptor-mediated phosphoinositide hydrolysis after agonist and cytokine pretreatment for 6 h. As shown in Fig. 8, the ability of BK, desArg^{10}KD, and IL-1β to promote an increase in desArg^{10}KD-stimulated phosphoinositide hydrolysis correlated closely with their ability to increase B1 receptor expression both individually and in combination. That the desArg^{10}KD-stimulated functional response was mediated through the B1 receptor was shown by the fact that 100 μM desArg^9[Leu^8]BK completely inhibited the desArg^{10}KD-mediated response, whereas this antagonist was unable to stimulate a response on its own (data not shown).

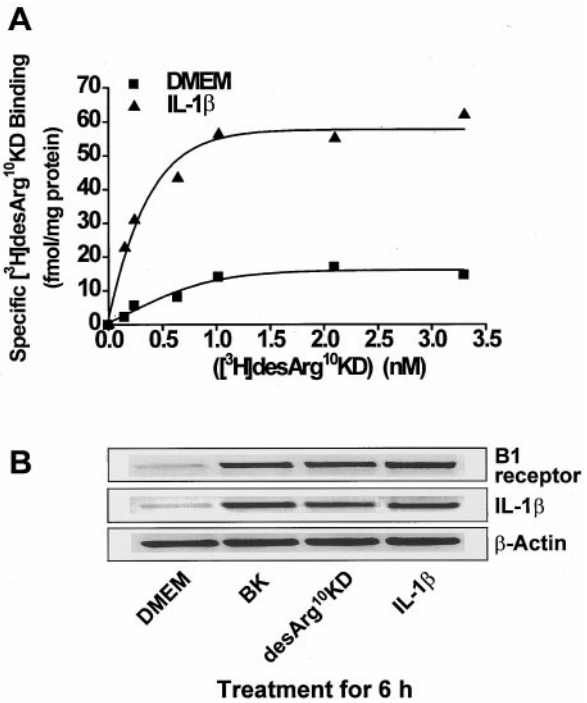

Fig. 3. The effect of IL-1β and kinins on the expression of B1 receptor mRNA and B1 receptors. A, IMR-90 cells were treated for 6 h at 37°C with DMEM or 500 pg/ml IL-1β as indicated and then assayed for specific [^{3}H]desArg^{10}KD binding by saturation binding analysis at 4°C as described in *Experimental Procedures*. The result is representative of two experiments. B, IMR-90 cells were treated for 6 h at 37°C with kinins (1 μM) or IL-1β (500 pg/ml) as indicated and then analyzed for B1 receptor mRNA and IL-1β mRNA as described in *Experimental Procedures*. β-Actin mRNA was used as a loading control. The result is representative of three experiments.

Discussion

Animal models suggest that B2 BK receptors participate in the acute phase of the inflammatory and pain response, whereas the B1 BK receptor participates in the chronic phase

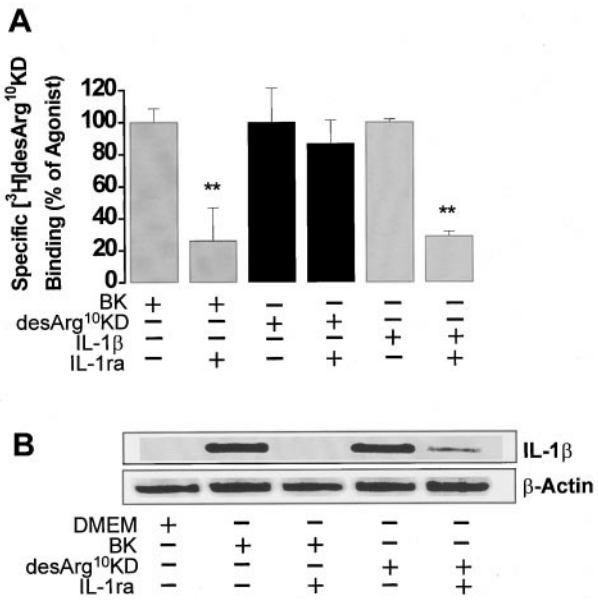

Fig. 4. The role of IL-1β in kinin-promoted B1 receptor expression. A, IMR-90 cells were pretreated for 1 h at 37°C with 200 ng/ml IL-1ra before treatment for 6 h at 37°C with 100 nM BK, 100 nM desArg^{10}KD, or 500 pg/ml IL-1β as indicated and then assayed for specific [^{3}H]desArg^{10}KD binding at 4°C as described in *Experimental Procedures*. The data shown are from three experiments. The results are presented as a percentage of agonist where 100% is the response to agonist treatment alone. Comparison with agonist treatment alone: $^{**}p < .01$. B, IMR-90 cells were treated for 6 h at 37°C as indicated and as described above and then analyzed for IL-1β mRNA as described in *Experimental Procedures*. β-Actin mRNA was used as a loading control. The result is representative of three experiments.

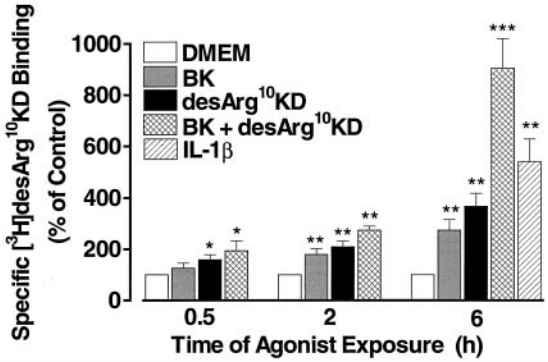

Fig. 5. Synergistic effect of kinins on B1 receptor expression. IMR-90 cells were treated for various times at 37°C with DMEM, 100 nM BK, 100 nM desArg^{10}KD, or 500 pg/ml IL-1β as indicated, washed with low pH buffer, and then assayed for specific [^{3}H]desArg^{10}KD binding at 4°C as described in *Experimental Procedures*. The data shown are from three experiments. The results are presented as a percentage of control where 100% refers to the response to DMEM treatment alone. Comparison with DMEM treatment at each time point: $^*p < .05$; $^{**}p < .01$; $^{***}p < .001$.

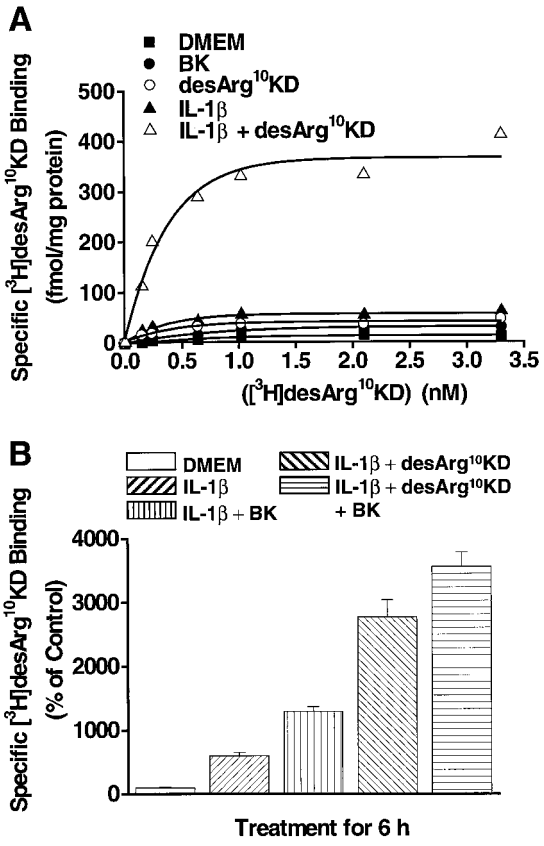

Fig. 6. Synergistic effect of kinins and IL-1β on B1 receptor expression. IMR-90 cells were treated for 6 h at 37°C with DMEM, 100 nM BK, 100 nM desArg^{10}KD, or 500 pg/ml IL-1β, washed with low pH buffer, and then assayed for specific [^{3}H]desArg^{10}KD binding at 4°C as described in *Experimental Procedures*. A, cells were treated for 6 h as indicated and then assayed for binding by saturation binding analysis. The result is representative of two experiments. B, cells were treated as indicated and then assayed for binding. The data shown are from three experiments. The results are presented as percent of control where 100% refers to the response to DMEM treatment alone.

of the response (Dray and Perkins, 1993). However, little, if anything, is known about the regulatory mechanisms underlying this sequence of receptor activities. The results presented in this report provide direct evidence that kinin agonists themselves regulate B1 and B2 receptors in vitro in IMR-90 cells, and this regulation matches that observed in vivo during inflammation.

IL-1β is believed to occupy a central position in the mechanism of B1 receptor up-regulation. IL-1β increases B1 receptor expression in vitro (Menke et al., 1994; Phagoo et al., 1997; Zhou et al., 1998), in vivo (Davis and Perkins, 1994), and ex vivo (Marceau, 1995), and this response appears to be mediated in part by the transcription factor nuclear factor κB (Ni et al., 1998; Schanstra et al., 1998; Zhou et al., 1998). Also, inflammatory conditions promote the release of IL-1β. In addition, both B1 and B2 agonists directly stimulate the release of IL-1β (Tiffany and Burch, 1989; Pan et al., 1996). Despite these observations, some models of B1 receptor induction show less dependence on IL-1β. For instance, the spontaneous sensitization to B1 agonists as a function of time in isolated rabbit aortic rings is insensitive to IL-1ra, even though this antagonist inhibits the potentiating effect of IL-1β on this process (Petitclerc et al., 1992). Furthermore, IL-1ra fails to prevent the expression of B1 receptor-mediated responses after bacterial lipopolysaccharide injection in rabbits (Whalley et al., 1993). Therefore, IL-1β does not seem to be the only mediator of B1 receptor induction in vivo during inflammation. Likewise, IL-1β cannot be responsible for the diminishing contribution of B2 receptors during the inflammatory response. IL-1β has been shown to regulate B2 receptors in vitro, but the change in the receptor number is relatively small and involves receptor up-regulation (Schmidlin et al., 1998).

BK and KD, the first set of kinin peptides formed after tissue damage, act on B2 receptors in a wide variety of tissues to cause a broad repertoire of physiological responses. The action of these kinins is probably limited to their site of production because they are subject to rapid degradation by both carboxyl- and amino-peptidases (Ward, 1991). The ac-

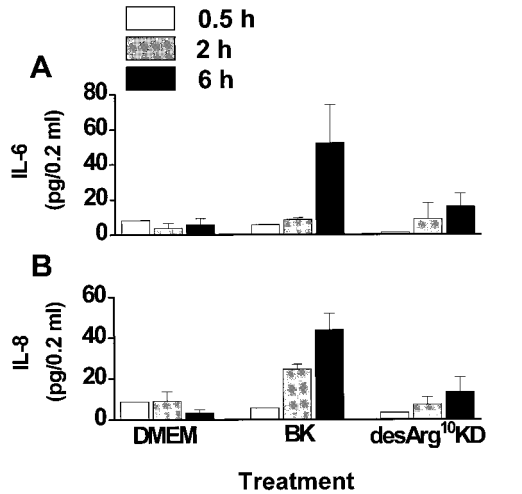

Fig. 7. The effect of kinins on the production of immunoreactive IL-6 and IL-8. IMR-90 cells were treated at 37°C with 100 nM BK or 100 nM desArg^{10}KD for 0.5, 2, or 6 h as indicated. Samples of the media were then removed for analysis of IL-6 (A) and IL-8 (B) as described in *Experimental Procedures*. The data shown are from three experiments.

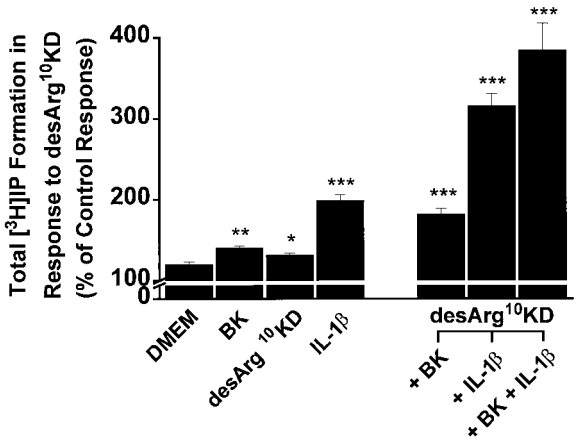

Fig. 8. The effect of kinins and IL-1β on B1 receptor-mediated phosphoinositide hydrolysis. Labeled IMR-90 cells were treated for 6 h at 37°C with DMEM, 100 nM BK, 100 nM desArg^{10}KD, or 500 pg/ml IL-1β and then washed with low pH buffer. The treated cells were stimulated in the absence or presence of 1 μM desArg^{10}KD for 20 min and then analyzed for phosphoinositide hydrolysis as described in *Experimental Procedures*. The results are presented as a percentage of control response where 100% refers to the phosphoinositide hydrolysis response for each treatment in the absence of stimulation with 1 μM desArg^{10}KD and was 5925 ± 615 dpm/well. The data shown are from three experiments. Comparison with DMEM treatment: *$p < .05$; **$p < .01$; ***$p < .001$.

tion of BK and KD is further limited by short-term mechanisms that negatively regulate the B2 receptor. Agonist binding to this receptor results in a transient signal that rapidly desensitizes (Mathis et al., 1996), and the agonist receptor complex is internalized (Munoz and Leeb-Lundberg, 1992; Munoz et al., 1993). In IMR-90 cells, this was observed as a rapid loss of the available cell surface B2 receptors. The agonist-promoted loss of B2 receptors is reversible because agonist removal results in a recycling of the receptors to the cell surface independently of protein synthesis (Munoz et al., 1993). There are examples of depressed B2 receptor function in chronic inflammatory conditions, but it is unclear how this occurs (Marceau et al., 1998). IL-1β caused only a minimal change (1.2-fold increase) in the number of B2 receptors in IMR-90 cells. Consequently, we propose that B2 receptor function is controlled primarily by short-term mechanisms involving receptor desensitization and internalization.

Despite the presence of regulatory mechanisms to limit the action of BK and KD on B2 receptors in IMR-90 cells, these peptides stimulate a small but significant increase in B1 receptor expression that is associated with an increase in B1 receptor-mediated phosphoinositide hydrolysis. This increase was sensitive to cycloheximide and matched by an increase in B1 receptor mRNA. Thus, B2 receptor-mediated up-regulation of B1 receptors appears to occur at the level of B1 receptor gene expression. That this type of kinin receptor cross-regulation occurs in vivo was supported by the fact that repeated administration of the B2 receptor agonist [Tyr^8]BK in a rat model of hyperalgesia resulted in an increase in the responsiveness to the B1 receptor agonist desArg^{9}BK (Campos et al., 1995). Furthermore, treatment with angiotensin-converting enzyme inhibitors, which inhibits the degradation of BK, also increased the responsiveness to desArg^{9}BK in rabbits (Whalley and Nwator, 1989).

BK and KD are degraded to desArg^{9}BK and desArg^{10}KD, respectively, via the action of arginine carboxypeptidases (kininase I), including carboxypeptidases N and M (Ward, 1991), and these metabolites represent the second set of kinin agonists formed after tissue damage (Dray and Perkins, 1993). The in vivo production of desArg^{9}BK and desArg^{10}KD is believed to be relatively inefficient because these enzymes have comparatively low affinities for BK and KD. However, desArg^{9}BK has a significantly longer half-life than BK (4–12-fold), suggesting that these metabolites have a greater capacity to accumulate than their parent peptides (Marceau et al., 1998). The action of desArg^{9}BK and desArg^{10}KD is also enhanced by the fact that the B1 receptor is not subject to short-term mechanisms that negatively regulate the receptor. DesArg^{9}BK or desArg^{10}KD binding to the B1 receptor elicits a signal that is highly sustained and subject to very limited desensitization (Mathis et al., 1996). Furthermore, this receptor is internalized only very slowly (Austin et al., 1997).

In this study, we show that the action of desArg metabolites through the B1 receptor subtype is also enhanced by autologous receptor up-regulation. DesArg^{9}BK and desArg^{10}KD stimulated a small but significant increase in the number of B1 receptors expressed in these cells. The rate of the increase was similar to that for BK through the B2 receptor. Furthermore, the increase was completely inhibited by cycloheximide and matched by an increase in B1 receptor mRNA. Together, these results indicate that B1 receptors are up-regulated in response to both B1 and B2 receptor activation, and the regulation occurs at the level of B1 receptor gene expression.

In agreement with previous studies (Menke et al., 1994; Schanstra et al., 1998; Zhou et al., 1998), treatment of IMR-90 cells with IL-1β for 6 h caused a significant increase in the amount of B1 receptor mRNA and B1 receptors. IL-1ra completely inhibited the IL-1β response, indicating that the response is mediated through the IL-1 receptor. The B2 receptor-mediated response was also inhibited by IL-1ra. Together with the fact that B2 agonists stimulated an increase in IL-1β mRNA, these results show that IL-1β is responsible for the B2 receptor-mediated increase in B1 receptor expression in IMR-90 cells. The lack of any significant synergism between BK and IL-1β in up-regulating the B1 receptor further emphasized the common mechanism of action of these factors in this response.

B1 agonists also stimulated an increase in the production of IL-1β mRNA in IMR-90 cells. However, the B1 receptor-mediated increase in B1 receptor expression was not inhibited by IL-1ra. Thus, autologous up-regulation of the B1 receptor involves a mechanism that is independent of the endogenous production of IL-1β. That the action of B1 agonist does not directly rely on IL-1β production was also emphasized by the remarkable synergism observed between B1 agonists and IL-1β delivered either exogenously or endogenously through stimulation of the B2 receptor. The magnitude of the increase in B1 receptor expression observed in response to the combined action of B1 and B2 agonists was at least 3-fold higher than that observed in response to a peak concentration of IL-1β alone, which was 4- to 6-fold. These results argue that the up-regulation of B1 receptors in vivo is not due to the individual action of any one factor alone but rather to the combined action of several factors. Together with the BK-promoted decrease in B2 receptors, the combined action of BK and desArg^{10}KD resulted in a shift in the ratio of expressed B1 and B2 receptors from ~1:85 under basal conditions to ~1:1 after exposure of the cells to BK and desArg^{10}KD for 6 h. Inclusion of a peak concentration of IL-1β further increased the ratio to ~4:1. Overall, the receptor ratio changes >300-fold in favor of the B1 receptor. Clearly, these conditions lead to a shift in the repertoire of kinin receptors from a predominantly B2 to a predominantly B1 receptor subtype on these cells. The increase in B1 receptor expression was matched by a 4-fold increase in B1 receptor-mediated stimulation of phosphoinositide hydrolysis indicating that the up-regulated B1 receptors are functionally coupled.

B1 and B2 agonists also stimulated the release of IL-6 and IL-8 in IMR-90 cells. Both factors have been strongly implicated in the mechanisms of inflammatory hyperalgesia (Cunha et al., 1991; Ferreira et al., 1993b). Additionally, IL-6 may exert anti-inflammatory effects by suppressing the production of IL-1β and TNF-α (Schindler et al., 1990). IL-8 has been reported to up-regulate B1 receptor-mediated responses in vivo, and this effect occurred through the release of IL-1β (Davis and Perkins, 1994). However, neither of these factors appears to be directly involved in the agonist-promoted up-regulation of B1 receptors in IMR-90 cells. Inflammation also involves the release of TNF-α (Ferreira et al., 1993a). Kinins have been reported to release TNF-α from macrophages (Tiffany and Burch, 1989; Ferreira et al., 1993a), and TNF-α

up-regulates B1 receptors in cultured human fibroblasts (Phagoo et al., 1997). However, the significance of TNF-α in this response is unclear because this cytokine is ineffective in inducing hyperalgesia in animal models (Davis and Perkins, 1994). In IMR-90 cells, TNF-α was as efficacious as IL-1β in up-regulating B1 receptor expression both by itself and in combination with B1 agonists.

In summary, our results present a cellular mechanism in which kinins themselves regulate the expression and activity of their receptors, and this mechanism is compatible with the sequence of activities of these receptors during the inflammatory process. In this mechanism, BK and KD, the first set of kinins formed in response to tissue injury, act on B2 receptors to prime the site of injury for inflammation. This involves the production of secondary mediators including the cytokines IL-1β and IL-6 and the chemokine IL-8. Furthermore, these kinins enhance the responsiveness of surrounding tissues to kinins by promoting an increase in the expression of B1 receptors. Because the B2 receptor is subject to both desensitization and internalization, progression of the inflammatory and pain response depends on BK and KD degradation to form sufficient amounts of desArg^{9}BK or desArg^{10}KD, the second set of kinins formed. These kinins act on B1 receptors to optimize the B1 receptor up-regulation. Kinin action is further enhanced by the limited desensitization of the B1 receptor. Indeed, the lack of negative regulation of the B1 receptor may be one reason why two kinin receptors have evolved: one constitutively expressed and rapidly desensitizing receptor, the B2 receptor, which acts to sense the tissue injury and to which kinin action is limited if the injury is less severe, and another inducible and nondesensitizing receptor, the B1 receptor, which acts to sustain the kinin response if the injury is more severe.

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