Stable Expression of the Human Kinin B₁ Receptor in Chinese Hamster Ovary Cells

CHARACTERIZATION OF LIGAND BINDING AND EFFECTOR PATHWAYS

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To delineate ligand binding and functional characteristics of the human B₁ kinin receptor, a stable clone of Chinese hamster ovary cells expressing a single class of binding sites for [³H]des-Arg¹⁰-lysylbradykinin with a Kᵦ of 0.3 nM and a B₅₀ of 38 fmol/mg protein (~40,000 receptors/cell) was isolated. Studies with peptide analogs showed that a lysine residue at position 1 (based on the lysylbradykinin sequence) of ligands was essential for high affinity binding to the human B₁ receptor. In marked contrast to cloned Chinese hamster ovary cells expressing the human kinin B₂ receptor, which internalized approximately 80% of the ligand within 5 min upon exposure to 2 nM [³H]bradykinin, exposure of cells expressing the B₁ receptor to 1 nM [³H]des-Arg¹⁰-lysylbradykinin resulted in minimal ligand internalization. Stimulation of the B₁ receptor led to inositol phosphate generation and transient increases in intracellular calcium, confirming coupling to phospholipase C, while immunoprecipitation of photoaffinity-labeled G-proteins from membranes indicated specific coupling of the receptor to Gα₁₁ and Gα₁₂. The B₁, unlike the B₂ receptor, does not desensitize (as demonstrated by continuous phosphoinositide hydrolysis), enhancing the potential role of this receptor during inflammatory events.

Bradykinin (BK)¹ and lysylbradykinin (LBK) are potent vasoactive peptides that have been implicated as mediators of inflammation, pain, and hyperalgesia (1, 2). Two subtypes of kinin receptors, designated B₁ and B₂ based upon their pharmacological properties, were described in animal tissues (3). BK and LBK are equipotent agonists at the B₂ receptor, but BK is ineffective at the B₁ receptor. The carboxypeptidase metabolites of BK and LBK, des-Arg²-BK and des-Arg¹⁰-LBK, are the prototypical B₁ receptor agonists (4). The existence of B₁ and B₂ receptors was confirmed using selective antagonists (3, 5, 6) and by cloning studies (7–9).

Kinin B₂ receptors are constitutively expressed on many cell types and are responsible for the majority of the observed effects of kinins (10), but B₁ receptor expression requires induction. Thus, des-Arg²-BK alters smooth muscle tone only after incubating tissues for several hours in vitro (3–5). Exposure in vitro to proinflammatory cytokines also induces B₁ receptor-mediated responses via a mechanism that requires protein synthesis (11, 12). Similarly, de novo expression of B₁ receptors in animals in vivo occurs upon exposure to noxious stimuli, including bacterial lipopolysaccharide (13) and ultraviolet light (14), or to proinflammatory cytokines (15, 16).

The ability of injurious and proinflammatory stimuli to induce B₁ receptors in animal models implies that this receptor may play a role in the actions of kinins in chronic inflammatory conditions (4, 17). Support for this concept comes from studies showing that B₁ receptor antagonists have antinociceptive effects in rodent models of persistent hyperalgesia (2, 18, 19).

Despite the potential importance of B₁ kinin receptors in chronic inflammation, little is known regarding either the distribution and importance of this receptor system or the signaling systems to which the receptor is coupled in humans. Although two human embryonic lung fibroblast cell lines, IMR90 and WI38, express small numbers of B₁ receptors (20, 21), they are poor models to characterize B₁ receptor properties because they also express much larger numbers of B₂ receptors (9). The recent cloning of the human B₁ receptor (9), therefore, provided the first opportunity to characterize this receptor. Binding studies, using transient expression in COS-7 cells, demonstrated species variation in B₁ receptor ligand affinities. In contrast to the rabbit receptor, the affinity of des-Arg²-BK for the human B₁ receptor was more than 1000-fold lower than that of des-Arg¹⁰-LBK (9), indicating that des-Arg²-BK is an ineffective agonist for the human receptor. This species variation emphasized the need to further define the properties of the human receptor to delineate its potential role in inflammatory disorders. To date, however, functional studies of the human B₁ receptor have been limited to studies in Xenopus laevis oocytes, showing that stimulation induced aequorin-mediated luminescence (9).

The aim of the present studies was to delineate ligand binding and functional characteristics of the human B₁ kinin receptor. To achieve this, we isolated a clone of Chinese hamster ovary (CHO) cells expressing the receptor in a stable manner, permitting binding and functional properties to be studied in the same cell type. Given the reported difference in affinities of des-Arg²-BK and des-Arg¹⁰-LBK for the human B₁ receptor (9), we tested the hypothesis that the presence of a lysine residue at position 1 of ligands is essential for high affinity binding. We also sought to establish whether the human B₁ receptor, like the B₂ receptor, is coupled to phospholipase C, and, if so, which
G-proteins are responsible for this coupling. Finally, we determined if human B$_2$ receptor-mediated actions are regulated by rapid ligand-induced desensitization or internalization.

**EXPERIMENTAL PROCEDURES**

**Materials**

The following materials were purchased: minimum essential medium-$\alpha$, Opti-MEM, trypsine, lipofectamine, and fetal calf serum from Life Technologies, Inc.; penicillin/streptomycin from Biofluids Inc; cell culture flasks and dishes from Costar Corp; G418, human serum albumin, and Fura-2AM from Molecular Probes, Inc.; bacitracin, captopril, PIPES, human serum albumin, MES, and 1,4-bis(2-methylamino)butane from Sigma; polyconal rabbit peptide antisera specific for Go$\alpha$(RII, NEI-805), Go$\alpha$(Q, NEI-809), Go$\alpha$(Q2, NEI-804), and Go$\alpha$(Q7, NEI-801), 1,3-proplyl-3,4-dihydcinnolin-2(1H)-one (98 Ci/ml) from ICN Pharmaceuticals Inc.; Fura-2AM from Molecular Probes, Inc.; des-Arg$_{10}$-Lbr, des-Arg$_{10}$-Leu$^5$-Lbr, BK, des-Arg$_{10}$-Leu$^5$-BK, des-Arg$_{10}$-HOE 140, and LBR from Bachem; and AG 1-X8 resin, formate form, 100–200 mesh from Bio-Rad. All other chemicals were obtained from Sigma.

The cDNA for the human kinin B$_1$ receptor was generously provided by Dr. J. Fred Hess (Merck); Hoe 140 was a kind gift of Drs. Bernard Scholkens and Klaus Wirth (Hoechst AG).

**Peptide Synthesis**

The following peptides were synthesized: D-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Pro-Arg; lys-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Pro-Arg; Arg-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Pro-Arg; d-Lys-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Pro-Arg; lys-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Pro-Arg; and d-Arg$_{10}$-Leu$_{9}$-Lrk, BK, des-Arg$_{10}$-Lbr, des-Arg$_{10}$-HOE 140, and LBR from Bachem; and AG 1-X8 resin, formate form, 100–200 mesh from Bio-Rad. All other chemicals were obtained from Sigma.

Characterization of the Human Kinin B$_1$ Receptor

**Expression and Cloning of Human Kinin Receptors**

A cDNA for the human kinin B$_1$ receptor was isolated from human synovial cell RNA using reverse transcriptase and the polymerase chain reaction. Recognition sequences for BarnHI and XhoI were inserted to allow for unidirectional cloning of the cDNA. The polymerase chain reaction product was cloned into pBluescript vector (Stratagene), transformed into XL-1 Blue cells (Stratagene) and shown to have a sequence identical to that previously published (8) by dideoxy sequencing (25). The cDNA was then cloned into the pcDNAeol eukaryotic vector (Invitrogen) and transformed in XL-1 Blue cells, and large scale preparations of the vector containing the cDNA (pcDNAeol/B$_2$) were purified using “maxi prep” kits (Qiagen). The cDNA for the human B$_1$ kinin receptor was provided in the eukaryotic vector pcDNA3 (Invitrogen).

The vectors containing the cDNA for each of the human kinin receptor transfected were transformed into CHO-DUKX21 cell monolayers of approximately 50% confluency in 100-mm dishes using lipofectamine reagent (Life Technologies, Inc.). Cells were incubated for 20–24 h with 20 $\mu$g of pcDNA3 containing the human B$_1$ cDNA or with 5–10 $\mu$g of pcDNAeol/B$_2$. Clonal selection was performed in G418 (0.8–1 g/ml). Stable transformants were cloned by limiting dilution and maintained in the same concentration of G418. Clones were selected on the basis of specific binding of the appropriate radiolabeled ligand. The clones selected for further analysis were designated CHO-B$_1$/3 for the B$_1$ receptor and CHO-B$_2$/20 for the B$_2$ receptor. The two clones expressed comparably numbers of their respective receptors (40,000 versus 35,000 sites/cell, respectively).

**Radioligand Binding Studies**

Binding assays were performed at 4 $^\circ$C in PIPES buffer, pH 7.4, containing 7.7 g/liter PIPES, 6.4 g/liter NaCl, 0.37 g/liter KCl, and 1.0 g/liter glucose, supplemented with 0.1% fatty acid-free human serum albumin, 10$^{-4}$ M bacitracin, 10$^{-4}$ M captopril, and 0.1% sodium azide. Cells from confluent flasks were washed with this buffer, removed from flasks by gentle scraping, and centrifuged at 1000 $\times$ g for 10 min. Cells were resuspended in buffer at a density of approximately 10$^6$ cells/ml. Binding assays were performed in a total volume of 400 $\mu$l. For saturation curves, $[^{3}H]$Dides-Arg$_{10}$-LBR was used in a concentration range from 0.1 to 10 nM. Displacement binding was performed using 1 nM $[^{3}H]$Dides-Arg$_{10}$-LBR and competing ligands in the range of 0.01–10 nM. Non-specific binding was determined in each case in the presence of 1 M des-Arg$_{10}$-LBR. Binding reactions were terminated as described previously (26), by filtration and rapid washing of the cells three times with ice-cold 14% 2-propanol using a cell harvester (Brandel). Radioactivity associated with the filters was quantified by scintillation spectrometry. Data were analyzed using GraphPad computer software (GraphPad Software Inc.).

**Ligand Internalization Studies**

Confluent monolayers of intact CHO-B$_2$/20 or CHO-B$_2$/3 in 12-well culture dishes were incubated at 37 $^\circ$C in incubation buffer (40 $\mu$m PIPES, 109 $\mu$m NaCl, 5 $\mu$m KCl, 0.1% glucose, 0.05% human serum albumin, 2 $\mu$m CaCl$_2$, 1 $\mu$m MgCl$_2$, 2 $\mu$m bacitracin, 10 $\mu$m phosphoramidon, 100 $\mu$m captopril, pH 7.4) containing 2 nM $[^{3}H]$Bk or 1 nM $[^{3}H]$Dides-Arg$_{10}$-LBR, respectively. At the indicated times, cells were washed four times with 0.5 ml of ice-cold buffer and treated for 10 min at 4 $^\circ$C with 0.3 M of a solution of 0.5 M NaCl and 0.2 M acetic acid, pH 2.7 (27, 28). The supernatant, containing the dissociated, formerly surface-bound, radioligand was then transferred with another 0.3 M of buffer to scintillation vials containing 6-ml scintillation fluid, and the radioactivity was quantified. The remaining cells were lysed in 0.3 M of 3 M NaOH, and the radioactivity measured was regarded as internalized ligand. Non-specific binding and internalization was determined in the presence of 3 M unlabeled ligand.

**Inositol Phosphate Measurement**

Total inositol phosphate (IP), defined as IP$_1$, IP$_2$, and IP$_3$, was measured based on the method of Berridge (29). Cells were grown to 80% confluency in six-well dishes and labeled overnight in minimum essential medium-$\alpha$ with HEPES containing 2 $\mu$Ci/ml myo-$[^{3}H]$inositol. Cells were washed and equilibrated for 20 min in medium containing 20 mM LiCl and then stimulated with the appropriate peptide. Reactions were terminated by removal of medium and the addition of ice-cold 15% trichloroacetic acid. Cell supernatants were collected and neutralized by diethyl ether extraction. Total inositol phosphates were isolated using AG 1-X8 anion exchange columns (formate form). Total inositol phosphates were eluted with 1.2 M ammonium formate, 0.1 M formic acid. The effects of pertussis toxin (PTX) on total IP generation were determined after preincubation with PTX (30). To examine receptor desensitization, additional experiments were performed in which CHO-B$_2$/3 or CHO-B$_2$/20 were exposed to buffer or to a maximal concentration (1 $\mu$m) of appropriate ligand, and total IP was measured after 5 min. Replicate incubations were then washed four times and exposed to either buffer or appropriate ligand for a second 5-min period, beginning 5 min after removal of the initial stimulus. Total IP was again measured, and the proportion of each 5-min stimulation period was compared. Data are expressed as mean $\pm$ S.D. (n = 3).

**Calcium Measurements**

Intracellular calcium changes were measured by digital video microscopy as described previously (30, 31).

**Immunoprecipitation Studies**

Membrane Preparations—Membranes were prepared from confluent monolayers of CHO-B$_2$/3 cells by a modification of previously described techniques (32). Cells were incubated for 30 min at 37 $^\circ$C in 5 mM HEPES, 0.5 mM EDTA buffer, pH 8.0, washed, and centrifuged (27,000 $\times$ g for 20 min) at 4 $^\circ$C. The pellets were resuspended in buffer containing 10 mM Tris-HCl and 1 mM EDTA (pH 8), sonicated for 12 s, and centrifuged as before. The pellet was resuspended in a solution of 10% sucrose with 20 mM Tris-HCl and 1 mM EDTA (pH 7.5), layered on a 44.5% sucrose cushion, and centrifuged at 75,000 $\times$ g for 30 min. The harvested membrane layer was resuspended in 20 mM Tris-HCl, 0.25 M sucrose, 1 mM MgCl$_2$, and frozen at –80 $^\circ$C.

**Photoblotting of Membrane Proteins**—$[^{32}P]$GTP-azidoanalalone was synthesized and purified as described (33). Photoblotting was carried out by a modification of previously described methods (34). Membrane protein (200 $\mu$g) was incubated with 2 $\mu$M des-Arg$_{10}$-LBR or vehicle and 8 $\mu$Ci of $[^{32}P]$GTP-azidoanalalone (specific activity 3000 Ci/mmole) in incubation buffer (50 mM HEPES, 5 mM MgCl$_2$, 30 mM KCl, 0.1 mM EDTA, 1 mM benzamidine, pH 7.4) for 3 min at 30 $^\circ$C. The reaction was stopped by placing the samples on ice. Samples were then centrifuged at 12,000 $\times$ g for 15 min, and pellets were resuspended in 200 $\mu$l of
Photolysis buffer (50 mM HEPES, 5 mM MgCl2, 30 mM KCl, 0.1 mM EDTA, 1 mM benzamidine, 2 mM glutathione, pH 7.4). The samples were irradiated for 15 s at 4 °C with an ultraviolet lamp (254 nm, 150 watts) from a distance of 3 cm. After irradiation, samples were again centrifuged (12,000 × g for 15 min) and subjected to immunoprecipitation.

**Immunoprecipitation—**Pellets of photolabeled membranes were solubilized in SDS (2%) prior to the addition of precipitating buffer (10 mM Tris-Cl, 1% (w/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.5% (w/v) SDS, 150 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, pH 7.4) and centrifugation at 4 °C for 15 min at 12,000 × g. Aliquots of the supernatant were incubated with an excess of each G protein antiserum at 4 °C for 4 h. Washed protein A-Sepharose beads (10% (w/v)) were added, and samples were incubated overnight. Thereafter, Sepharose beads were precipitated by centrifugation (12,000 × g for 15 min). Supernatants were removed, and aliquots were used to compare levels of total [α-32P]GTP-labeled proteins in each sample using two methods; 1) an aliquot was counted directly via scintillation counting, and 2) an aliquot of each sample was subjected to SDS-polyacrylamide gel electrophoresis and analyzed using a PhosphorImager. By both methods, there was <10% variation in total [α-32P]GTP incorporation among samples. Meanwhile, the precipitated protein A-Sepharose beads were washed with buffer A (50 mM Tris-HCl, 1% (w/v) Nonidet P-40, 0.5% (w/v) SDS, 600 mM NaCl, pH 7.4), followed by a wash with buffer B (100 mM Tris-HCl, 300 mM NaCl, 10 mM EDTA, pH 7.4). Samples were prepared for SDS-polyacrylamide gel electrophoresis according to standard protocols (35, 36). SDS-polyacrylamide gel electrophoresis was performed using 12% acrylamide gels (37). Gels were stained with Coomassie Brilliant Blue, dried, and imaged on a Molecular Dynamics PhosphorImager plate. Results were analyzed as the densitometric ratio of bands in representative lanes.

**RESULTS**

Initial association kinetic experiments showed that specific binding of [3H]des-Arg10-LBK to CHO-B1/3 cells reached equilibrium by 60 min at 4 °C (Fig. 1). All subsequent binding studies were, therefore, performed using incubation periods of at least 60 min. Analysis of data from saturation binding indicated the presence of a single population of binding sites with a dissociation constant (Kd) of 0.5 nM and a Bmax of 38 fmol/mg protein, equivalent to approximately 40,000 sites/cell (Fig. 2). Mock-transfected CHO cells failed to show specific binding of [3H]des-Arg10-LBK (not shown).

An evaluation of the ability of analogs of bradykinin to displace [3H]des-Arg10-LBK from the cloned human kinin B1 receptor demonstrated that the presence of a lysine residue at position 1 of the LBK sequence was essential for high affinity binding (Fig. 3). This is exemplified not only by confirmation of the previously published finding (9) of the much higher affinity (Kd = 0.15 nM) of des-Arg10-LBK for the receptor compared with des-Arg9-BK (Kd = 180 nM) but also by the similar differences in potency between the antagonists des-Arg10-Leu6-LBK and des-Arg9-Leu6-BK. In addition, LBK showed a moderate affinity (Kd = 88 nM) for the human B1 receptor, while BK was totally ineffective in displacing [3H]des-Arg10-LBK. In contrast to the striking increases in binding affinity observed upon extension of the amino terminus of BK, or of des-Arg9-BK, with a lysine residue, extension with a trans-l-lysine residue had minimal effects (Table I). Extension of the bradykinin sequence with an amino-terminal arginine did improve binding affinity, but not to the same degree as lysine. Although the lysine at position 1 is important, further extension of the LBK sequence with an amino-terminal arginine did improve binding affinity, but not to the same degree as lysine. Although the lysine at position 1 is important, further extension of the LBK sequence with a neutral l-lysine residue had little additional effect on binding affinity. Finally, as expected, truncation of the carboxyl terminus of des-Arg10-LBK resulted in a striking loss of binding affinity.

Previous studies using conformationally constrained analogs of BK containing an alkyl ether of D-4-hydroxyproline in either the cis or trans geometric state at position 7 of the BK sequence demonstrated that analogs containing trans-propyl ethers had a much higher affinity for the B3 receptor than corresponding cis analogs (38). We examined, therefore, the ability of similar...
analogs of des-Arg⁹-BK to displace [³H]des-Arg¹⁰-LBK from the cloned B₁ receptor (Table II). In contrast to the B₂ receptor, the human B₁ receptor showed no marked specificity for cis- or trans-propyl ether derivatives.

Marked differences between cloned human B₁ and B₂ receptors were also observed with regard to receptor-mediated ligand internalization. Exposure of cloned CHO-B₁/3 cells to 2 nM [³H]bradykinin resulted in internalization of approximately 80% of the ligand within 5 min, but exposure of CHO-B₁/3 cells to 1 nM [³H]des-Arg¹⁰-lyseylbradykinin resulted in minimal ligand internalization (Fig. 4). These data were confirmed in additional experiments (data not shown). Exposure of nontransfected CHO cells to either radiolabeled ligand resulted in no internalization.

Stimulation of CHO-B₁/3 cells with des-Arg¹⁰-LBK resulted in a dose-dependent generation of total IP (Fig. 5), confirming coupling of the human B₁ receptor to phospholipase C activation. Preincubation of cells with 100 ng/ml of PTX had no effects on the dose-dependent generation of total IP in response to des-Arg¹⁰-LBK. Consistent with the coupling of the human B₁ receptor to phospholipase C, stimulation of CHO-B₁/3 cells with 10 nM des-Arg¹⁰-LBK induced a robust transient increase in intracellular Ca²⁺ levels (Table III). The effect of des-Arg¹⁰-LBK was receptor-mediated, since it was inhibited by a brief preincubation of the cells with the B₁ receptor antagonist, des-Arg¹⁰-Leu⁵-LBK. BK did not significantly elevate Ca²⁺ above basal levels, even at a concentration of 1 μM.

In light of the lack of receptor-induced ligand internalization observed for the B₁ receptor, we used IP generation as a tool to examine functional desensitization. Exposure of CHO-B₁/3 cells to 1 nM des-Arg¹⁰-LBK led to a continuous, linear accumulation of total IP over a 60-min period, indicating a lack of desensitization (Fig. 6). In contrast, BK-induced generation of total IP in CHO-B₂/20 cells reached a plateau within minutes (not shown). These data were further supported by studies of repeat stimulation. Exposure of CHO-B₂/20 cells to 1 μM BK resulted in an increase in total IP (1371 ± 136 cpm) over a 5-min incubation, compared with exposure to buffer alone (389 ± 20 cpm). After washing, a second 5-min exposure to buffer led to neither a significant increase nor decrease in total IP levels (1261 ± 235 cpm). Repeated stimulation with BK caused only a modest further increase in total IP (to 1607 ± 131 cpm) during the second incubation, consistent with significant receptor desensitization. By contrast, a different pattern of response was seen with CHO-B₁/3 cells. Exposure to 1 μM des-Arg¹⁰-LBK resulted in a generation of 1313 ± 101 cpm of IP in 5 min, compared with 577 ± 56 cpm in cells exposed to buffer alone. After repeated washing, however, there was no significant difference in IP levels after a second 5-min exposure period to either buffer (2171 ± 110 cpm) or 1 μM des-Arg¹⁰-LBK (2206 ± 321 cpm), but both were markedly elevated compared with the first 5-min period. Binding studies using radiolabeled ligand under similar conditions revealed that these data can be explained by a low off-rate of des-Arg¹⁰-LBK from the human B₁ receptor, since over 95% of the ligand remained on the cell surface after 5 min, despite repeated washing. Thus, continued production of IP occurring during the washing and second incubation periods is due to continued receptor occupancy and confirms the lack of B₁ receptor desensitization.

To determine which G-proteins may be involved in coupling the B₁ receptor to functional responses, we performed immunoprecipitation studies using membranes incubated in the presence or absence of des-Arg¹⁰-LBK. A representative blot (Fig. 7) shows enhanced GTP binding to Goα₁/11 and Goα₁₂, but not to Gαi₁/2, indicating receptor-mediated activation of the former two Go subunits. In seven such experiments, des-Arg¹⁰-LBK stimulation led to a 2.6 ± 0.7-fold increase in the intensity of the immunoprecipitated Goα₁/11 band compared with that in unstimulated membranes and a 1.5 ± 0.2-fold increase in Goα₁₂, but no changes in Gαs or Goαq.

### DISCUSSION

The cloning of the human B₁ kinin receptor provided the first opportunity to examine the properties of this receptor. Initial studies, however, relied on the use of transient transfection of the receptor into COS-7 cells for binding studies, while limited functional data were obtained in X. laevis oocytes (9). The development of a stable clone of CHO cells expressing the human B₁ receptor has permitted us to analyze, for the first time, both ligand binding characteristics and functional responses of the receptor in the same cell.

Our studies clearly demonstrate that agonist binding to the human B₁ receptor leads to the generation of IP and transient increases in intracellular calcium, demonstrating that the receptor is coupled to phospholipase C activation. This is consistent with studies also demonstrating B₁ receptor-mediated activation of phospholipase C in cells from other species (39–42) and confirms that both B₁ and B₂ receptors couple to this

### TABLE I

| Ligand structure | $K_i$ (nM) |
|------------------|------------|
| Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe (des-Arg¹⁰-LBK) | 0.15 ± 0.03 |
| Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe (des-Arg⁹-BK) | 1.0 ± 0.4 |
| Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro | 710 ± 120 |
| Lys-Pro-Pro-Gly-Phe-Ser-Pro | 1460 ± 190 |
| Lys-Pro-Pro-Gly-Phe-Ser-Pro Pro-Pro-Gly-Phe-Ser-Pro-Phe (trans) | 92 ± 4 |
| Lys-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Pro-Phe-Pro-Phe-Pro-Phe-Pro (cis) | 38 ± 0.06 |
| Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (BK) | >10,000 |
| Lys-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg (LBK) | 85 ± 7 |
| Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg | 925 ± 200 |
| Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg | >10,000 |
| Lys-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg | 160 ± 26 |
| Antagonists | |
| Lys-Pro-Pro-Gly-Phe-Ser-Pro-Leu | 0.66 ± 0.12 |
| Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu | 78 ± 18 |
| Des-Arg⁹-HOE140 | 14 ± 1.2 |

### TABLE II

| Ability of ligands with cis- or trans-propyl ether residues at the 7-position of the bradykinin sequence to displace 1 nM [³H]des-Arg¹⁰-LBK from the cloned human B₁, kinin receptor | $K_i$ (nM) |
|------------------|------------|
| t-Arg-Pro-Hyp-Gly-Phe-Ser-d-Hype(trans-3-phenylpropyl)-Oic | 3 ± 0.6 |
| t-Arg-Pro-Hyp-Gly-Phe-Ser-d-Hype(cis-3-phenylpropyl)-Oic | 12 ± 2.5 |
| Arg-Pro-Hyp-Gly-Phe-Ser-d-Hype(trans-3-phenylpropyl)-Oic | 17 ± 1.5 |
| Arg-Pro-Hyp-Gly-Phe-Ser-d-Hype(cis-3-phenylpropyl)-Oic | 40 ± 8.0 |
signaling pathway (43–45).

Immunoprecipitation studies indicate that the human B₁ receptor is coupled to Gaq/11 and Gaα₁₂ but not to Gaα₉. The fold increase in GTP uptake upon receptor stimulation was most marked for Gaq/11. This pattern of G-protein coupling is very similar to that observed upon stimulation of human B₂ receptors.² Although the observation that BK-induced increases in IP can be modified by PTX exposure in some cell types demonstrates that proteins of the Gi family can contribute to B₂ receptor-mediated activation of phospholipase C, the failure of PTX to modify IP generation in response to des-Arg₁⁰-LBK stimulation in our studies indicates that Gi₁/2 does not contribute to B₁ receptor-mediated activation of phospholipase C. Rather, members of the Gq/11 subfamily of G-proteins have been shown to link cell surface receptors to the known β-isofoms of phospholipase C (48–51), and our data show that these G-proteins serve a similar role for the human B₁ receptor.

Our data also provide insight into the regulation of B₁ receptor-mediated responses. Exposure of cloned human B₂ receptors to ligand results in a rapid receptor-mediated activation of phospholipase C, the failure of PTX to modify IP generation in response to des-Arg₁⁰-LBK stimulation in our studies indicates that Gi₁/2 does not contribute to B₂ receptor-mediated activation of phospholipase C. Rather, members of the Gq/11 subfamily of G-proteins have been shown to link cell surface receptors to the known β-isofoms of phospholipase C (48–51), and our data show that these G-proteins serve a similar role for the human B₁ receptor.

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TABLE III

| Stimulus | Net maximal Ca²⁺ (mean ± S.E.; n = 5) | Inhibition |
|----------|----------------------------------------|------------|
| 10 nM des-Arg₁₀-LBK alone | 1900 ± 458 | % |
| With des-Arg₁₀-Leu₉-LBK (0.1 nM) | 878 ± 143 | 54 |
| With des-Arg₁₀-Leu₉-LBK (10 nM) | 410 ± 111 | 78 |
| 1 μM BK alone | 86 ± 9 | |
affinity. This is not simply a requirement for a charged amino acid, since a bulky arginine residue is much less effective than lysine. Moreover, there is a stereoechemical requirement for the lysine to be in the L-configuration, since D-lysine does not confer the same high affinity. This requirement for a lysine residue at position 1 probably explains why des-Arg9-BK, the “classical” B1 receptor agonist, has a 10-fold lower affinity for the human receptor than has been reported for the rabbit B1 receptor (41). The 1000-fold difference in affinity for the human B1 receptor between des-Arg10-LBK and des-Arg9-BK is in agreement with previously published data (9) and indicates that des-Arg10-LBK must be considered the natural ligand for the human B1 receptor in vivo. Although we find that LBK is a much better ligand than BK, the affinity of LBK for the human B1 receptor observed by us was 10-fold lower than that reported by Menke and colleagues (9). The reasons for this are unclear, but one potential explanation could be that these authors performed binding studies at 23 °C. If carboxypeptidase activity was not fully inhibited at this temperature, conversion of a small percentage of LBK to des-Arg10-LBK could lead to a higher apparent affinity.

The knowledge that des-Arg10-LBK is the likely natural ligand for the human B1 receptor has significant implications. First, those studies that have used des-Arg9-BK to try to delineate the role of B1 receptors in human tissue responses in vivo must now be considered flawed and should be repeated using the appropriate ligand. As mentioned above, even if future studies demonstrate expression of B1 receptors in human tissues during inflammatory diseases, this will not, by itself, constitute proof of biological importance but must be considered in light of the fact that receptor-mediated activity is also dependent upon ligand availability. To generate des-Arg10-LBK, an extremely specific environment must exist. First, tissue kallikrein must be expressed in the tissue in question in an active form, since this is the only enzyme that is known to produce LBK in humans (1). Then, LBK must be converted to des-Arg10-LBK by the actions of an appropriate carboxypeptidase that must also be present. Moreover, this latter step must occur before the LBK is acted upon by other peptidases, such as aminopeptidase M (which would remove the latter step must occur before the LBK is acted upon by other peptidases, such as aminopeptidase M (which would remove the carboxyl terminus of the ligand to a form that would be inactive on the human B1 receptor. These extremely specific requirements provide exquisite control mechanisms for activation of the receptor.

In summary, we have used a stable clone of CHO cells expressing the human B1 receptor to characterize the binding properties and functional responses of this receptor. Des-Arg10-LBK appears to be the natural ligand for the human receptor. Activation of the receptor by this ligand results in inositol phosphate generation and increases in intracellular calcium levels via mechanisms that are coupled to phospholipase C via Gq/11. In contrast to the B2 receptor, activation of the human B1 receptor is not associated with ligand-induced receptor internalization, nor does the receptor show desensitization. The availability of a stable clone expressing the human B1 receptor should greatly facilitate future studies of the regulation of this receptor, its interaction with G-proteins, and its potential role in inflammation.

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