Expression Cloning of a Human B₁ Bradykinin Receptor*

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A cDNA clone encoding a human B₁ bradykinin receptor was isolated from a human embryonic lung fibroblast cDNA library by expression cloning. The photoprotein aequorin was utilized as an indicator of the ability of the B₁ receptor agonist [des-Arg⁷]kallidin to mediate Ca²⁺ mobilization in Xenopus laevis oocytes injected with RNA. A clone was isolated with a 1307-nucleotide insert which contains an open reading frame encoding a 353-amino acid protein with the characteristics of a G-protein-coupled receptor. The amino acid sequence of the B₁ bradykinin receptor is 36% identical to the amino acid sequence of the B₂ bradykinin receptor. The cloned B₁ bradykinin receptor expressed in mammalian cells exhibits high affinity binding for H-labeled [des-Arg⁷]kallidin and low affinity for bradykinin. The B₁ receptor antagonist [des-Arg⁷,Leu⁸]kallidin effectively displaces H-labeled [des-Arg⁷]kallidin from the cloned receptor, whereas the B₂ receptor antagonist Hoe-140 (0-Arg⁴-Hyp⁵,Thi⁶-P-Tic⁷,Oic⁸)bradykinin, where Thi is L-[3-(2-thienyl)alanin], Tic is O-[(2,3,4-tetrahydroisoquinolin-3-yl)carbonyl], and Oic is 1-(3aS,7aS)-octahydroin-2-yl-carbonyl) does not. Therefore, the expressed receptor has the pharmacological characteristics of the B₁ receptor subtype. The availability of both the cloned human B₁ and B₂ bradykinin receptors should allow the elucidation of the relative contributions of these two receptor subtypes in acute and chronic inflammatory processes.

Two mammalian bradykinin receptor subtypes, B₁ and B₂, have been defined based on their pharmacological properties (1, 2). The B₂ receptor is synthesized de novo following tissue injury and has recently been shown to mediate hyperalgesia in animal models of chronic inflammation (1). The B₁ bradykinin receptor is normally present in smooth muscle and certain neurons, where activation of B₁ receptors causes pronounced hypotension, bronchoconstriction, pain, and inflammation (1, 2). The agonists for the B₁ and B₂ bradykinin receptors are generated by the proteolytic action of kallikreins which release the nonapeptide bradykinin (BK) and the decapeptide Lys-BK (kallidin) from large protein precursors, low and high molecular weight kininogens. BK and kallidin are equipotent agonists at the B₂ receptor. In contrast, BK is inactive at the B₁ bradykinin receptor subtype. Degradation of the B₂ receptor agonists by a carboxypeptidase produces the B₁ receptor agonists, [des-Arg⁷]BK and [des-Arg⁹]kallidin. The phenomenon of proteolytic transformation of a peptide from B₂ to B₁ selectivity has been observed not only for the endogenous kinin agonists but also for several synthetic peptide antagonists (3, 4).

The B₁ receptor was originally discovered through a contractile response to [des-Arg⁷]BK that was observed in rabbit aortic strips only after a prolonged in vitro incubation (5-7). The de novo synthesis of B₁ receptors has been reported in vivo following treatment with bacterial lipopolysaccharide (8) and in animal models of antigen arthritis (9). In vitro studies have implicated a number of cytokines, most notably interleukin-1 (IL-1) and IL-2, as mediators that induce the expression of B₁ receptors (6, 10-12). Furthermore, the activation of a B₁ bradykinin receptor on mouse macrophages causes the release of cytokines (13, 14). Significantly, the B₁ bradykinin receptor antagonist [des-Arg⁷,Leu⁸]BK was recently found to alleviate hyperalgesia in animal models of persistent inflammation (1, 15, 16). Thus, a body of evidence implicates the B₁ bradykinin receptor in the pathophysiology of chronic inflammation. Relatively little is known about the role of the B₂ receptor in healthy tissues, although both B₁ and B₂ receptors may play a physiological role in renal function (17, 18).

The cloning of the B₁ bradykinin receptor has revealed that this receptor is a member of the superfamily of G-protein-coupled receptors (19-22), definitive evidence that the B₁ receptor couples to G-proteins has not been forthcoming. The rat B₂ bradykinin receptor was cloned (19) using a Xenopus oocyte expression system that exploited the ability of the B₂ receptor to act through G-proteins to activate phospholipase C and mobilize Ca²⁺ (23, 24). Recently, the B₂ bradykinin receptor has also been shown to activate phospholipase C in primary cultures of rabbit aorta smooth muscle cells, rabbit mesenteric artery smooth muscle cells, and rat mesangial cells (25-27). Furthermore, both B₁ and B₂ bradykinin receptor activities were detected when mRNA from the human fibroblast cell line WI-38 was injected into X. laevis oocytes (28, 29). Although the similarity of Egands for the two bradykinin receptor subtypes suggests a similarity between the B₁ and B₂ receptor genes, the results of genomic Southern analyses indicated that these two receptors are not highly homologous (19, 30). Therefore, to clone the human B₁ receptor, we pursued an expression cloning strategy in Xenopus oocytes utilizing the photoprotein aequorin as an indicator of Ca²⁺ mobilization (31, 32). We isolated a cDNA clone that encodes a G-protein-coupled receptor with an amino acid sequence that is 36% identical to that of the B₁ bradykinin receptor. The pharmacological properties of this cloned receptor expressed in mammalian cells demonstrate that it is a B₁ bradykinin receptor.

MATERIALS AND METHODS

Oocyte Injections—Injection of mRNA or cRNA into Xenopus oocytes was performed by a modification of established protocols (33, 34). The excised ovarian lobes were teased apart with jeweler's forceps and then placed into OR-2 medium (82.5 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM
HEPES, pH 7.4) containing 2 mg/ml collagenase B (Boehringer Mannheim) for 2 h at room temperature. Oocytes were selected and cultured overnight in supplemented OR-2 (OR-2 containing 1.8 mM CaCl₂, 0.5 mg/ml gentamycin, and 0.5 mg/ml theophylline). Initially, oocytes were injected with 46 nl of RNA at a concentration of 1 or 2 mg/ml in H₂O. Once the pool became less than 30 clones, the cRNA concentration was decreased to 40 ng/ml. RNA was injected using a Nanoinject automatic oocyte injector (Drummond Scientific), and injection needles were pulled from 3.5-inch Drummond capillaries using a Flaming/Brown Micropipette puller (Sutter Instruments). Two to three days after the RNA injection, oocytes were injected with 92 ng of aequorin (Pharos Harber Photoprotein) resuspended in 46 nl of 1 mg/ml EDTA, as described previously (31, 32). The following day, individual oocytes placed in wells of a microtiter dish containing 225 µl OR-2 were challenged with peptide agonists, and the aequorin photo response was measured using a ML3000 microtiter plate luminometer (Dynatech).

RNA Fractionation—IMR-90 cells (ATCC CCL 186) were grown in minimal essential medium supplemented with 10% fetal calf serum, glutamine, nonessential amino acids, sodium pyruvate, penicillin, and streptomycin (Life Technologies, Inc.). Two and a half hours prior to mRNA extraction, IMR-90 cells were exposed to 200 pg/ml IL-1β (R & D Systems). mRNA was purified from these cells using the poly(A) tract mRNA isolation system (Promega) and resuspended in H₂O at a concentration of 2 mg/ml.

IMR-90 mRNA was size-fractionated on a continuous 6–20% sucrose gradient in 15 mM PIPES, pH 6.5, 5 mM EDTA, and 0.25% N-lauroyl-sarcosine. 480 µg of mRNA from the IL-1β-induced IMR-90 cells was loaded on the gradient and size-fractionated by centrifugation at 18°C for 19 h at 77,000 x g. Fractions (450 µl) from each gradient were collected from the bottom of the tube. Fractions were ethanol-precipitated twice and resuspended to a final concentration of 1 µg/ml. RNA size determination was based on the migration pattern of 80 µg of 9.49-0.24-kb RNA standards (Promega) and resuspended in H₂O at a concentration of 2 mg/ml.

Mammalian Cell Expression and Pharmacological Characterization—COS-7 cells were transfected by electroporation using a Bio-Rad gene pulser. Three days post-transfection, cells were processed for either whole cell or membrane binding assays as described previously (27, 36). Displacement studies were done with 1 nM [de-Arg¹,Leu³]-kallidin (20 nM). This pool was then subdivided into 25 pools, with an average insert size of 1.9 kb. The apparent size of the mRNA enabling the BK response is consistent with the previously determined size of the B₂ bradykinin receptor transcript (19, 30).

The RNA fraction from the sucrose gradient which gave the greatest response to [de-Arg¹]kallidin was utilized to generate a cDNA library. The library contained greater than 90% inserts, with an average insert size of 1.9 kb. The library was plated in pools of approximately 5000 clones that were used to synthesize cRNA. Of the 25 pools of cRNA that were injected into Xenopus oocytes, 11 exhibited aequorin-mediated luminescence in response to either the B₁ agonist [de-Arg¹]kallidin or the B₂ agonist BK. The mRNA was size-fractionated over a sucrose gradient, fractions were injected into oocytes, and the oocytes were assayed for their ability to respond to either BK or [des-Arg¹]kallidin. The B₁ and B₂ receptor transcripts were clearly separated by the size fractionation (Fig. 1). The mRNA mediating the response to [des-Arg¹]kallidin exhibited an apparent size of 1.6–1.8 kb, whereas the mRNA mediating the response to BK had an apparent size of 4.4–4.6 kb. The apparent size of the mRNA enabling the BK response is consistent with the previously determined size of the B₂ bradykinin receptor transcript (19, 30).

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FIG. 2. Functional response of clone 33E9 in Xenopus oocytes. Luminometer tracing of the response to 20 nM [des-Arg10]kallidin of an individual Xenopus oocyte injected with IMR 90 mRNA (closed circles) or cRNA prepared from clone 33E9 (open squares). The B2 receptor agonist was added at time 0 and the aequorin response measured as described under "Materials and Methods."

FIG. 3. Comparison of the amino acid sequence of the human B2 bradykinin receptor and the human B1 bradykinin receptor. The alignment was performed using the GAP program in the GCG software package. Seven putative transmembrane domains are underlined. The symbol * indicates a potential N-linked glycosylation site, and indicates potential protein kinase C phosphorylation sites, and V indicates potential CAMP-dependent protein kinase sites. The highly conserved cysteine residues that are proposed to be involved in a disulfide bond are connected by a dotted line.

Clone 33E9 contains an insert of 1307 nucleotides with an open reading frame of 1059 nucleotides. We isolated several different clones that encompassed the same DNA sequence as clone 33E9 but began and ended at different locations, indicating that they were independently derived. The sequence surrounding the proposed initiator methionine codon at nucleotide 209 conforms to the Kozak consensus sequence in the +4 position but not at the -3 position (37). The open reading frame encodes a protein that is 36% identical to the B2 bradykinin receptor (Fig. 3). The sequence identity at the nucleotide level, 54%, probably explains the failure to clone this receptor by low stringency hybridization with DNA encoding the B1 receptor. A homology search of the Swiss Protein data base indicates that the B2 receptor is 30% identical to the angiotensin type 2 receptor and 29% identical to the angiotensin type 1 receptor (38-40) and less homologous to other G-protein-coupled receptors.

The ability of several bradykinin receptor agonists to displace [3H]-labeled [des-Arg10]kallidin from the cloned receptor was assessed (Fig. 4B, Table I). The IC50 for displacement of 1 nM [3H]-labeled [des-Arg10]kallidin from the cloned receptor by (41, 42). Two conserved Cys residues that are proposed to form a disulfide bond between the second and third extracellular domains in nearly all G-protein-coupled receptors are also present in this sequence (Fig. 3). There are two consensus sites for N-linked glycosylation in the NH2-terminal domain and one in the third extracellular domain. Potential phosphorylation sites for protein kinase C and CAMP-dependent protein kinase are present in intracellular domains 2 and 3 and the carboxyl-terminal domain. Similar potential phosphorylation sites in other G-protein-coupled receptors have been implicated in short term desensitization of the receptor following agonist stimulation (41, 42).

Clone 33E9 was transfected into COS-7 cells, and the pharmacological properties of the expressed receptor were determined. Scatchard analysis of saturation binding data with [3H]-labeled [des-Arg10]kallidin indicated a Kd of 0.4 nM and a Bmax of approximately 100 fmol/mg of protein (Fig. 4A). Mock-transfected COS-7 cells did not demonstrate any specific binding for [3H]-labeled [des-Arg10]kallidin (data not shown).

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The IC$_{50}$ was determined by the displacement of 1 nM $^{3}H$-labeled [des-Arg$^9$]kallidin in the membrane binding assay described under "Materials and Methods." The data presented are the average of three experiments.

| Peptide          | IC$_{50}$ human B$_2$ nM | IC$_{50}$ IMR 90 nM |
|------------------|--------------------------|---------------------|
| [des-Arg$^9$]Kallidin | 0.2                      | 0.5                 |
| [des-Arg$^9$][Leu]$^6$Kallidin | 1.3                      | 1.3                 |
| Kallidin         | 42                       | 62                  |
| Bradykinin       | 2000                     | 7800                |
| [des-Arg$^9$]BK  | 720                      | 590                 |
| [des-Arg$^9$, Leu]$^6$BK | 440                      | 130                 |
| Hoo-140          | >10,000                   | >10,000             |
| [des-Arg$^9$]Hoo-140 | 60                       | 20                  |
| [Met,Lys]$^2$BK | 70                       | 96                  |

BK is $>2 \mu M$. The low affinity of this receptor for BK and high affinity for [des-Arg$^9$]kallidin argues strongly that this cloned bradykinin receptor is of the B$_2$ receptor subtype. Competition binding studies yielded a rank order of affinity for kinin agonists of: [des-Arg$^9$]kallidin > kallidin > [des-Arg$^9$]BK $>>$ BK at the cloned human receptor. This is very similar to the rank order of potency reported for the rabbit B$_2$ bradykinin receptor. Therefore, the interaction of the cloned receptor with [des-Arg$^9$]kallidin in the membrane binding assay described under "Materials and Methods" was also analyzed. The cloned human B$_2$ receptor has relatively high affinity binding for the B$_2$-specific antagonists [des-Arg$^9$, Leu]$^6$Kallidin and [des-Arg$^9$, Leu]$^6$BK (Table I). By contrast, the cloned receptor has a very low affinity for the potential B$_2$-specific antagonist Hoe-140. Significantly, the removal of the COOH-terminal Arg from Hoe-140 results in a dramatic increase in affinity (Table I), as would be expected for a B$_3$ receptor (3). Therefore, the interaction of the cloned receptor with bradykinin antagonists is consistent with the B$_3$ receptor subtype classification.

In summary, we have utilized an expression cloning strategy to isolate a cloned human B$_2$ bradykinin receptor.

and hyperalgesia, whereas the B$_2$ receptor appears to mediate acute inflammatory and algesic responses. The availability of cloned human B$_1$, B$_2$, and B$_3$ receptors should lead to a greater understanding of the role of these receptors in both normal and pathophysiological conditions.

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