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Cloned Murine Bradykinin Receptor Exhibits a Mixed B_1 and B_2 Pharmacological Selectivity

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SUMMARY

We have isolated DNA clones encoding functional bradykinin receptors from human, rat, and mouse sources. Genomic bradykinin receptor clones have been isolated from mouse and human cosmid libraries and cDNA clones have been isolated from the human lung fibroblast cell line WI38, from the neuroblastoma/glioma hybrid NG108–15, and from rat dorsal root ganglion cells. The receptor protein is encoded by an intronless region of the gene in both mouse and human. There is evidence of a splice acceptor site 8 bases upstream from the initiation codon in all three species. The function of the expressed receptor proteins from mouse, rat, and human was tested by electrophysiological assays after injection of cRNA into *Xenopus laevis* oocytes and also by binding assays with membranes from COS-7 cells transfected with cloned receptor-encoding DNA. The receptors from human and rat showed the pharmacological properties of B_2 receptors in both expression systems when tested with a variety of bradykinin analogues, but receptors from mouse divided into two populations, one population with pharmacological properties of B_1-like receptors and another, larger, population with properties of B_2 receptors.

The peptide hormone bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) is produced in response to tissue damage by the action of kallikrein proteases on high molecular weight precursors, the kininogens. It is a potent mediator of a variety of responses in mammalian tissues, including smooth muscle contraction, vasodilatation, increased vascular permeability, neurotransmitter release, and mediation of pain (1, 2). These effects are a consequence of bradykinin binding to specific receptors, which are found in a wide range of tissues (3–6).

Three pharmacological bradykinin receptor subtypes have been reported in the literature. B_1 receptors were originally described by Regoli *et al.* (7). The B_1 receptor has higher affinity for [des-Arg^9]bradykinin than for bradykinin and has a limited tissue distribution in normal tissues, being found primarily in vascular tissues (1). However, there are reports that B_1 receptor expression can be induced in various tissues after insult or injury, with a time course of several hours (8, 9). We previously reported that the human lung fibroblast cell line WI38 mRNA encodes B_1 and B_2 receptor subtypes (10), based on an electrophysiological assay of receptors expressed in *Xenopus* oocytes.

B_2 receptors have high affinity for bradykinin but, in contrast to B_1 receptors, they have low affinity for [des-Arg^9]bradykinin and are blocked by low concentrations of selective B_2 antagonists. B_2 receptors are found on a wide variety of tissue types including peripheral neuronal cells (5). A B_3 receptor subtype has recently been proposed in trachea and lung, principally due to a lack of activity of B_2 antagonists (11).

Because many of the studies on pharmacological subtypes have been done in tissues from different species, it is possible that significant differences in the functional characteristics of receptors from diverse species could exist. One approach to investigate this possibility would be to study the functional characteristics of receptors from several species. However, to enable direct comparisons to be made, the receptors should be expressed in the same cell type.

A bradykinin receptor has been cloned from rat uterus, using a *Xenopus* oocyte expression assay (12), and shown to have the pharmacological profile of a B_2 receptor in oocytes; 10 μM [des-Arg^9]bradykinin is inactive at the expressed receptor, bradykinin is an agonist and can be antagonized by the B_2-specific antagonist [Thi^{5,8},D-Phe^7]bradykinin with an IC_{50} value of about 400 nM, and this compound produces weak agonism at 10 μM to 1 mM. Subsequently, a human bradykinin receptor was cloned from the fibroblast cell line CCD-16Lu by PCR and cDNA library screening (13) and the human gene was cloned (14). The deduced amino acid sequences of the two receptors show extensive similarity, which is consistent with their belonging to the superfamily of receptors with seven membrane-spanning regions. The human receptor was characterized by binding studies on COS cells transiently transfected with the receptor (13). These studies indicate that the human receptor has the characteristics of a B_2 receptor, with minimal affinity for the B_1-selective agonist [des-Arg^9]bradykinin (18% displacement at 10 μM) or the B_1-selective antagonist [Leu^8,des-

ABBREVIATIONS: PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; kb, kilobase(s); G protein, guanine nucleotide-binding protein; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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Arg[9]]bradykinin (1) (11% displacement at 10 μM) but high affinity (IC_{50} value of 65 pM) for the selective B_2 antagonist HOE140 (D-Arg[Hyp[3],Thi[5],dTic[7],Oic[8]]bradykinin) (15).

In this study, the pharmacology of bradykinin B_2 receptors from different species was compared, the genes for the human and mouse bradykinin receptors were isolated, and cDNAs encoding human and rat receptors were cloned. Receptors encoded by these DNAs were characterized after expression in two heterologous expression systems. Comparison of the genomic DNA and cDNA sequences from mouse and human indicated that there are no introns in the protein-coding regions of the gene and there are no differences between the cDNA sequence and the genomic sequence in the protein-coding region. However, there is a potential splice acceptor site (16) 8 bases 5′ to the putative intitation codon.

Full length coding domain clones from human, rat, and mouse species were subcloned into an expression vector and cRNA from these clones was injected into *Xenopus* oocytes. Oocytes expressing the mouse receptor responded to a variety of bradykinin analogues, including both B_1 and B_2 agonists (and antagonists), whereas oocytes expressing human and rat receptors responded reliably only to bradykinin. Mammalian expression constructs of the receptors were expressed in COS-7 cells and membranes containing the expressed receptor proteins were analyzed in a [^{3}H]bradykinin binding assay. The human and rat receptors bound bradykinin and related ligands with pharmacology indicative of a B_2 subtype, whereas the expressed mouse receptor clone appears to produce two populations, one with the pharmacology of B_2 receptors and the other with B_1-like properties.

Materials and Methods

Cell culture. NG108–15 hybrid neuroblastoma-glioma cells and WI38 human fibroblasts were grown as described (10).

RNA preparation. Total RNA was prepared by the method of Chomczynski and Sacchi (17). Poly(A)$^+$ RNA was prepared by one or two cycles of binding and elution from oligo(dT)-cellulose (18).

cDNA synthesis. First-strand cDNA was synthesized using a Boehringer cDNA synthesis kit with either the oligo(dT) primer supplied with the kit or random hexamer primers (Pharmacia). One to 2 μg of RNA were used in each reaction. [^{32}P]dCTP (5 μCi, 0.5 μl, 3000 Ci/mmol; Amersham) was added to each 20-μl reaction to enable quantitation and tracing of the labeled cDNA. The cDNA was purified by gel filtration on a 1-ml Sepharose CL-4B column (Pharmacia).

PCR. PCR was carried out on cDNA samples using a Techne temperature-controlled heating block and a Perkin Elmer/Cetus Amplitaq PCR kit, according to the manufacturer's instructions. The rat bradykinin receptor cDNA sequence (12) was used to generate PCR primers to amplify the bradykinin receptor from various sources. Deoxynucleoside triphosphates were used at 200 μM and primers at 1 μmol/reaction, with 2.5 units of Amplitaq polymerase/reaction being added after the first denaturation step. Amplified cDNA was size-fractionated on agarose gels, and DNA in the required size range was recovered on NA45 paper (Schleicher and Schuell).

RACE PCR (19) was used to clone 3′ and 5′ cDNA ends from rat, NG108–15, and human sources. An oligo(dT)$_{17}$ primer with *Xho*I, *Cla*I, and *Sal*I restriction sites was used to prime cDNA synthesis for 3′ RACE experiments, and random primers were used in cDNA synthesis for 5′ RACE. Spun columns (Clontech) were used to remove excess primers from the cDNA, and a terminal transferase DNA tailing kit (Boehringer) was used to add a poly(A)$^+$ tail with an average length of 30 bases to the 3′ end of the random-primed cDNA.

5′ RACE for both the NG108–15 and WI38 mRNAs used the primer CAGTCGAAGTTATTGGCGATG, from the rat bradykinin B_2 sequence between transmembrane domains 2 and 3, using the following temperature cycle for NG108–15: 94° for 1 min, 55° for 1.5 min, and 72° for 2 min, for 30 cycles, plus 72° for 7 min. The same conditions were used for 5′ RACE of WI38 poly(A)$^+$ RNA, except that the annealing temperature was reduced to 53°. These reactions yielded ethidium bromide-staining cDNA of about 600-base pair length in each case.

The entire length of the protein-coding domain of a bradykinin receptor from rat dorsal root ganglion mRNA was obtained by 3′ RACE using the oligo(dT) primer and the specific 5′ oligonucleotide TACAACACAGAACCGGCT (25 cycles of 95° for 1 min, 57° for 2 min, and 72° for 2 min).

3′ RACE was carried out for NG108–15 poly(A)$^+$ RNA using an oligonucleotide based on a sequence between transmembrane domains 1 and 2 of the rat sequence (CTGGCCGCATTGGAGAACATCTT). The following temperature cycle was used: 94° for 1 min, 57° for 2 min, and 72° for 2 min, for 30 cycles, plus 72° for 7 min. This yielded a fragment of 2300-base pair length. The RACE products were ligated into pKS II$^+$ Bluescript vector, prepared for the direct cloning of unmodified PCR products as described by Marchuk *et al.* (20).

The 5′ and 3′ RACE products derived from NG108–15 poly(A)$^+$ RNA (subsequently shown to be of rat origin) were ligated together at a *Bgl*II site approximately 200 base pairs downstream of the initiation codon, to give a construct that was functional in oocytes and COS cells. The predicted amino acid sequence was identical to that of the receptor from rat dorsal root ganglion cells and that reported by McEachern *et al.* (12).

The 5′ RACE products from WI38 were cloned and sequenced, and the sequence information was used to make a specific 5′ primer that included an *Eco*RI cloning site, the putative initiation codon, and an additional 4 bases of predicted coding domain (GAGAGAATTCGACTGAAGTGCCCATGCCGC). 3′ RACE was carried out on cDNA made from WI38 mRNA. Temperature cycle conditions were 94° for 1 min, 56° for 2 min, and 72° for 3 min, for 30 cycles, plus 72° for 9.9 min.

After gel purification, the full length coding domains of the human and rat bradykinin receptor cDNAs were cloned into the mammalian expression vector pKS1 (21).

Genomic cloning. A 1.4-kb DNA fragment that contained the entire protein-coding domain of the NG108–15-derived rat B_2 bradykinin receptor was used to screen a human placental genomic DNA cosmid library and a mouse genomic DNA cosmid library in pWE15 (Stratagene). In each case, approximately 400,000 colony-forming units were plated on nitrocellulose filters on four 200-mm square plates. Replica filters were made and probed with the rat cDNA probe. Positive colonies were cloned and their DNA was analyzed by restriction analysis and Southern blotting. The coding domain of the mouse gene was subcloned into pKS II$^+$ as a 4.4-kb *Eco*RI fragment. A 1.4-kb *Alw*26I fragment containing the mouse protein-coding domain was cloned into pKS1 and pKS II$^+$ after addition of *Eco*RI linkers (New England Biolabs). The human coding domain was subcloned into pKS II$^+$ as two *Bgl*II fragments of 1.8 kb and 4.6 kb.

Sequencing. cDNA clones were sequenced by the dideoxy method (22) using Sequenase 2 (United States Biochemicals). Sequence analysis was carried out using the Intelligenetics programs. All other manipulations were carried out by standard techniques (18). The DNA sequences covering the protein-coding domains of the mouse genomic clone, the rat and human cDNA clones, and a mouse partial cDNA clone have been deposited in the EMBL database under the following accession numbers: X69676, X69680, X69681, and X69682, respectively.

Analysis of cloned cDNAs by expression in *Xenopus* oocytes. RNA transcripts of cDNAs cloned into pKS1 (21) were translated in *Xenopus laevis* oocytes and analyzed for function. *In vitro* transcription with T7 RNA polymerase was carried out on 1 μg of linearized DNA using a transcription/capping kit (Stratagene). The product of each transcription reaction was dissolved in 15 μl of water, and 50 nl were injected per oocyte. The maintenance of *Xenopus* frogs, the culture of

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oocytes derived from them, and the electrophysiological assay of expression were as described by Phillips *et al.* (10).

COS-7 cell expression. COS-7 cells were obtained from Flow Laboratories and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. cDNAs cloned in the expression vector pKS1 were introduced into the cells by electroporation (23) using a Bio-Rad Gene-pulsar apparatus, according to the manufacturer's instructions. Approximately 10^7 cells were used per transfection. These were transfected with 50 μg of nonlinearized DNA in sucrose buffer (240 mM sucrose, 7 mM potassium phosphate, pH 7.5), using 25-μF capacitance at 220 V. The cells were recovered and plated on a 200-mm square plate. Typically, four transfections were carried out in parallel to generate sufficient material for membrane preparations. The cells were cultured for 3 days and then harvested.

Plates were washed rapidly with two changes of ice-cold harvesting buffer (25 mM potassium phosphate, pH 6.5) and then scraped into a total of 15 ml/plate of the same buffer at 4°. No protease inhibitors were added at any stage, but care was taken to keep the preparation at 4° throughout and to process the membranes as rapidly as possible. The pooled cells were homogenized with a Polytron homogenizer and then centrifuged for 30 min at 30,000 × *g*. The pellets were washed twice by homogenization and centrifugation, and the final pellet was homogenized in a volume of approximately 3 ml. Protein concentrations were determined by the Bio-Rad version of the Bradford assay (Bio-Rad Laboratories), and membranes were either used immediately or aliquoted and stored at −70°.

Binding assays were carried out in 25 mM potassium phosphate buffer, pH 6.5, supplemented with 0.2% bovine serum albumin, 1.0 mM EGTA, 0.1 mM bacitracin, and 50 μg/ml chymostatin. [^{3}H]Bradykinin (65 Ci/mmol; Amersham) and either displacer or buffer were added in a volume of 100 μl each to assay tubes, and the reaction was begun by the addition of membranes (5 μg/tube) in a total volume of 800 μl. The tubes were incubated at 4° for 60 min, and the assay was terminated by filtration through a GF/B filter (previously soaked overnight in 0.1% polyethyleneimine) on a Brandel harvester. Scintillation counting was carried out in a Beckman counter. Data were analyzed by the LIGAND program (24). The LIGAND program was used to compare one- and two-site models using the *F* test.

Results

Receptor DNA cloning. A mouse genomic DNA fragment encoding a bradykinin receptor was isolated by screening a mouse genomic cosmid library with a rat B_2 bradykinin receptor fragment of 1.4 kb, which covered the entire protein-coding region. The mouse bradykinin receptor-coding domain was subcloned and sequenced as described in Materials and Methods, and this was used for subsequent expression studies.

Several rat bradykinin receptor cDNAs were cloned from different sources by PCR. A clone with a full length protein-coding domain was obtained from rat dorsal root ganglion RNA using a PCR primer that covered the putative initiation codon (12). Overlapping rat bradykinin receptor 5′ and 3′ cDNAs were also cloned from NG108–15 cells using RACE PCR (19), and these were then cut at a unique *Bgl*II site in the overlapping DNA and ligated to produce a full length cDNA, which was used for functional studies.

DNA encoding a human bradykinin receptor was isolated from a human placental DNA library, and the receptor-coding domain was subcloned and sequenced. A cDNA sequence was cloned from WI38 human lung fibroblast cells using PCR. The predicted protein sequences from the cDNA isolated from the WI38 cells and from the human DNA clone were identical to each other and to two previously reported human bradykinin receptor sequences (13, 14).

The mouse and human genomic sequences contained a potential 3′ splice acceptor site upstream of the putative initiation codon. Eggerickx *et al.* (14) noted a difference at this point between their human genomic sequence and the human cDNA sequence reported by Hess *et al.* (13), and they suggested that it may be a potential splice junction. To determine whether mRNAs with different 5′ ends existed, several rodent partial cDNA clones were isolated from rat lung, rat uterus, and NG108–15 cells (a hybrid rat glioma/mouse neuroblastoma cell line), using the 5′ RACE technique. All of our rat cDNA clones had a predicted protein sequence that was identical to that of the rat uterine bradykinin receptor (12). Several of the rat 5′ cDNA clones we isolated differed from the published rat sequence 8 bases upstream from the putative initiation codon (Fig. 1A), suggesting that these sequences represent alternatively spliced forms of the mRNA. Four 5′ RACE cDNA clones that obviously encoded the mouse bradykinin receptor were identified from NG108–15 cells. The sequences of three of these mouse cDNA clones deviated from the mouse genomic sequence at a point 8 bases upstream from the putative initiation codon (Fig. 1B). The existence of a consensus splice acceptor site in the genomic DNA in this region (16) suggests that this is a splice junction. The longest divergent mouse cDNA had 267 bases of sequence 5′ to the splice junction.

The bradykinin receptor sequences from each of the species contained a single methionine codon in a context favorable for initiation of translation (25), which was assumed to define the protein reading frame in each case. The mouse genomic clone had no methionine codons upstream of the proposed splice acceptor site to the *Bgl*II site used for subcloning. The longest putative splice variant had four methionine codons; however, the first was followed by an in-frame stop codon and the rest were in unfavorable contexts for initiation of translation (25).

The predicted human receptor protein is shorter than the rodent receptors by two amino acids in the amino-terminal region, corresponding to amino acids 8 (alanine) and 9 (leucine) in the rat sequence. The deduced amino acid sequences for the human, rat, and mouse clones are compared in Fig. 2. The similarity at the amino acid level is 87% between receptors from rat and mouse and 77% between receptors from either rodent species and human. There is 91% identity between rat and mouse at the DNA level over the protein-coding domain and 82% identity between both rodent species and the human DNA sequence. The amino acid similarity between the rodent and human sequences is greatest in the transmembrane domains (88% identity) and is least in the amino-terminal region of the receptors, where the human receptor shares only 51% amino acid identity with the rat sequence. The predicted molecular weights are as follows: human, 41,443; rat, 41,713; and mouse, 41,293.

Sites of possible functional significance are conserved in all three receptors that we have studied. There are two potential *N*-linked glycosylation sites in the amino-terminal region and one in the second extracellular loop. There is also a potential palmitoylation site in the carboxyl-terminal intracellular portion of the polypeptide, similar to those found and shown to be palmitoylated in the β_2-adrenergic receptor (26) and rhodopsin (27). Palmitoylation may serve to anchor this region of the polypeptide in the membrane. Potential sites of phosphorylation by cAMP-dependent protein kinase and by other protein kinases, within the third intracellular loop and the carboxyl-

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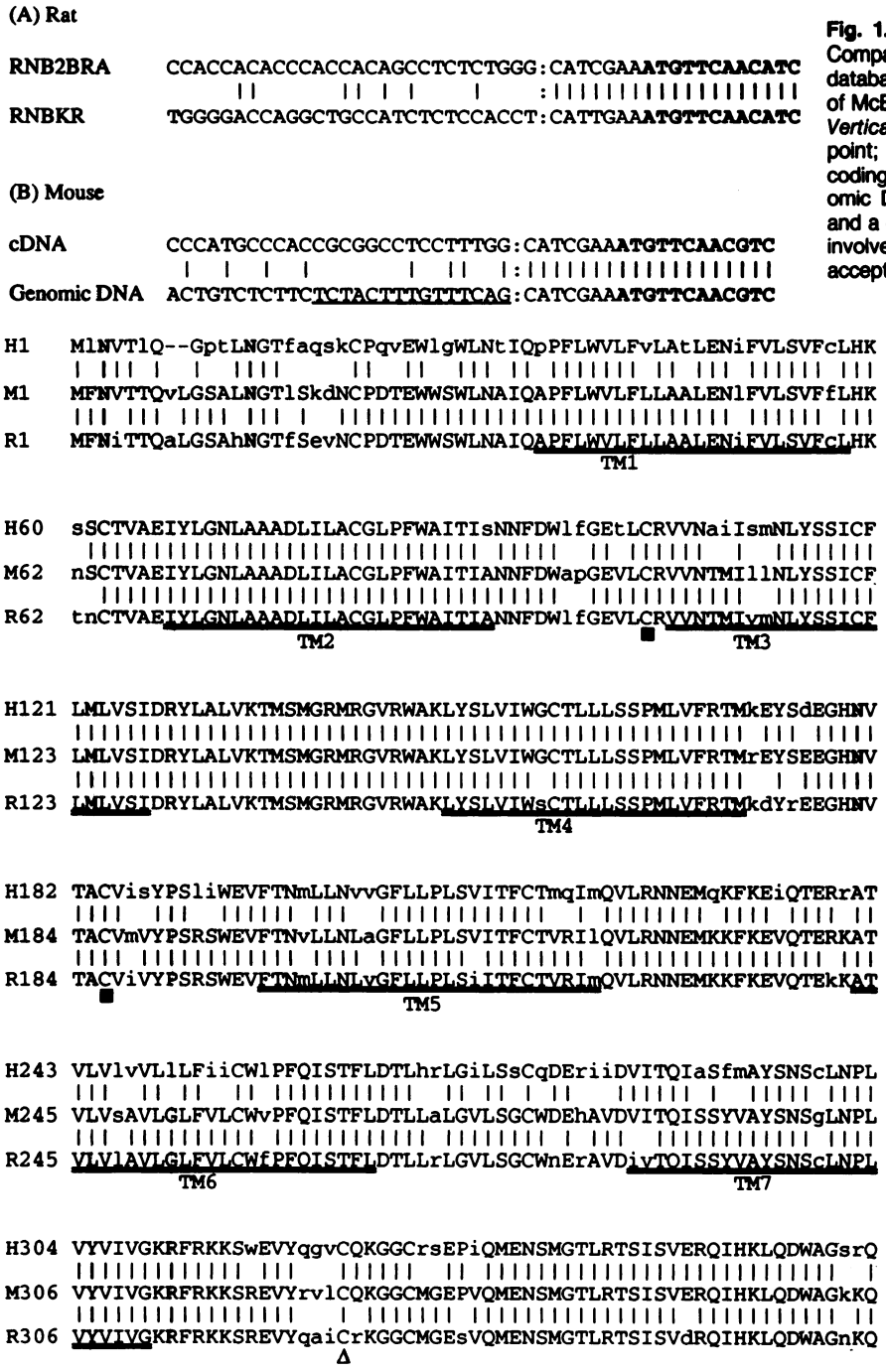

Fig. 1. Identification of 3′ RNA splice acceptor sites. A, Comparison of rat cDNA sequences from this study (EMBL database accession number X69681) and from the report of McEachern *et al.* (12) that are involved in RNA splicing. *Vertical lines*, base identity; *colon*, RNA splice junction point; *bases in bold*, begining of the predicted protein-coding domain. B, Comparison of a region of mouse genomic DNA (EMBL database accession number X69676) and a cDNA (EMBL database accession number X69682) involved in RNA splicing. The concensus 3′ RNA splice acceptor site in mouse genomic DNA is *underlined*.

Fig. 2. Comparison of the amino acid sequences of human, rat, and mouse bradykinin receptors. The predicted amino acid sequences of the human, rat, and mouse bradykinin receptors are shown using the single-letter amino acid code. The amino acids are numbered on the *left*, with numbers prefixed with H, M, or R for human, mouse, or rat, respectively. *Vertical line*, residues in the rat and human receptors that are identical to those present in the mouse receptor. Putative transmembrane domains are *underlined*, and potential *N*-linked glycosylation sites are marked in *bold*. ■, Two cysteines that may be involved in a disulfide bridge; Δ, a third cysteine in the carboxyl-terminal tail that may be a site of palmitoylation.

terminal region, exist in the three receptors and may be involved in the phenomenon of ligand-induced desensitization that has been reported for bradykinin receptors (28).

Characterization of cloned receptors in *Xenopus* oocytes. We carried out a preliminary characterization of the receptors from mouse, rat, and human by injecting *in vitro* cRNA transcripts of the cloned DNAs into *Xenopus* oocytes. Electrophysiological responses to applied agonist were measured 2–4 days after injection, as described by Phillips *et al.* (10). Oocytes injected with transcripts derived from each of the three receptor clones produced large inward currents upon exposure to 100 nM bradykinin (Fig. 3, A, B, and D).

The response to bradykinin by oocytes expressing the human or rat receptors could be completely inhibited by prior application of the B_2 antagonist NPC567 (D-**Arg[Hyp**^{3},D-Phe^7] bradykinin) (5 μM) (5, 6). The inhibition of the response produced by NPC567 was reversible upon removal of the compound (Fig. 4). The inhibition could also be overcome by increasing the agonist concentration (data not shown) and therefore had the characteristics of competitive antagonism.

Oocytes expressing the cloned receptors from human and rat were unresponsive to the bradykinin receptor B_1 agonist [des-Arg^9]bradykinin (2 μM), although the same oocytes responded to a subsequent exposure to bradykinin (Fig. 3, A and B). In contrast, oocytes injected with cRNA encoding the mouse bradykinin receptor responded both to 100 nM bradykinin and to 2 μM [des-Arg^9]bradykinin (Fig. 3, C and D). We also observed responses to [des-Arg^9]bradykinin at 200 nM (see Fig. 5). How-

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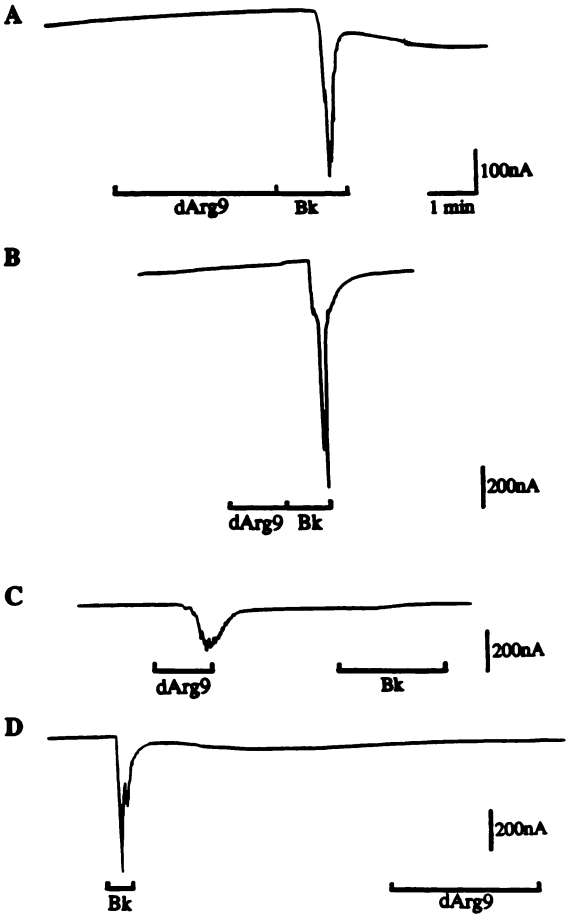

Fig. 3. Responses from *Xenopus* oocytes injected with RNA transcripts of bradykinin receptor clones. A-C, Human (A), rat (B), and mouse (C) transcripts during exposure to [des-Arg^9]bradykinin (*dArg9*) (2 μM), followed by exposure to bradykinin (*Bk*) (0.1 μM). D, Mouse transcript during exposure to bradykinin (0.1 μM), followed by exposure to [des-Arg^9]bradykinin (2 μM). Oocytes were injected with 50 nl (1 ng) of cRNA generated by *in vitro* transcription (see Materials and Methods). Responses were recorded on the third day after injection. Oocytes were impaled with two electrodes and voltage clamped at −55 mV as described (10). They were perfused with test compounds for the periods indicated.

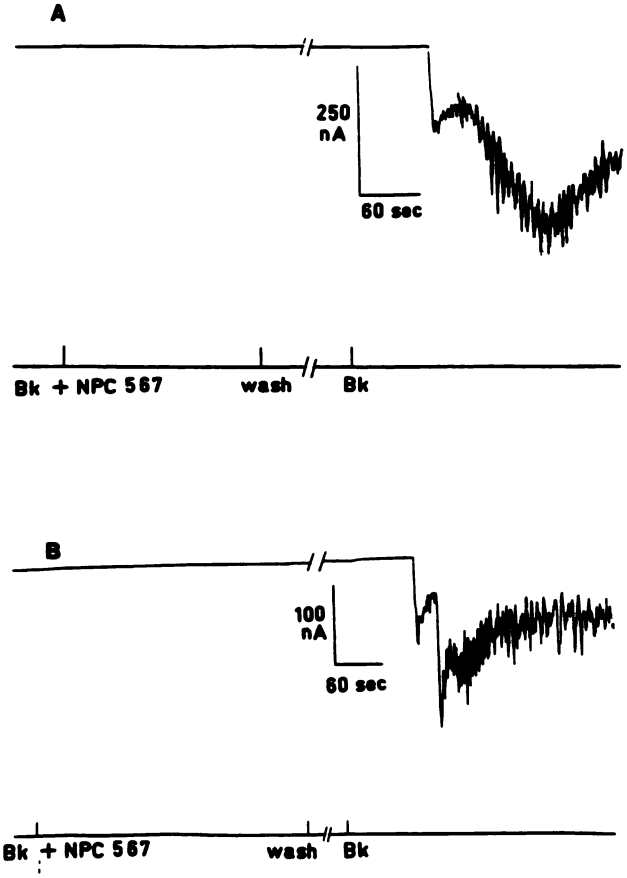

Fig. 4. NPC567 antagonism of the bradykinin (*Bk*) response of oocytes expressing human (A) or rat (B) bradykinin receptors. Oocytes were exposed to NPC567 (5 μM) for 15 min, and then bradykinin (100 nM) was applied in the continuing presence of the antagonist. Responses to bradykinin alone (100 nM) were recorded after the antagonist was washed out.

ever, oocytes that had previously responded to either agonist appeared to be unable to respond when the alternate agonist was applied shortly after the initial response (Fig. 3, C and D).

The mouse bradykinin receptor exhibited weak agonism in response to both the B_2 antagonist NPC567 (Fig. 5B) and the selective B_1 antagonist [Leu^8,des-Arg^9]bradykinin (Fig. 5A). In spite of this weak agonism, NPC567 could antagonize bradykinin, and [Leu^8,des-Arg^9]bradykinin could antagonize [des-Arg^9]bradykinin responses in oocytes expressing the mouse receptor (Fig. 5). NPC567 agonism was not observed in oocytes expressing either human (0 of 6 eggs) or rat (0 of 11 eggs) bradykinin receptors. [Leu^8,des-Arg^9]Bradykinin agonism was not observed in oocytes expressing human bradykinin receptors (0 of 2 eggs), but a weak response was seen once in an oocyte expressing the rat receptor (1 of 3 eggs).

Receptor expression in transfected COS cells. We examined the binding characteristics of the cloned mouse, human, and rat bradykinin receptors expressed transiently in COS cells. Crude membrane preparations were used for these experiments and the level of receptor expression was typically 1–2 pmol/mg of crude membrane protein. We measured the specific binding of [^{3}H]bradykinin at various concentrations. These experiments (three or more) yielded a calculated K_d of 37 ± 14 pM (mean ± standard error) for the mouse receptor, 79 ± 36 pM for the human receptor, and 144 ± 46 pM for the rat receptor. LIGAND analysis indicated that a two-site model was a statistically significant improvement over a one-site model. This was seen most clearly when homologous displacement using [^{3}H]bradykinin (1 nM) diluted with unlabeled bradykinin (3–1000 nM) was included in the analysis. The second site had a K_d in the range of 5–10 nM in all three species, but the value could not be accurately determined in the current experiments.

Displacement studies were carried out using 1 nM [^{3}H]bradykinin and various concentrations of displacing compounds that are selective for B_1 and B_2 receptor subtypes. The B_1 agonist [des-Arg^9]bradykinin failed to displace bradykinin significantly from the human receptor at concentrations up to 10 μM (Fig. 6A). [des-Arg^9]Bradykinin gave weak displacement at rat receptors (K_i value of 363 ± 54 nM, mean ± standard error) (Fig. 6B). The B_1 agonist also displaced [^{3}H]bradykinin weakly from the mouse receptor but there was also an apparent high affinity component of the binding, making the mouse receptor binding curve more complex than either the human receptor curve or the rat receptor curve (Fig. 6C). The data for the mouse receptor could be fitted to a two-site model ($F = 7.5$, $p = 0.001$) where approximately 30% of the bradykinin binding

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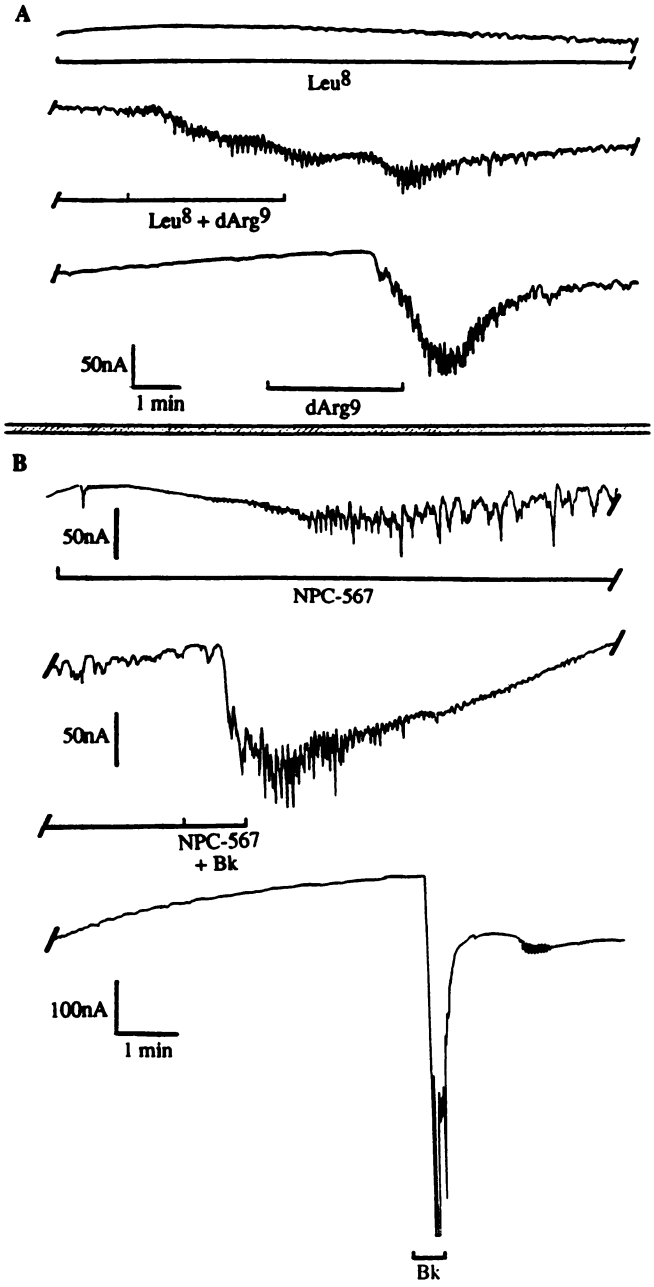

Fig. 5. Partial blocking of responses from *Xenopus* oocytes injected with RNA transcripts of mouse bradykinin receptor clone. A, During exposure to [Leu^8,des-Arg^9]bradykinin (*Leu^8*) (1 μM for 15 min), followed by exposure to [des-Arg^9]bradykinin (*dArg^9*) (0.2 μM), in the continuing presence of antagonist. After the antagonist was washed out for 30 min, [des-Arg^9]bradykinin (0.2 μM) was applied in the absence of antagonist. B, Mouse transcript during exposure to NPC567 alone (0.5 μM for 15 min) and then together with bradykinin (*Bk*) (0.1 μM), followed by a 30-min wash and re-exposure to bradykinin alone (0.1 μM).

was displaced very potently by [des-Arg^9]bradykinin (<1 nM) and about 70% was displaced less potently (K_i value of 114 ± 67 nM, mean ± standard error) (Fig. 6C).

The B_1 antagonist [Leu^8,des-Arg^9]bradykinin at 10 μM did not significantly displace [^{3}H]bradykinin from either the human or rat receptor (Fig. 6, A and B). It gave a maximum of 30% inhibition at 1–30 μM in mouse preparations. LIGAND analysis of [Leu^8,des-Arg^9]bradykinin data simultaneously with [^{3}H]bradykinin saturation data was consistent with a two-site

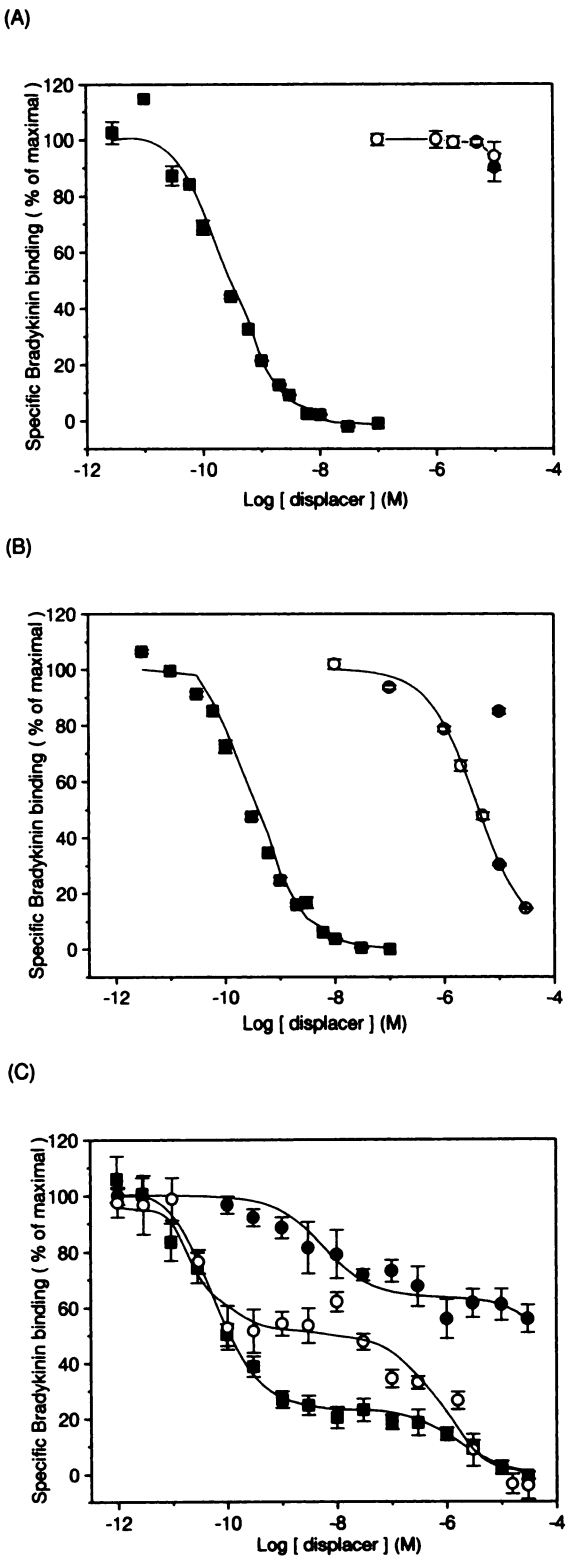

Fig. 6. Displacement of [^{3}H]bradykinin binding to COS-7 cell membranes expressing cloned bradykinin receptors from human (A), rat (B), and mouse (C) by HOE140 (■), [des-Arg^9]bradykinin (○), and [Leu^8,des-Arg^9] bradykinin (●). [^{3}H]Bradykinin (1 nM) and membranes (5 μg of protein) were incubated as described in Materials and Methods in the presence of increasing concentrations of displacer. The *points* represent the mean ± standard error of at least three separate determinations in triplicate. *Curves* were fitted by the simultaneous analysis of the data sets by LIGAND.

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model where the majority of the receptors had low affinity for [Leu^8,des-Arg^9]bradykinin (>30 μM) and a small proportion had very high affinity (<1 nM) (Fig. 6C).

The recently described compound HOE140 (15) has been reported to be a highly potent and specific antagonist of B_2 receptor-mediated functions. This compound displaced [^{3}H] bradykinin (1 nM) from human and rat receptors, with K_i values of 40 ± 3 pM for the human receptor and 36 ± 5 pM for the rat receptor (three experiments) (Fig. 6, A and B). However, in mouse preparations about 70% of the binding of bradykinin was displaced by HOE140 with picomolar affinity (9.0 ± 2.4 pM). The remainder could be ascribed to a binding site with micromolar affinity for HOE140 (F = 20.8, p = 0.00, six experiments) (Fig. 6C).

When the displacement of [^{3}H]bradykinin from the mouse cloned receptor by [des-Arg^9]bradykinin was repeated in the presence of 10 nM HOE140, there was a reduction in the B_{max} for bradykinin of about 70% and all the remaining binding sites had high affinity for [des-Arg^9]bradykinin (~10 nM) (Fig. 7A). A concentration of 10 nM HOE140 was sufficient to give complete displacement of [^{3}H]bradykinin from human and rat B_2 subtype receptors (Fig. 6, A and B), whereas for the mouse receptor 10 nM HOE140 was able to displace bradykinin only from the high affinity HOE140 binding site (Fig. 6C). In addition, when bradykinin displacement by HOE140 was examined in the presence of 30 nM [des-Arg^9]bradykinin, there was a 20–30% reduction in the B_{max} for bradykinin and the remaining binding sites had only high affinity for HOE140 (K_i value of 8.12 ± 1.54 pM, mean ± standard error) (Fig. 7B). [des-Arg^9]Bradykinin at 30 nM had no effect on bradykinin binding to the cloned receptors from human and rat (Fig. 6, A and B), whereas this concentration was sufficient to occupy completely the high affinity site on mouse bradykinin receptors (Fig. 6C).

Discussion

We have isolated genomic DNA encoding a functional mouse bradykinin receptor. A cosmid clone encoding the human bradykinin receptor gene and a cDNA encoding the same receptor from the human lung fibroblast cell line WI38 were isolated and rat cDNA clones for the receptor were obtained from rat dorsal root ganglion RNA and NG108–15 RNA. The rat cDNA clones encode the same predicted receptor protein as does a previously cloned rat B_2 bradykinin receptor (12). Interestingly, in NG108–15 cells we identified the 5′ end of a second cDNA sequence that is homologous to the mouse receptor sequence in the protein-coding domain. This cDNA is most likely derived from the mouse N18 neuroblastoma parental cells of the hybrid NG108–15 cells, whereas the rat sequence that we identified is probably derived from the rat C6 glioma parental cells.

Sequence differences in cDNAs and genomic DNAs 5′ to the receptor-coding domain suggest that rat and mouse bradykinin receptor mRNAs can be spliced at a point 8 bases from the initiation codon at a consensus splice acceptor site (Fig. 1), as suggested by Eggerickx *et al.* (14) for the human receptor. Neither mouse nor human genomic DNA contained any obvious intron sequences in the protein-coding domain. Several other G protein-coupled receptor genes have been found to lack introns within the coding sequence (29, 30).

When a human genomic library was screened at moderate stringency with a cDNA probe containing the B_2 sequence, two overlapping clones were isolated. These contained a sequence

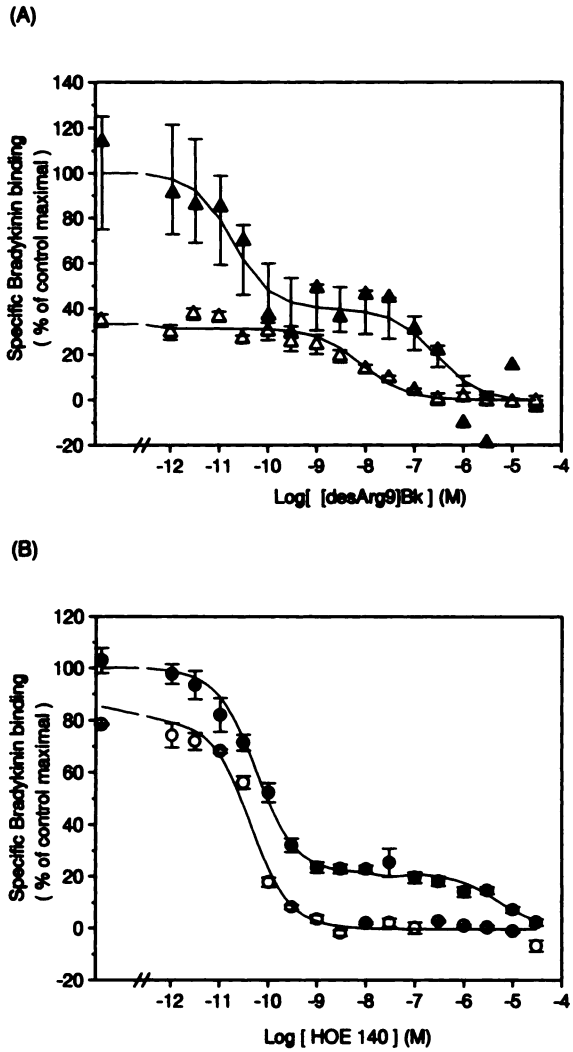

Fig. 7. Displacement of [^{3}H]bradykinin binding to COS-7 cell membranes expressing cloned bradykinin receptors from mouse, by [des-Arg^9]bradykinin in the absence (▲) and presence (△) of HOE140 (10 nM) (A) or by HOE140 in the absence (●) and presence (○) of [des-Arg^9]bradykinin (30 nM) (B). [^{3}H]Bradykinin (1 nM) and membranes (10 μg of protein) were incubated as described in Materials and Methods, in the presence of increasing concentrations of displacer. The *points* represent the mean ± standard error of three separate determinations (each one a seperate transfection), in triplicate. In the case of the [des-Arg^9]bradykinin control curve, the *error bars* represent the variance of the fitted data. *Curves* were fitted by the simultaneous analysis of the data sets by LIGAND.

identical to that of the coding domain of the human cDNA sequence.

Southern blotting analysis (12) suggests that there is a single gene with high homology to the B_2 receptor-encoding cDNAs in rat, human, and guinea pig. Similar results are found with mouse DNA (data not shown). In addition, the sequences of the mouse and human genomic clones reveal no intron/exon structure within the B_2 receptor protein-coding domain. We cannot rule out the possibility that there is a 5′ exon upstream of the initiation codon for the B_2 receptor-encoding region that can be spliced onto the 5′ end of the B_2 receptor-encoding species to enable the use of an alternative, upstream, initiation codon, but there is no evidence that this occurs.

The function and pharmacology of the expressed cloned human, rat, and mouse receptors were examined in *Xenopus* oocytes and in COS-7 cells. cRNA from each of the receptor

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clones was able to direct the expression of receptors in *Xenopus* oocytes. These receptors couple via intrinsic G proteins to the inositol trisphosphate pathway, resulting in the release of calcium from internal stores and the consequent opening of calcium-sensitive chloride channels (31, 32). In the oocyte system, the human and rat receptors were responsive to bradykinin. In oocytes expressing rat or human receptors, this response could be blocked by the well characterized B_2 antagonist NPC567 (5, 6). This antagonist rather than HOE140 was chosen because in a previous study we found that extremely long wash times (6 hr or longer) were required to reverse the antagonism by HOE140 (10). In contrast, the response to bradykinin was recovered after a 15-min washout of NPC567. The blockade given by NPC567 was reversible and could be overcome by increasing the agonist concentration, thus showing the characteristics of competitive antagonism. Oocytes expressing either human or rat receptors failed to respond to high concentrations (2 μM) of the B_1 agonist [des-Arg^9]bradykinin, although the same oocytes responded to subsequent application of bradykinin. These data confirm those of McEachern *et al.* (12) for the rat receptor and provide additional confirmation that the human receptor cloned by others (13, 14) and ourselves is of the B_2 type.

In contrast, oocytes expressing the mouse receptor responded both to bradykinin and to the B_1 agonist [des-Arg^9]bradykinin. The bradykinin response could be partially blocked with NPC567, but this compound exhibited weak agonist activity in these oocytes. Such agonist activity has been seen previously with this compound and related peptide antagonists (14, 33). In the present study, similar results were found with the B_1-specific antagonist [Leu^8,des-Arg^9]bradykinin.

These experiments demonstrated that the clones we have isolated encode functional bradykinin receptors that are able to couple to heterologous G proteins endogenous to the oocyte. They also reveal that the mouse receptor differs from the rat and human receptors in its ability to respond to both B_1 and B_2 agonists. Although both classes of antagonist displayed a small degree of agonism at this receptor, they were nevertheless able to antagonize the responses to the appropriate agonist. To obtain quantitative data with which to characterize this receptor more rigorously, we carried out binding experiments on receptors expressed in COS cells.

All three receptor DNAs were introduced into COS-7 cells in the pKS1 vector (21) under the control of a cytomegalovirus promoter. The levels of expression of the receptor (1–2 pmol/mg of crude membrane protein) were slightly higher than the levels of expression reported by Hess *et al.* (13) for a similar crude membrane preparation from COS cells that had been liposome-tranfected with the human bradykinin receptor (0.21–0.45 pmol/mg). The binding of the natural ligand bradykinin was studied by both binding isotherm analysis and displacement techniques. Binding isotherm studies yielded K_d values of 79 ± 36 pM, 144 ± 46 pM, and 37 ± 14 pM for the human, rat, and mouse receptors, respectively. These values are not significantly different from each other by Student's t test ($p > 0.05$). The second low affinity site observed with all three receptors may be explained by the presence of receptors uncoupled from G proteins (13). The observation that this second site is present in all three receptors makes it unlikely that the low affinity binding of bradykinin corresponds to the "B_1-like" site seen only in mouse receptors. Additional experiments with GTP analogues and B_1-selective radioligand are underway to investigate these effects in more depth.

The recently described B_2-selective antagonist HOE140 (15) displaced radiolabeled bradykinin from all three receptors at low concentrations (K_i values of 9 ± 2 pM for mouse, 40 ± 3 pM for human, and 36 ± 5 pM for rat). However, in mouse preparations only about 70% of the bradykinin binding was displaced at these low concentrations. The remainder had an affinity for HOE140 in the micromolar range, consistent with the affinity of HOE140 at B_1 receptors (34).

We also examined the ability of B_1-selective ligands to displace [^{3}H]bradykinin from all three receptors. The antagonist [Leu^8,des-Arg^9]bradykinin gave no [^{3}H]bradykinin displacement from either human or rat receptors. The agonist [des-Arg^9]bradykinin was also inactive at 10 μM with the human receptor but displaced the radioactive ligand from the rat receptor with a calculated K_i value of 363 ± 54 nM.

The high affinity of HOE140, low affinity or inactivity of [des-Arg^9]bradykinin, and inactivity of [Leu^8,des-Arg^9]bradykinin for human and rat receptors indicate that these receptors are of the B_2 subtype. The data for the mouse cloned receptor indicate two populations of binding sites. A fraction (~30%) of mouse bradykinin receptor binding was displaced by very low concentrations of the B_1 antagonist [Leu^8,des-Arg^9]bradykinin ($K_i < 10$ nM). This small fraction of high affinity binding could be quantified only by co-analysis with [^{3}H]bradykinin saturation data, used to define the B_{max}. Approximately 30% of the bradykinin receptors also had very high affinity for the agonist [des-Arg^9]bradykinin ($K_i < 1$ nM), whereas the remaining receptors showed a K_i value of 114 ± 67 nM, consistent with the affinity of [des-Arg^9]bradykinin at B_2 receptors (35). The data with HOE140, [des-Arg^9]bradykinin, and [Leu^8,des-Arg^9]bradykinin indicate that ~70% of expressed mouse receptors show a B_2 pharmacology. The remaining ~30% of the mouse bradykinin receptors show a low affinity for HOE140 but high affinity for [des-Arg^9]bradykinin and [Leu^8,des-Arg^9]bradykinin. It is possible that this proportion represents a B_1-like population of receptors.

The hypothesis that the mouse bradykinin receptors were a mixed population, with a smaller fraction (~30%) like the B_1 subtype and the majority (~70%) of the B_2 subtype, was supported by experiments where one or the other of the binding site subtypes was blocked with low concentrations of selective B_1 or B_2 ligands. [des-Arg^9]Bradykinin at 30 nM blocked approximately 30% of the bradykinin receptors, and the remaining bradykinin receptors had high affinity for HOE140. HOE140 at 10 nM spared only approximately 30% of the mouse bradykinin receptors, which had high affinity for [des-Arg^9]bradykinin. The affinity of the population of receptors that exhibited high affinity [des-Arg^9]bradykinin binding was apparently lower in the presence of HOE140 (Fig. 6C), compared with that seen in the absence of HOE140 (Fig. 7A). We are currently trying to determine whether this receptor population corresponds to the same B_1-like site seen in the absence of HOE140 or whether the shift is due to inaccuracy in determination of affinities at the B_1-like site using [^{3}H]bradykinin (see below).

The K_i values for 30% of the mouse receptors, which show high affinity for [des-Arg^9]bradykinin and [Leu^8,des-Arg^9]bradykinin, are reported as <1 nM or <10 nM, because the blocking experiments indicated that this proportion of the mouse bra-

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dykinin receptors are B_1-like. The radioligand used in this study ([^{3}H]bradykinin) is B_2 subtype preferring. The K_i values for B_1-selective ligands displacing a B_2-preferring radioligand from a B_1 bradykinin receptor subtype would be underestimated and, therefore, only an maximum estimate can be given.

We intend to characterize the fraction of mouse bradykinin receptors that appear to be B_1-like by using the B_1-selective radioligand [^{3}H][des-Arg^{10}]kallidin. This will allow us to quantitate the K_i values for bradykinin, [des-Arg^9]bradykinin, and [Leu^8,des-Arg^9]bradykinin at the B_1-like site.

These experiments show that the expressed cloned receptors from the human and rat sources encode functional receptors that are of the B_2 subtype. The mouse receptor differs in that binding studies indicate the presence of a small population of B_1 subtype-like receptors, in addition to a population of receptors that are of the B_2 subtype. This is confirmed by the observation that the mouse receptor expressed in oocytes responds to both 200 nM [des-Arg^9]bradykinin and bradykinin. Thus, homologous bradykinin receptors from two closely related rodent species exhibit very different properties in the expression systems we have used. Small sequence differences can account for large differences in pharmacological characteristics of receptors; for instance, a single amino acid difference has been shown to be responsible for the interspecies variation in pharmacology of two α_2-adrenergic receptors (36). Similarly, a single amino acid difference is responsible for differences in the pharmacology of rodent and human 5-HT_{1B} receptors (37). These examples are distinct from what we report here for the mouse bradykinin receptor.

It is apparent from binding data that the mouse receptor gene can give rise to two populations of receptors with different types of pharmacology when expressed in COS cells and this is not apparently due to RNA splicing, although we cannot exclude the possibility of RNA editing (38). The experiments described have not enabled us to distinguish whether this is a specific property of the mouse receptor that is somehow masked in the case of the expressed rat and human bradykinin receptor clones. One possible explanation for the observed binding data is that the mouse receptor, but neither the rat nor the human receptor, is incompletely post-translationally modified to produce two bradykinin receptor subtypes. The COS cell expression system allows the mouse bradykinin receptor to be distinguished from the rat and the human receptors, so it should be possible to identify the region of the mouse receptor that gives rise to the mixed populations of two receptor subtypes by making bradykinin receptor chimeras between the mouse receptor and either the rat or the human bradykinin receptor. This approach was used successfully to identify single amino acid residue differences in distinct gene products of α_2-adrenergic receptors (36) and 5-hydroxytryptamine$_{1B}$ receptors (37) that confer large differences in pharmacological behavior on those receptors.

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