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Differential Pharmacology of Cloned Human and Mouse B_2 Bradykinin Receptors

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SUMMARY

The pharmacology of cloned B_2 bradykinin receptors heterologously expressed in cell lines lacking any endogenous bradykinin receptors was analyzed. The possibility of B_2 bradykinin receptor heterogeneity had been proposed on the basis of numerous studies in various tissue preparations. The results reported here permit a direct evaluation of some of these hypotheses by examining the pharmacological properties of cloned bradykinin receptors. A cloned human B_2 bradykinin receptor was stably transfected into Chinese hamster ovary cells. The data suggest that in response to bradykinin (BK), the cloned receptor activates both phosphatidylinositol hydrolysis and arachidonic acid release by independent pathways. Thus, the activation of these two second messenger pathways does not require the existence of two B_2 bradykinin receptor subtypes. A mouse gene encoding the B_2 bradykinin receptor was isolated, and the coding region was expressed in COS-7 cells. This murine receptor exhibited the pharmacological properties of a "classical" B_2 bradykinin receptor. A comparison of the pharmacological profiles of cloned human and murine homologs of the B_2 bradykinin receptor indicates that both receptors bind agonists with similar properties. However, the two receptors differ dramatically in their affinity for some peptide antagonists. The mouse receptor has a 60- to 80-fold higher affinity for [D-Arg^{0}Hyp^3, Thi^{5,8},D-Phe^7]BK and [D-Arg^0,Hyp^3,D-Phe^7]BK than its human homolog. Thus, the species of a bradykinin receptor can have a significant effect on its pharmacology. The cloning, expression, and pharmacological comparison of human and mouse B_2 bradykinin receptor genes indicate that some of the previous reports of B_2 receptor subtypes can be explained by species differences in a single B_2 bradykinin receptor gene.

BK is a nonapeptide (RPPGFSPFR) paracrine hormone that appears to play a physiological role in mediating pain and inflammation (1–3). Two types of bradykinin receptors, B_1 and B_2, have been characterized pharmacologically (1–3). The physiological agonists for the B_2 receptor subtype are BK and kallidin (lys-BK), which are equipotent at the receptor. The enzymatic removal of the C-terminal arginine from these agonists generates [des-Arg^9]BK and [des-Arg^{10}]kallidin, which are the preferred agonists for the B_1 receptor.

Subtypes of the B_2 bradykinin receptor have been proposed based on the ability of BK to stimulate multiple second messenger pathways (4–6). The B_2 bradykinin receptor subtype is coupled through G proteins to at least two separate pathways of phospholipid metabolism, the hydrolysis of phosphatidylinositol by PLC and the release of arachidonic acid by PLA_2 (5, 7, 8). Previous studies have indicated that arachidonic acid can be released in PLC-dependent pathways (9–12). The release of arachidonic acid by a pathway directly dependent on PLC activity can be distinguished from a PLC-independent pathway by examining the effect of phorbol esters on both phosphatidylinositol hydrolysis and arachidonic acid release. In Swiss 3T3 fibroblast cells and MDCK cells, phorbol ester treatment was found to inhibit BK-induced phosphatidylinositol hydrolysis and to stimulate BK-mediated arachidonic acid release, suggesting that the activation of PLA_2 is independent of PLC activation (7, 8). This hypothesis is supported further by apparent simultaneous activation of both PLC and PLA_2 in response to BK (13). Based on these findings it has been suggested, because BK appears to activate independently two parallel signal transduction pathways, that one bradykinin receptor subtype is responsible for stimulating the PLC pathway and another subtype is responsible for stimulating the PLA_2 pathway (4–6).

The existence of subtypes of the B_2 bradykinin receptor has been proposed also on the basis of variations in the interaction of bradykinin receptors from different tissue preparations with

ABBREVIATIONS: BK, bradykinin; PLC, phospholipase C; PLA, phospholipase A_2; MDCK, Madin-Darby canine kidney; Hoe 140, [D-Arg^0,Hyp^3,Thi^5,D-Tic^7,Oic^8]BK; CHO, Chinese hamster ovary; ES, embryonic stem; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; bp, base pair; kb, kilobase; PBS-EDTA, phosphate-buffered saline-ethylenediaminetetraacetate; IP_1, inositol monophosphate; IP_2, inositol biphosphate; phorbol ester, phorbol 12-myristate 13-acetate.

a series of peptide analogs of BK that were first synthesized by Vavrek and Stewart (5, 14–21). However, recent experiments with the more potent B_2 bradykinin receptor antagonist Hoe 140 (22) argue against some of the previously proposed B_2 bradykinin receptor heterogeneity (23).

The recent cloning of B_2 bradykinin receptors now affords the tools to begin to define unambiguously bradykinin receptor pharmacology. The DNA encoding a B_2 bradykinin receptor was obtained first by expression cloning from rat uterine mRNA (24). Subsequently, a cDNA clone (25) and a genomic clone (26, 27) of a human B_2 bradykinin receptor were isolated. These receptors have the putative seven transmembrane helix topography characteristic of G protein-coupled receptors (28, 29). Expression of the human B_2 bradykinin receptor in CHO cells demonstrated that the cloned receptor activates PLC to increase intracellular CA^{2+} (26). Expression of the cloned rat B_2 bradykinin receptor was shown to activate PLA_2 to stimulate prostaglandin synthesis in Chinese hamster lung CCL39 fibroblasts (30). In the present study, we report that the human B_2 bradykinin receptor stably expressed in CHO cells apparently can stimulate both PLC and PLA_2.

We also report the cloning, expression, and pharmacological characterization of a genomic clone encoding the mouse B_2 bradykinin receptor. A comparison of the pharmacological profiles of cloned mouse and human B_2 bradykinin receptor homologs reveals that, although they have very similar properties with respect to binding peptide agonists, they differ significantly in their ability to bind some of the peptide antagonists synthesized by Vavrek and Stewart (14, 15). These results demonstrate that pharmacological variations in B_2 bradykinin receptors can result from minor alterations in the primary structure of species homologs. Furthermore, our data suggest that species differences, rather than additional B_2 bradykinin receptor subtypes, could account for some of the previously reported pharmacological diversity of this receptor.

Materials and Methods

Cloning and transfections. The gene encoding the mouse B_2 bradykinin receptor was isolated from a sCOS cosmid (31) library constructed from genomic DNA of ES cells derived from mouse strain J129, which was kindly provided by Dr. John Mudgett (Merck Research Lab, Rahway, N.J.). This library was plated on Colony/Plaque Screen hybridization transfer membrane (Dupont-NEN, Boston, MA) at a density of approximately 30,000 colonies/plate. Replicas of master plates were lysed and processed for hybridization using standard protocals (32). The DNA was UV-crosslinked to the membrane with a Stratalinker (Stratagene, La Jolla, CA). The filters were incubated overnight at 42° with probe in 50% formamide hybridization solution, [5× SSC, 5× Denhardt's solution, 100 μg/ml DNA (Sigma Chemical Co., St. Louis, MO)]. The probe, a 1.3-kb fragment containing the coding sequence of the human B_2 bradykinin receptor, was generated by random prime labeling (Boehringer-Mannheim, Indianapolis, IN) in the presence of [a-^{32}P]dCTP (3000 Ci/mmol). Filters were washed at a final stringency of 0.1× SSC, 0.1% SDS at 42°. Positives were rescreened to isolate single colonies. Cosmid clone 3A was chosen for further analysis. A 7.5-kB *Eco*RI by *Hind*III fragment was subcloned into pSP72 (Promega, Madison, WI). The coding sequence of the mouse B_2 bradykinin receptor was isolated by PCR (Perkin-Elmer, Norwalk, CT) using a Techne thermocycler, with the template being the *Eco*RI by *Hind*III genomic fragment in pSP72 with the 5′-primer CCTCAAGCTTCTCCTTCTGCTATCC and the 3′-primer AGCTCTAGACCCACCAATCCTCACAC. The PCR product was subcloned into the *Hind*III and *Xba*I sites of the eukaryotic expression vector pcDNAI-Neo (Invitrogen, San Diego, CA). The PCR product begins 99 bp upstream of the initiator methionine codon and ends 49 bp downstream of the stop codon. The DNA sequence analysis, using Sequenase (US Biochemical Corp., Cleveland, OH) of the subcloned PCR product, confirmed that the sequence was identical to that in the original genomic fragment.

Stable cell lines expressing the human B_2 bradykinin receptor were isolated by transfecting CHO cells by electroporation. Approximately 20 μg of a plasmid DNA, pcDNAI-Neo (Invitrogen), with an insert containing the cDNA encoding the human B_2 bradykinin receptor was mixed with 500 μg of Herring sperm DNA and added to 7×10^6 CHO cells resuspended in phosphate buffered saline in a volume of 0.7 ml. This mixture was electroporated to a 0.4-mm cuvette using a BioRad GenePulser (BioRad, Richmond, CA). Cells were plated and grown in DMEM high glucose media (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum for 2 days, at this point G418 (GIBCO) was added to a final concentration of 1 mg/ml. After 2 weeks of selection with G418, individual colonies were selected. Colonies were expanded, and the level of bradykinin receptor expression was determined by ligand binding assay. COS-7 cells were transfected as previously described (25).

Genomic Southerns. Genomic Southern blot analysis was performed according to established protocals (32), with the following modifications. Mouse genomic DNA (Promega) was digested with restriction enzymes at 37° overnight. Approximately 10 μg of digested genomic DNA was loaded per lane on a 1% agarose gel. After electrophoresis, the gel was transferred to HybondN membrane (Amersham Intl., Buckinghamshire, England) with a Stratagene Posiblot (Stratagene, La Jolla, CA) at 75 PSI for 1 hr and then was UV-crosslinked to the membrane. Membranes were hybridized in 50% formamide hybridization solution [5× SSC, 5 × Denhardt's solution, 100 μg/ml DNA, (Sigma)] at 42° overnight in the presence of probe. The random prime-labeled probes were a 1.2-kb *Bgl*II fragment isolated from the 7.5-kb *Eco*RI by *Hind*III fragment of mouse Cosmid 3A, and the 1.3-kb human B_2 bradykinin receptor cDNA fragment (25). Filters hybridized with the mouse probe were washed at 0.1 X SSC, 0.1% SDS at 42°. Filters hybridized with the human probe were washed 1 × SSC, 0.1% SDS at room temperature.

Northerns. Northern blot analysis was done by modifying established procedures (32). mRNA was prepared from mouse tissues using the PolyAtract mRNA isolation system (Promega). mRNA (approximately 5 μg/lane) was separated on a 1.2% agarose gel containing formaldehyde and was transferred to HybondN. Probes labeled by random priming were the mouse 1.2-kb *Bgl*II fragment described above and a 200-bp PCR product from mouse β-actin. Both probes were hybridized in 50% formamide hybridization solution at 50° overnight, and the filters were washed at 50° in 0.5× SSC, 0.1% SDS.

Binding assays. COS-7 cells were transfected, and membranes were prepared as previously described (25). Binding assays using intact cells were done by first pelleting the cells by centrifugation at 200 × *g* for 5 min. The cells were then resuspended in whole cell binding assay buffer (5 mM KCl, 20 mM HEPES, 100 mM *N*-methyl-D-glucamine, 0.1% BSA, 140 μg/ml bacitracin, 1 mM 1.0 phenanthroline, and 100 nM enalaprilat, pH 7.4). The cells were washed one time. Binding assays with 100-pm [^{3}H]BK using whole cells were performed at room temperature for 60 min in a final volume of 400 μl with 0.5×10^4 to 2×10^5 cells/tube. The assay was terminated and analyzed as previously described (25). Membrane binding assays were done as previously described (25), except that the buffer utilized in the assay was the whole cell binding assay buffer described above lacking the *N*-methyl-D-glucamine and KCl.

Phospholipid assays. CHO cells were labeled for 18 hr with 3 μCi/ml [^{3}H]*myo*-inositol in culture media. The cells were harvested using PBS-EDTA, centrifuged, and resuspended in phosphoinositide assay buffer (116 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM HEPES, 11 mM glucose, 50 μg/ml bacitracin, and 100 nM enalaprilat, pH 7.4) at 37°C. The cells were

washed one time with the same buffer and one time with buffer containing 10 mM LiCl. Approximately 1.5×10^6 cells were used in a final assay volume of 0.5 ml. Cells were incubated with agonist at 37° in a shaking water bath for 15 min, and reactions were terminated by the addition of 0.4 ml of an ice-cold solution of 12% perchloric acid, 3 mM EDTA, 1 mM diethylenetriaminepentaacetic acid. After 15 min on ice, the mixture was neutralized with approximately 0.32 ml of a solution containing 3 M KOH, 0.25 M 2-(*N*-morpholinoethanesulfonic acid, and 0.25 M 3-(*N*-morpholino) propanesulfonic acid. Tubes were centrifuged at $2000 \times g$ for 15 min, and 0.75 ml of the supernatant was diluted with 3 ml of H_2O. [^{3}H]Inositols were separated according to established protocols (33).

CHO cells were seeded at a density of 5×10^5 cells/well in 6-well culture plates and were labeled for 24 hr with 1 μCi/ml [^{3}H]arachidonic acid. Before assay each well was washed 4 times with 2 ml of the buffer described for the phosphoinositide assay. Incubations with agonist were performed for 15 min at room temperature in 2 ml of buffer. Incubations were terminated by removal of 1 ml of media, which was centrifuged 5 min at $1000 \times g$ to pellet any suspended cells. One-half ml of the supernatant was taken for the determination of [^{3}H]arachidonate released from the cells.

Phorbol ester was added to cells at 37° 30 min before the addition of agonist for assaying either phosphatidylinositol hydrolysis or arachidonic acid release as described above.

Results

Characterization of the human B_2 bradykinin receptor. A cDNA fragment encoding the human B_2 bradykinin receptor (25) was transfected into CHO cells. A clonal cell line was isolated that stably expresses a specific [^{3}H]BK binding site with a K_d of 209 ± 22 pM (mean ± standard error, $n = 6$) in intact cells and a maximal binding density (B_{max}) of approximately 140,000 receptors/cell. [^{3}H]BK binding was undetectable in untransfected CHO cells at ligand concentrations up to 10 nM.

The ability of the cloned human B_2 bradykinin receptor expressed in CHO cells to couple to a G protein was suggested by competition binding studies with several BK analogs in the presence and absence of guanine nucleotide in membrane preparations (Table 1). The approximately 4-fold increase in the binding affinity of the human B_2 receptor for [^{3}H]BK in membrane preparations relative to the intact cell was likely caused by the higher ionic strength in the intact cell binding assay and the presence of GTP in the whole cell. In the absence of GTP, agonists, but not antagonists, exhibited shallow displacement curves as demonstrated by Hill coefficients significantly less than one. Inclusion of GTP, guanosine 5′-*O*-(3-thiotriphosphate), or 5′-guanylylimidodiphosphate, but not ATP, resulted in an increase in the slope of the displacement curve and a Hill coefficient close to one. These properties are characteristic of G protein-coupled receptors in that high affinity agonist binding is sensitive to GTP analogs, whereas antagonist binding is not. The rank order of potency of agonists and antagonists determined for the human B_2 bradykinin receptor expressed in CHO cells (Table I) is very similar to that previously found upon transient expression of the cloned receptor in COS-7 cells (25).

The ability of the human B_2 bradykinin receptor expressed in CHO cells to stimulate phospholipid metabolism was examined. PLC activity was assayed by measuring the accumulation of [^{3}H]IP_1 in cells labeled with *myo*-[^{3}H]inositol. In CHO cells expressing the human B_2 bradykinin receptor, BK stimulated increases in [^{3}H]IP_1 formation with an EC_{50} of 0.5 nM and a maximal stimulation of 4- to 5-fold over the basal value (Fig. 1A). In control CHO cells 100 nM BK had no effect on phosphoinositol hydrolysis. These results indicate that the human B_2 bradykinin receptor, when expressed in CHO cells, effectively couples to a G protein that activates PLC. To test for PLA_2 activation, cells were labeled with [^{3}H]arachidonic acid, and the release of radioactivity in response to BK was measured. BK increased [^{3}H]arachidonic acid release in CHO cells expressing the human B_2 bradykinin receptor in a dose-dependent manner, with an EC_{50} of 0.7 ± 0.3 nM ($n = 3$) and a maximal stimulation that was 6-fold over the basal level (Fig. 1B). BK had no effect on the release of [^{3}H]arachidonate from control CHO cells. The B_2 bradykinin receptor antagonist Hoe 140 behaved as a competitive antagonist of both BK-stimulated PLC and PLA_2 activation.

The ability of the cloned human B_2 bradykinin receptor expressed in CHO cells to activate both arachidonic acid release and PLC suggests that one receptor acts through two different signaling pathways. However, as discussed previously, it is possible that arachidonic acid release is a direct consequence of PLC activation (11, 12). To test whether arachidonic acid release is independent of PLC activation in these cells, the cells were treated with phorbol ester before stimulation with BK (Fig. 2). Relative to untreated controls, short term treatment with phorbol ester before agonist addition reduced the maximal level of BK-stimulated [^{3}H]IP_1 accumulation to 55 ± 6% ($n = 3$) of the maximum. In contrast, the release of [^{3}H]arachidonic acid was stimulated by 132 ± 10% ($n = 3$) compared to control values. Thus, it appears that the cloned human B_2 bradykinin

TABLE 1

Inhibition of [^{3}H]BK binding to membrane preparations from CHO cells expressing the human B_2 bradykinin receptor in the absence and presence of GTP

Results are presented as means ± standard error from at least three independent determinations for each peptide.

Peptide	IC_{50} (−GTP)	N_H[a]	IC_{50} (+GTP)[b]	N_H
	pM		pM	
Agonists				
BK	52 ± 6	0.51 ± 0.07	145 ± 13	0.98 ± 0.04
Lys-BK	117 ± 20	0.57 ± 0.04	376 ± 31	1.10 ± 0.09
Met,Lys-BK	276 ± 34	0.55 ± 0.13	993 ± 68	0.90 ± 0.06
Antagonists				
Hoe 140	38 ± 8	0.95 ± 0.03	29 ± 7	1.01 ± 0.04
[D-Arg^0,Hyp^{2,3},Thi^{5,8},D-Phe^7]BK	6220 ± 1380	0.91 ± 0.04	8800 ± 1696	1.01 ± 0.09
[D-Arg^0,Hyp^3,D-Phe^7]BK	15100 ± 2600	0.88 ± 0.03	23500 ± 3000	0.92 ± 0.03

[a] N_H: Hill coefficient.
[b] The concentration of GTP added was 100 μM.

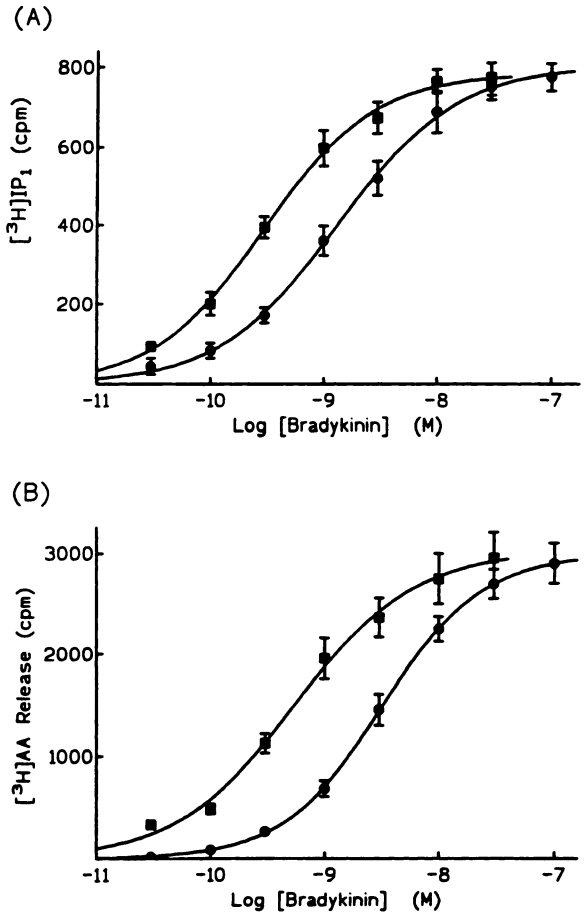

Fig. 1. A, BK stimulation [^{3}H]IP$_1$ accumulation in CHO cells expressing the human B_2 bradykinin receptor. The assay was performed as described in Materials and Methods. The curves represent the absence (■) and presence (●) of 3 nM Hoe 140. B, BK stimulation of [^{3}H]arachidonic acid release. The symbols and conditions are the same as in *A*.

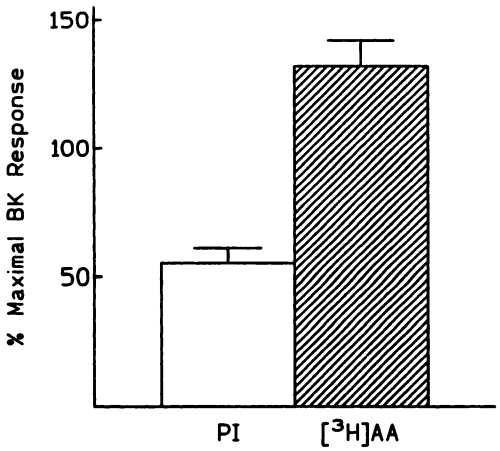

Fig. 2. Phorbol ester treatment of CHO cells expressing the human B_2 bradykinin receptors. The amount of [^{3}]IP$_1$ accumulation (*open bar*) and [^{3}H]arachidonic acid release (*hatched bar*) in cells treated with phorbol 12-myristate 13-acetate 30 min before BK addition as described in Materials and Methods. These values are expressed relative to the response to 10 nM BK (100%) in untreated cells. The basal values for a representative experiment were approximately 180 ± 11 cpm for phosphotidylinositol hydrolysis and approximately 461 ± 87 cpm for arachidonic acid release.

receptor is capable of activating PLA_2 to release arachidonic acid independently of PLC activation.

The activity of the BK analog [Thi^{5,8},D-Phe^7]BK at the cloned human B_2 bradykinin receptor was assessed. This peptide has been reported to act as a partial agonist on B_2 bradykinin receptors in smooth muscle and as a full agonist in neuronal tissue, suggesting the possibility of B_2 bradykinin receptor subtypes (18). At the cloned human B_2 bradykinin receptor, [Thi^{5,8},D-Phe^7]BK behaved as a weak partial agonist. At a concentration of 30 μM, the maximal activation of phosphatidylinositol hydrolysis by [Thi^{5,8},D-Phe^7]BK was 15% of the maximal BK response (data not shown). These data indicate that the cloned receptor is similar to that found in smooth muscle, consistent with its reported tissue distribution (25) and with the pharmacological characterization of the rat homolog (24).

Cloning of the mouse B_2 bradykinin receptor gene. A genomic clone encoding the mouse B_2 bradykinin receptor was isolated by hybridization screening of a cosmid library prepared from ES cells derived from mouse strain J129 using the human B_2 bradykinin receptor cDNA as a probe (25). Cosmid 3A, containing 35 kb of mouse genomic DNA, was isolated, and an approximately 7.5-kb *Hin*dIII by *Eco*RI fragment containing the DNA encoding the mouse B_2 bradykinin receptor was subcloned. DNA sequence analysis of a portion of the *Hin*dIII by *Eco*RI fragment (Fig. 3) revealed an uninterrupted open reading frame of 1101 bp that is 92% identified to the coding sequence of the rat B_2 bradykinin receptor (24), 84% identical to the human B_2 bradykinin receptor (25–27), and 98% identical to a recent mouse B_2 bradykinin receptor isolated by McIntyre *et al.* (34). The tissue distribution of the message encoding the mouse B_2 bradykinin receptor was examined by Northern blot analysis (Fig. 4). The message was most abundant in uterus, but was also present in ileum, kidney, heart, and brain, which was similar to previous studies with the rat and human B_2 bradykinin receptors (24, 25).

The coding sequence for the mouse B_2 bradykinin receptor lacks introns, as does the human B_2 bradykinin receptor (26). However, a potential intron was found in the 5′-untranslated region of the mouse gene 8 bp upstream of the initiator methionine codon; this is the same position as seen with a mouse B_2 bradykinin receptor recently isolated by McIntyre *et al.* (34). A comparison of the human B_2 bradykinin receptor genomic and cDNA clones also indicated the presence of an intron in the 5′-untranslated sequence (26). A similar gene structure has been proposed for the human angiotensin II type I receptor gene (35). Further experiments are needed to determine the role of the introns in the 5′-untranslated region of these genes.

The high degree of DNA and amino acid sequence identity suggests that the clone described here is the murine homolog of the B_2 bradykinin receptor. This hypothesis was supported by genomic Southern blot analysis. Mouse genomic DNA digested with either *Eco*RI or *Hin*III was probed with either a *Bgl*II fragment of the mouse B_2 bradykinin receptor clone or the human B_2 bradykinin receptor cDNA. The human and mouse probes both hybridized to a single restriction fragment of the same size, an *Eco*RI fragment of 8 kb and a *Hin*dIII fragment of 7.5 kb (Fig. 5). These data indicate that the mouse gene isolated here is the sequence in the mouse genome that has the highest degree of similarity to the human B_2 bradykinin receptor.

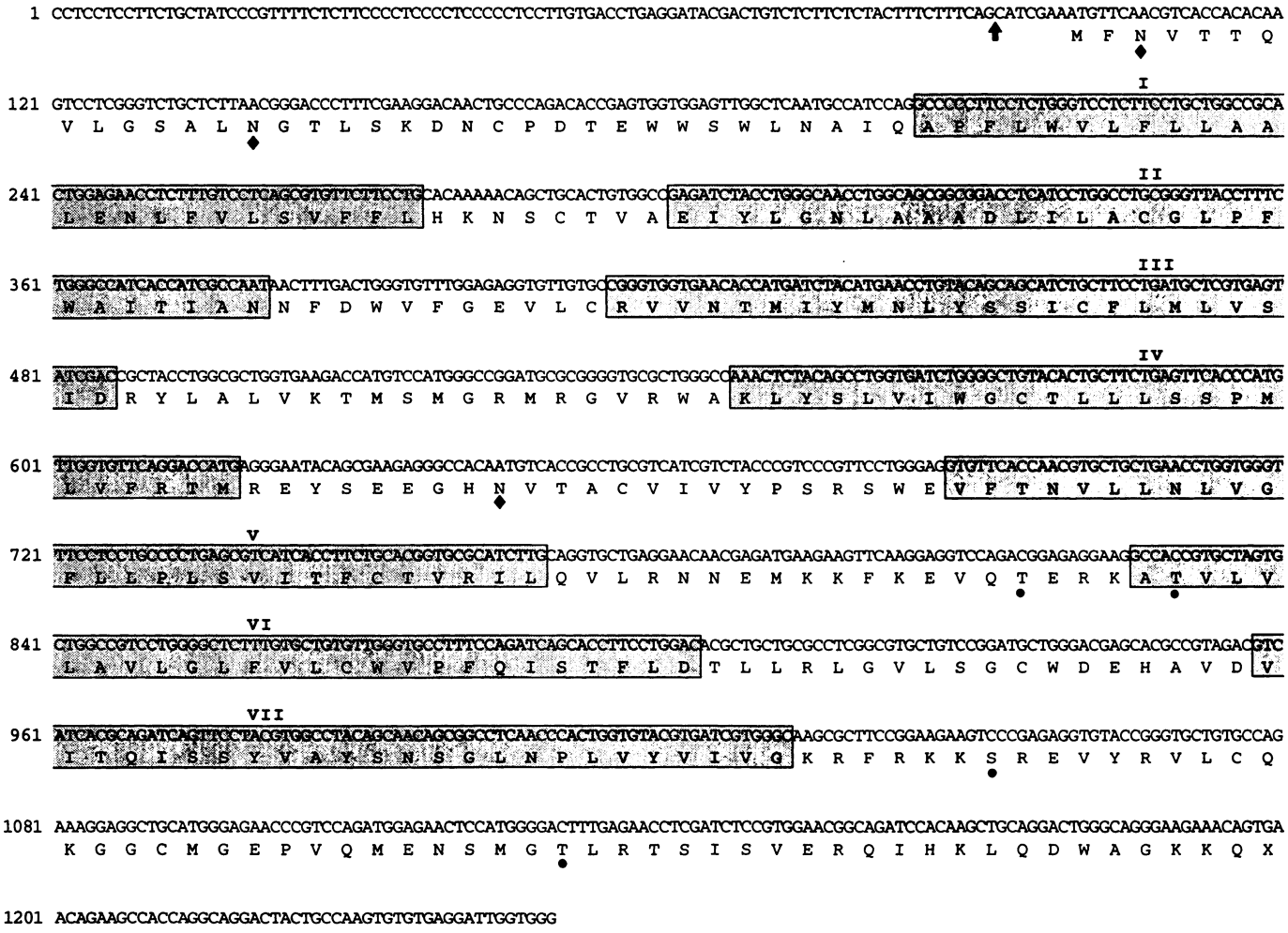

Fig. 3. DNA sequence and the corresponding amino acid sequence of the mouse B_2 BK receptor. (↑) site of potential intron/exon boundary, (♦) sites of potential N-linked glycosylation, (●) sites of potential phosphorylation by cAMP-dependent protein kinase or protein kinase C.

After completion of these studies, the cloning of a murine bradykinin receptor was reported by McIntyre *et al.* (34). The gene described in that publication appears to be nearly identical to the one we have isolated. However, the pharmacological properties of the expressed receptors differ (see below). The predicted amino acid sequence of these two gene products is 98% identical, with the following amino acids reported in McIntyre *et al.* (34) differing in our sequence: A99V, P100F, L113Y, L114M, M118I, A206V, S248L, and A272R. In nearly all of the cases where the two sequences differ, the amino acid that we report is identical to that of the rat B_2 bradykinin receptor. A comparison of 250 bp of genomic sequence 5′ to the initiator ATG reported by McIntyre *et al.* (34) with the gene isolated here indicates a nucleotide sequence identity of 98.8% in the untranslated region. Thus, it appears likely that these two clones represent the same murine allele.

Pharmacological characterization of the murine B_2 bradykinin receptor. The pharmacological properties of the murine B_2 bradykinin receptor were analyzed by heterologous expression in a mammalian cell line lacking any endogenous bradykinin receptor, COS-7. Saturation binding to whole COS cells transiently expressing the mouse B_2 receptor indicated a single high affinity site with a K_d for [^{3}H]BK of 200 ± 30 pM and a B_{max} that ranged from 650 to 1700 fmol/mg of protein. The binding of BK to membranes was reduced by increasing concentrations of GTP, guanosine 5′-*O*-(3-thiotriphosphate), or 5′guanylylimidodiphosphate (data not shown). This decrease of BK binding in the presence of guanine nucleotides suggests that the mouse receptor is coupled to a G protein in the COS-7 cells.

Competitive binding experiments using 100 pM [^{3}H]BK revealed that the expressed mouse B_2 bradykinin receptor possessed a high (subnanomolar) affinity for the B_2 receptor agonists BK and kallidin (Table 2). In contrast, the receptor had a very low affinity for the B_1 bradykinin receptor agonists [des-Arg^9]BK (IC_{50} = 6 μM) and [des-Arg^{10}]kallidin (IC_{50} = 25 μM) (Table 2 and Fig. 6). Therefore, the mouse bradykinin receptor reported here exhibited the classic pharmacological properties of a B_2 bradykinin receptor. These results differed dramatically from the pharmacological properties of the mouse bradykinin receptor recently isolated and characterized by McIntyre *et al.* (34), in which both a high affinity (subnanomolar) site and a low affinity (114 ± 67 nM) site for [des-Arg^9]BK were detected. To further examine the potential for any B_1 receptor properties of the murine bradykinin receptor clone reported here, we performed binding assays with [^{3}H][des-Arg^{10}]kallidin. No specific binding was detected at concentrations of up to 30 nM [^{3}H][des-Arg^{10}]kallidin (data not shown). We also examined

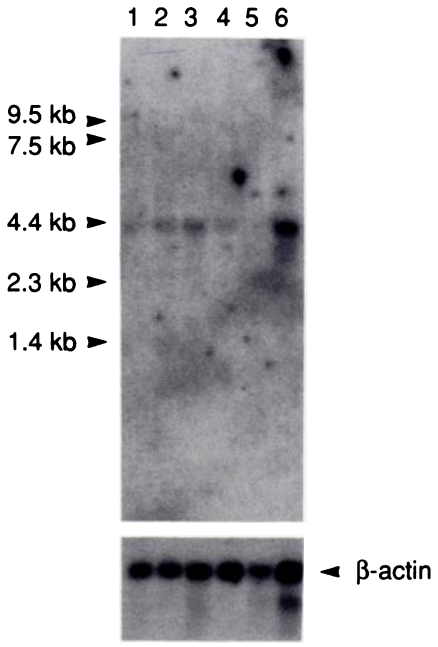

Fig. 4. Northern blot of tissues expressing B_2 bradykinin receptor mRNA. *Top panel* is an autoradiogram of a Northern blot in which approximately 5 μg of mRNA/*lane* was separated on a formaldehyde agarose gel, transferred to a filter, and probed with the mouse B_2 bradykinin receptor *Bgl*II fragment as described in Materials and Methods. *Lanes*: *1*, brain; *2*, heart; *3*, ileum; *4*, kidney; *5*, liver; *6*, uterus. *Lower panel* is the same filter probed with a mouse β-actin probe as described in Materials and Methods. Molecular weight standards are as indicated.

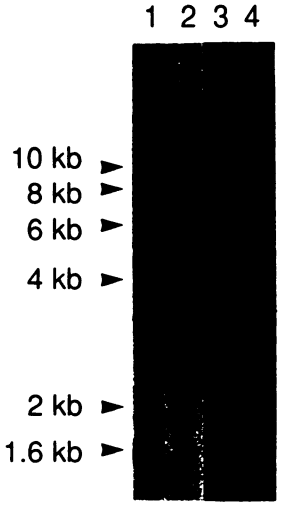

Fig. 5. Genomic Southern of mouse genomic DNA probed with either mouse or human B_2 bradykinin receptor probes. Approximately 10 μg of mouse genomic DNA was digested with *Eco*RI (*lanes 1* and *3*) or *Hin*dIII (*lanes 2* and *4*). The filter containing *lanes 1* and *2* was probed with the mouse B_2 bradykinin receptor *Bgl*II probe, and the filter containing *lanes 3* and *4* was probed with the human B_2 bradykinin receptor cDNA as described in Materials and Methods.

the ability of Hoe 140 to displace [^{3}H]BK from the murine bradykinin receptor (Fig. 6). A single high affinity site of 0.23 nM was detected for Hoe 140. This result is in contrast to results obtained by McIntyre *et al.* (34), in which both a high affinity site (picomolar) and a low affinity site (micromolar) for Hoe 140 were detected. Further experiments will be necessary to determine the basis for the differences in the pharmacology reported for these two systems.

TABLE 2

Comparison of peptide binding properties of cloned human and mouse B_2 bradykinin receptors

The IC_{50} was determined from the displacement of 100 pM [^{3}H]BK in a whole cell binding assay as described in Materials and Methods.

	IC_{50}	
	Human	Mouse
	nM	
Agonists		
BK	0.54	0.48
Kallicin (lys-BK)	0.63	0.52
Met,Lys-BK	2.1	3.6
[Tyr^8]BK	10.7	8.1
[des-Arg^9]BK	8,100	6,400
[des-Arg^{10}]kallidin	>30,000	25,000
Antagonists		
Hoe 140	0.41	0.23
[D-Arg^0,Hyp^{2,3},Thi^{5,8},D-Phe^7]BK	98	1.2
[D-Arg^0,Hyp^3,D-Phe^7]BK	120	2.1
[Thi^{5,8},D-Phe^7]BK	470	89
[des-Arg^9,Leu^8]BK	>30,000	>30,000
[des-Arg^{10},Leu^9]kallidin	>30,000	>30,000

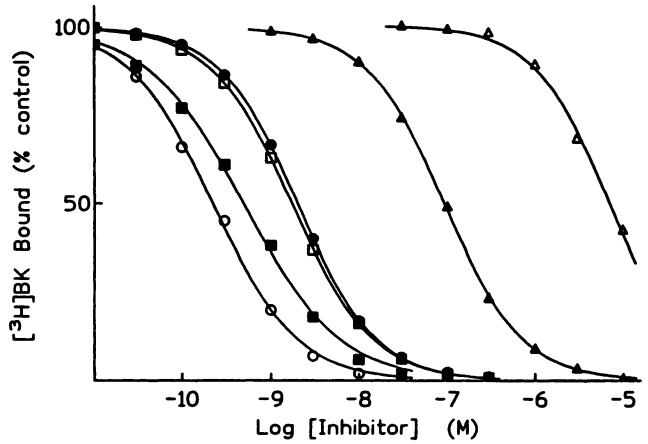

Fig. 6. Displacement curves of 100 pM [^{3}H]BK binding to the cloned mouse B_2 bradykinin receptor expressed in COS-7 cells. [^{3}H]BK binding was determined using intact cells as described in Materials and Methods in the presence of increasing concentrations of compounds. (○) Hoe 140; (■) BK; (□) [D-Arg^0,Hyp^{2,3},Thi^{5,8},D-Phe^7]BK; (●) [D-Arg^0,Hyp^3,D-Phe^7]BK; (▲) [Thi^{5,8},D-Phe^7]BK; and (△) [des-Arg^9]BK.

The ability of the mouse B_2 bradykinin receptor to bind a number of peptide antagonists was analyzed by competition binding (Fig. 6). Interestingly, a comparison of the cloned mouse B_2 receptor expressed in COS cells and the human receptor B_2 receptor expressed in CHO cells indicates that the mouse receptor has a significantly higher affinity for several of these compounds (Table 2). Several experiments comparing the pharmacology of the human B_2 receptor expressed in COS cells to that of the receptor expressed in CHO cells have indicated that there are no pharmacological differences attributable to the cell background (R. Ransom, unpublished observations). Relative to the human B_2 bradykinin receptor, the mouse receptor has approximately an 80-fold greater affinity for [D-Arg^0,Hyp^{2,3},Thi^{5,8},D-Phe^7]BK and a 60-fold greater affinity for [D-Arg^0,Hyp^3,D-Phe^{7}BK (Table 2). Only a 5-fold increase in affinity for the mouse receptor is observed for the related peptide [Thi^{5,8},D-Phe^7]BK. In contrast, the mouse and human B_2 bradykinin receptors show similar affinities for the peptide antagonist Hoe 140.

Discussion. To investigate the potential for pharmacologically distinct subtypes of the B_2 bradykinin receptor, we have expressed and characterized the receptors from two different species. It has been proposed that one subtype of B_2 bradykinin receptors could be responsible for activation of PLC, and a second subtype could be responsible for activation of PLA_2 (4–6). The present study suggests that the human B_2 bradykinin receptor expressed in CHO cells is capable of activating both phosphatidylinositol hydrolysis and arachidonic acid release. The BK-stimulated release of arachidonic acid could be mediated by a PLC-dependent pathway (11). To begin to dissociate the PLC pathway from the release of arachidonic acid, the effect of phorbol ester treatment on the BK-mediated activation of these two signaling pathways was examined. Phorbol ester treatment has been shown to interfere with G protein activation of PLC, possibly by a protein kinase C-mediated phosphorylation of the G protein that reduces the ability to activate PLC (36,37). Short-term treatment of CHO cells expressing the cloned human B_2 bradykinin receptor with phorbol ester inhibits BK-stimulated phosphatidylinositol hydrolysis and stimulates BK-mediated arachidonic acid release. These results suggest that the B_2 bradykinin receptor can couple to two independent second messenger pathways and implies that a single bradykinin receptor has the ability to interact with at least two different G proteins. Alternatively, the activation of arachidonic acid release may occur in a PLC-dependent pathway involving PKC-mediated influx of Ca^{2+}, which in turn activates PLA_2 (9, 10). Although our data does not address this possibility, our results obviate the need to invoke additional bradykinin receptor subtypes to explain the activation of both PLC and PLA_2. In addition, these results raise the interesting possibility that the actions of a B_2 bradykinin receptor in different cell types are affected by the complement of G proteins in a particular cell.

The differences in the affinities of BK analogs synthesized by Vavrek and Stewart (14, 15) for the bradykinin receptor(s) in different tissue preparations have been suggestive of further bradykinin receptor subtypes (5, 16, 18–21, 38). In some cases the pharmacology of bradykinin receptors from different species was compared (5, 17, 20, 21), making it difficult to discern the contribution of species differences to the observed bradykinin receptor pharmacology. To determine whether species divergence contributes significantly to these pharmacological differences, we compared the pharmacological profiles of the cloned human and mouse B_2 bradykinin receptors. Both the human and mouse B_2 bradykinin receptors bind agonists with the same affinity and rank order of potency. This conservation of function probably reflects the selective pressure of maintaining a physiological response to endogenous kinins. However, there are significant pharmacological differences in the binding of certain synthetic peptide antagonists, particularly [D-Arg^0,$Hyp^{2,3}$,$Thi^{5,8}$,D-Phe^7]BK and [D-Arg^0,Hyp^3,D-Phe^7]BK, to the human and mouse receptors. Although these data do not exclude the potential for further bradykinin receptor subtypes, they do demonstrate that species divergence can explain, at least in part, the differential pharmacology that has been observed with synthetic peptides. In addition, these results emphasize the need to assess the ability of synthetic compounds to act on human receptors when developing B_2 bradykinin receptor antagonists as therapeutic agents for human health.

The mouse B_2 bradykinin receptor that we have isolated has a very high degree of similarity (98.9% identity over 1415 nucleotides) to the gene recently isolated by McIntyre *et al.* (34). However, the pharmacology of the receptors encoded by the two clones is quite different. The mouse bradykinin receptor characterized in this report exhibits the "classical" pharmacological properties of a B_2 receptor, whereas the mouse bradykinin receptor characterized by McIntyre *et al.* (34) exhibits a mixed B_1 and B_2 pharmacology. The two clones differ by eight amino acids. The amino acids that differ lie in regions of the receptor that might have the potential to affect its ligand binding properties; four of these differences lie in putative transmembrane spanning domains, and the other four differences lie in the extracellular domains. However, the lack of changes in putative intracellular domains argues against the possibility of the appearance of B_1 pharmacological properties caused by a post-translational modification mediated by a cytoplasmic protein. The eight amino acid differences could be a consequence of different bradykinin receptor genes, allelic variation between strains of mice, or DNA sequencing artifacts. We believe that it is unlikely that these clones represent different genes, because of the high degree of sequence identity throughout the coding region, the high degree of nucleotide sequence identity in putative intron sequences, and the results of genomic Southerns that indicate that there is a single murine gene with high homology to the DNA encoding a B_2 bradykinin receptor. We have examined the pharmacology of the B_2 bradykinin receptor in membranes prepared from several tissues from a different strain of mouse, Swiss Webster, and found the same "classical" B_2 pharmacology as was observed for the cloned murine B_2 receptor expressed in COS cells. (R. Ransom, unpublished observations). Further experiments will be necessary to determine whether the reported amino acid differences represent allelic variation between mouse strains and whether any of these substitutions are responsible for the pharmacological differences observed between the two murine bradykinin receptor clones.

Alternatively, the differential pharmacology reported for these two murine B_2 bradykinin receptor clones could be a consequence of differences in experimental conditions and procedures. We have addressed several of these differences, including the concentration of radioligand in displacement experiments (100 pM versus 1 nM), the temperature of binding assays (room temperature versus 4°), and the buffering conditions (pH 7.4 versus 6.5). Altering our experimental procedures to match those presented by McIntyre *et al.* (34) had no effect on the pharmacology profile shown in Fig. 6. Further work is necessary to investigate the contributions of various structural and procedural differences to the observed pharmacology.

In conclusion, a direct comparison of the expressed human and murine B_2 bradykinin receptors shows significant species differences in their interaction with synthetic peptide antagonists. These differences are sufficient to account for some of the previous reports of multiple subtypes of the B_2 bradykinin receptor. Also, we have no evidence to indicate that B_1 receptor pharmacology can be generated by species differences in B_2 bradykinin receptors, suggesting that the gene encoding the B_1 bradykinin receptor remains to be cloned. Expression and pharmacological characterization of cloned bradykinin receptors will be necessary for a definitive characterization of bradykinin receptor subtypes.

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