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CLONING AND PHARMACOLOGICAL CHARACTERIZATION OF A HUMAN BRADYKININ (BK-2) RECEPTOR

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A human BK-2 bradykinin receptor was cloned from the lung fibroblast cell line CCD-16Lu. The cDNA clone encodes a 364 amino acid protein that has the characteristics of a seven transmembrane domain G-protein coupled receptor. The predicted amino acid sequence of the human BK-2 receptor is 81% identical to the smooth muscle rat BK-2 receptor (1). Transfection of the human BK-2 receptor cDNA into COS-7 cells results in the expression of high levels of specific BK binding sites. Saturation binding analysis indicates that the human BK-2 receptor expressed in COS-7 cells binds BK with a K_D of 0.13 nM. Pharmacological characterization of the expressed BK receptor is consistent with the cDNA encoding a receptor of the BK-2 subtype. The BK-2 receptor antagonist Hoe 140 (2), D-Arg^0[Hyp^3, Thi^5, D-Tic^7, Oic^8]BK has a high affinity (IC_{50}=65 pM) for the cloned human receptor. The tissue distribution of the human BK-2 receptor was analyzed by competitive PCR with human tissue cDNA and is similar to that determined for the BK-2 receptor in the rat.

Bradykinin (BK) is a nonapeptide hormone (RPPGFSPFR) that is an important mediator of the physiological response to injury and trauma (3,4). The local release of BK causes pain, plasma extravasation, and vasodilation, as well as smooth muscle contraction. BK may play a role in the pathogenesis of asthma, since it appears to be a mediator of hyperallergenic bronchoconstriction in asthmatic patients (5,6). Bradykinin receptors exert their effect by coupling to G-proteins and activating phospholipid metabolism by phospholipase C or phospholipase A_2 (7).

Pharmacological studies suggest the existence of several subtypes of bradykinin receptors. The BK-1 receptor subtype preferentially binds the agonist Des-Arg^9 BK, a natural proteolytic product of BK generated by removal of the C-terminal Arg (3,4). The precise physiological role of BK-1 receptors is unclear. Most of the physiological effects of BK appear to be mediated through the BK-2 receptor, which is characterized by a high affinity for BK and a low affinity for Des-Arg^9 BK. Two subtypes of the BK-2 receptor,

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a smooth muscle and a neuronal, have been postulated from pharmacological studies (8-11). The smooth muscle BK-2 receptor has a lower affinity for bradykinin and a higher affinity for the bradykinin analogue [Thi^{5,8}, D-Phe^{7}]BK than the neuronal subtype. Furthermore, [Thi^{5,8}, D-Phe^{7}]BK acts as an antagonist at the smooth muscle subtype and as an agonist at the neuronal subtype. A pulmonary subtype, BK-3, has been described in guinea pig lung and trachea and is characterized by a reduced affinity for some BK-2 antagonists (12).

The cDNA for a smooth muscle subtype of the rat BK-2 receptor was recently reported (1). Analysis of the amino acid sequence indicates that the BK receptor is a member of the superfamily of seven transmembrane domain receptors that couple to G-proteins (13,14). In the present report we describe the cloning, pharmacological characterization, and tissue distribution of a human BK-2 receptor from a human lung fibroblast cell line, CCD-16Lu.

Materials and Methods

PCR. Reverse PCR with *Thermus thermophilus* DNA polymerase (Perkin Elmer, Cetus) was performed using human uterine mRNA (Clonetech). Annealing of the reverse primer and reverse transcription was done by incubating the PCR reaction minus the forward primer for 10 min. each at room temperature, 42° C, and 60°C. PCR was performed for 35 cycles of 1 min. each 94°C, 40°C, and 60°C. Two rounds of PCR were performed using degenerate primers with the restriction site adapters, NotI on the forward primer CGGCGGCCGCGCNAAYAAYTTYGAYTGG and XhoI on the reverse primer CGCTCGAGCGYTTYTTYTCNGTYTG. These primers were removed using a Centricon 30 (Amicon) and a third round of PCR was performed using a second pair of nested primers (with restriction site adapters) GCGCGGCCGCAAYACNATGATHTA and CGCTCGAGACYTCYTTRAAYTTYTTCAT. PCR products were analyzed on a 3.5% Nusieve (FMC Bioproducts) gel. A 386 nucleotide PCR product was subcloned into pBluescript (Stratagene) and characterized by DNA sequence analysis.

Isolation of cDNA. mRNA was isolated from the human cell line CCD-16Lu (CCL 204 obtained from the ATCC) using the Invitrogen Fast Track system. cDNA was prepared from 4 µg mRNA using the BRL cDNA Synthesis System. BstXI adapters were added and the cDNA ligated into pcDNA II (Invitrogen). Colonies were plated at a density of 30,000 colonies per plate and transferred to duplicate Durulose-UV (Stratagene) filters using standard techniques (15). The probe utilized for screening was generated by random primed synthesis (Boehringer Mannheim Biochemicals) of the 386 nucleotide PCR product described above in the presence of [α-^{32}P]dCTP (400 Ci/mmole) . Duplicate filters were hybridized with 1.5 x 10^{6} cpm/ml [^{32}P] labeled probe in 50% formamide hybridization solution, [5X SSC, 5X Denhardt's, 100 µg/ml DNA, (Sigma)], at 50°C for 12 hours. The filters were washed at high stringency in a final wash of 0.1X SSC, 0.1% SDS at 60°C. Positive colonies were rescreened as before. Plasmid was isolated from second round positives and the DNA sequence was determined by double strand DNA sequencing using the Sanger Method with Sequenase (US Biochemicals).

Transfection and Membrane Preparation. Cos-7 cells were transfected using Lipofectin (BRL) with 50 µg/10^{7} cells of the BK-2 receptor cDNA subcloned into the eukaryotic expression vector pcDNA I-Neo (Invitrogen). Cells were harvested at 72 hours and membranes prepared by scraping the cells in phosphate buffered saline and centrifuging 10 min. at 500 x g. The cell pellet was resuspended and homogenized with a Polytron in 20 mM N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES, pH 6.8 at room temperature) buffer containing 1 mM 1,10 phenanthroline. The homogenate was centrifuged 15 min. at 48,000 x g and the resulting pellet rehomogenized in fresh buffer and centrifuged as before. The final membrane pellet was resuspended in assay buffer (TES plus 0.1% protease free bovine serum albumin, 5 µM MK-422 (enalaprilat) and 140 µg/ml bacitracin using a motor-driven Teflon-glass tissue homogenizer. Protein

determinations were performed by the method of Bradford (16) using bovine IgG as the standard.

Binding Assay. Membrane binding assays were done according to published protocols (17). [^{3}H]BK (at the concentrations indicated in the Fig. legends) was incubated for 60 min. at 25°C with approximately 50µg membrane protein in a volume of 1 ml. The assay was terminated by filtration over Whatman GF/B filters presoaked for 3 hr. in 0.1% polyethyleneimine using a Brandel M-24 cell harvester. The tubes were rinsed two times with 4 ml ice-cold 10 mM TES and the filter bound radioactivity was quantitated by liquid scintillation counting. Nonspecific binding was determined by performing incubations in the presence of 1 µM BK and represented less than 5% of the total binding at 100 pM [^{3}H]BK. Competition binding experiments, in the presence of 100 pM [^{3}H]BK, were done with varying concentrations of the test compound, as described in the Fig. legends . Competition and saturation experiments were analyzed using EBDA-Ligand (18) or GraphPAD InPlot (GraphPAD Software).

Competitive PCR. Human mRNA (2.5 µg) from brain, heart, lung, kidney, pancreas, testis and uterus (Clonetech) was digested with 10 units RNase free DNase (Stratagene) for 30 min. at 37°C to remove genomic DNA. Random primed cDNA was prepared according to the BRL cDNA synthesis system. The cDNA was phenol chloroform extracted and ethanol precipitated in the presence of ammonium acetate. To adjust the amounts of cDNA from different tissues, the ability to generate a 206 nucleotide PCR product using the β-actin primers, the forward primer CCTTCCTGGGCATGGAGTC CTG and the reverse primer GGAGCAATGATCTTGATCTTC (19) was analyzed by agarose gel electrophoresis at 20,24,26,28, and 30 cycles of 1 min. each at 94°C, 55°C, and 72°C.

The BK-2 receptor PCR primers, the forward primer GCTCTACAGCTTGGTG ATCTGG and the reverse primer AGCATGTTGGTGAACACTTCC generate a 155 nucleotide product. The 155 nucleotide rat competitor was obtained by reverse PCR from rat lung mRNA using these BK-2 primers. Competitive PCR was done for 45 cycles at 1 min. each 94°C, 55°C, and 72°C in a volume of 50 µl with a constant amount of human tissue cDNA (adjusted based on the results of the β-actin experiment described above) in the absence of competitor or in the presence of 3 concentrations (approximately .006 amoles, .001 amoles, and .0006 amoles) of the rat competitor. PCR products were then digested with an enzyme that cuts the rat but not the human PCR product (AccI) and the restriction digests were analyzed on a 4% Nusieve Gel (FMC Bioproducts). In order to verify that the PCR products were derived from the human BK-2 receptor described in this report, the reaction containing no competitor was digested with SacI, an enzyme that recognizes the human but not the rat PCR product. In PCR reactions performed for 45 cycles as described above on an aliquot of DNase treated mRNA, no bands were detected in the absence of a reverse transcriptase.

Results and Discussion

A cDNA clone encoding a human BK-2 receptor was isolated by a combined approach involving PCR and cDNA library screening. Reverse PCR from human uterus mRNA using degenerate primers based on the amino acid sequence of the rat BK-2 receptor (1) was first used to obtain a 386 nucleotide partial cDNA for a human BK-2 receptor. This partial cDNA, encompassing nucleotides 540 to 926 (Fig. 1), is 87% identical to the corresponding region of the rat BK-2 cDNA (nucleotides 703 to 1089). The 386 nucleotide partial cDNA was then used to screen, by nucleic acid hybridization, a cDNA library prepared from CCD-16Lu cells. CCD-16Lu is a human fibroblastic lung cell line that contains 20,000-30,000 BK receptors per cell (R.Ransom, unpublished data). Two positive clones, CCD-16-2 (0.78 kb) and CCD-16-6 (1.1 kb) were isolated from the CCD-16Lu library and characterized by DNA sequence analysis. The DNA sequence of clone CCD-16-2 indicates that this clone begins in the 5' untranslated region

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1    ctccgaggaggggtggggacggtcctgacggtggggacatcaggctgccccgcagtacca
61   gggagcgacttgaagtgcccatgccgcttgctccgggagaagcccaggtgtggcctcact
121  cacatcccactctgagtccaaatgttctctccctggaagatatcaatgtttctgtctgtt
181  cgtgaggactccgtgcccaccacggcctctttcagcgccgacATGCTCAATGTCACCTTG
                                              M  L  N  V  T  L

241  CAAGGGCCCACTCTTAACGGGACCTTTGCCCAGAGCAAATGCCCCCAAGTGGAGTGGCTG
     Q  G  P  T  L  N  G  T  F  A  Q  S  K  C  P  Q  V  E  W  L

301  GGCTGGCTCAACACCATCCAGCCCCCCTTCCTCTGGGTGCTGTTCGTGCTGGCCACCCTA
     G  W  L  N  T  I  Q  P  P  F  L  W  V  L  F  V  L  A  T  L

361  GAGAACATCTTTGTCCTCAGCGTCTTCTGCCTGCACAAGAGCAGCTGCACGGTGGCAGAG
     E  N  I  F  V  L  S  V  F  C  L  H  K  S  S  C  T  V  A  E

421  ATCTACCTGGGGAACCTGGCCGCAGCAGACCTGATCCTGGCCTGCGGGCTGCCCTTCTGG
     I  Y  L  G  N  L  A  A  A  D  L  I  L  A  C  G  L  P  F  W

481  GCCATCACCATCTCCAACAACTTCGACTGGCTCTTTGGGGAGACGCTCTGCCGCGTGGTG
     A  I  T  I  S  N  N  F  D  W  L  F  G  E  T  L  C  R  V  V

541  AATGCCATTATCTCCATGAACCTGTACAGCAGCATCTGTTTCCTGATGCTGGTGAGCATC
     N  A  I  I  S  M  N  L  Y  S  S  I  C  F  L  M  L  V  S  I

601  GACCGCTACCTGGCCCTGGTGAAAACCATGTCCATGGGCCGGATGCGCGGCGTGCGCTGG
     D  R  Y  L  A  L  V  K  T  M  S  M  G  R  M  R  G  V  R  W

661  GCCAAGCTCTACAGCTTGGTGATCTGGGGGTGTACGCTGCTCCTGAGCTCACCCATGCTG
     A  K  L  Y  S  L  V  I  W  G  C  T  L  L  L  S  S  P  M  L

721  GTGTTCCGGACCATGAAGGAGTACAGCGATGAGGGCCACAACGTCACCGCTTGTGTCATC
     V  F  R  T  M  K  E  Y  S  D  E  G  H  N  V  T  A  C  V  I

781  AGCTACCCATCCCTCATCTGGGAAGTGTTCACCAACATGCTCCTGAATGTCGTGGGCTTC
     S  Y  P  S  L  I  W  E  V  F  T  N  M  L  L  N  V  V  G  F

841  CTGCTGCCCCTGAGTGTCATCACCTTCTGCACGATGCAGATCATGCAGGTGCTGCGGAAC
     L  L  P  L  S  V  I  T  F  C  T  M  Q  I  M  Q  V  L  R  N

901  AACGAGATGCAGAAGTTCAAGGAGATCCAGACGGAGAGGAGGGCCACGGTGCTAGTCCTG
     N  E  M  Q  K  F  K  E  I  Q  T  E  R  R  A  T  V  L  V  L

961  GTTGTGCTGCTGCTATTCATCATCTGCTGGCTGCCCTTCCAGATCAGCACCTTCCTGGAT
     V  V  L  L  L  F  I  I  C  W  L  P  F  Q  I  S  T  F  L  D

1021 ACGCTGCATCGCCTCGGCATCCTCTCCAGCTGCCAGGACGAGCGCATCATCGATGTAATC
     T  L  H  R  L  G  I  L  S  S  C  Q  D  E  R  I  I  D  V  I

1081 ACACAGATCGCCTCCTTCATGGCCTACAGCAACAGCTGCCTCAACCCACTGGTGTACGTG
     T  Q  I  A  S  F  M  A  Y  S  N  S  C  L  N  P  L  V  Y  V

1141 ATCGTGGGCAAGCGCTTCCGAAAGAAGTCTTGGGAGGTGTACCAGGGAGTGTGCCAGAAA
     I  V  G  K  R  F  R  K  K  S  W  E  V  Y  Q  G  V  C  Q  K

1201 GGGGGCTGCAGGTCAGAACCCATTCAGATGGAGAACTCCATGGGCACACTGCGGACCTCC
     G  G  C  R  S  E  P  I  Q  M  E  N  S  M  G  T  L  R  T  S

1261 ATCTCCGTGGAACGCCAGATTCACAAACTGCAGGACTGGGCAGGGAGCAGACAGTGAgca
     I  S  V  E  R  Q  I  H  K  L  Q  D  W  A  G  S  R  Q  *

1321 aacgccagcagggctgctgtgaatttgtgtaaggattgagggacagttgcttttcagg
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Fig. 1. DNA Sequence of Human BK-2 Receptor. DNA sequence of human BK-2 receptor isolated from CCD-16Lu cell line. The proposed coding region is capitalized with the predicted amino acid sequence shown in one letter code. The underlined sequence corresponds to the probe obtained by reverse PCR from human uterus mRNA that was used to screen the CCD-16Lu cDNA library. The sequence shown was derived from two overlapping partial clones: clone CCD-16-2 begins at base 1 and ends at the closed circle symbol and CCD-16-6 begins at the closed diamond symbol and ends at base 1378.

of the BK-2 receptor cDNA and ends in the middle of the coding sequence (Fig. 1). The second clone, CCD-16-6, begins in the coding region and contains an in-frame translation termination codon (Fig. 1). These two clones overlap for 312 nucleotides and are 100% identical in the overlap region. In addition, both clones are 100% identical in the region spanned by the probe derived from human uterine mRNA. These results

indicate that these clones are derived from the same mRNA transcription unit and that this transcript is also present in human uterus.

A unique restriction site (SacI) in the overlap region of CCD-16-2 and CCD16-6 permitted the construction of a cDNA clone encoding a full length BK-2 receptor (Fig. 1). The human BK-2 receptor cDNA clone shown in Fig. 1 contains an open reading frame from nucleotide 136-1314. The initiator methionine codon is believed to be at nucleotide 223, which is analogous to the proposed initiator methionine in the rat BK-2 cDNA (1). Although two in-frame methionine codons, at nucleotides 142 and 166, occur upstream of the proposed initiator methionine, only the methionine codon at nucleotide 223 contains the elements described by Kozak that are required for efficient initiation of translation (20). Furthermore, the sequence similarity upstream of the methionine codon at nucleotide 223 between the human BK-2 receptor cDNA and the rat BK-2 cDNA is relatively low (40% data not shown), suggesting that this represents an untranslated region. Thus, it seems likely that the translational start site is at nucleotide 223. The in-frame translation termination codon at nucleotide 1315 is in the analogous position to the termination codon in the rat BK-2 receptor cDNA. Therefore, the predicted size of the human BK-2 receptor is 364 amino acids corresponding to a predicted molecular weight of 41,140 Daltons.

The human and rat BK-2 receptors have an overall amino acid identity of 81% (Fig 2). The most striking difference between the human and rat BK-2 receptors is a two amino acid deletion that occurs in the N-terminal extracellular region of the human receptor. The significance of this deletion and the other amino acid substitutions on BK receptor function remain to be determined. The three potential sites of N-glycosylation seen in the rat BK-2 receptor (1) (two in the N-terminal domain and one in the putative extracellular loop between transmembrane helices 4 and 5) are all conserved in the human receptor. The human BK-2 receptor contains several consensus sites for phosphorylation by cAMP dependent protein kinase and protein kinase C in the third intracellular loop and in the carboxyl terminal tail. In the β-adrenergic receptor these regions appear to be involved in receptor coupling to G-proteins, and phosphorylation at analogous sites occurs during receptor desensitization (13,14). Thus, phosphorylation of these intracellular Ser and Thr residues may affect the ability of the BK receptor to communicate with G-proteins. The highest degree of overall identity seen between this receptor and other known proteins is with the rat angiotensin receptor (32%).

Functional expression of the human BK-2 receptor was obtained by placing the entire clone under the control of the CMV promoter in the eukaryotic expression vector, pcDNA I-Neo. This construct was transfected into COS-7 cells and membranes from these cells analyzed for expression of the BK receptor. Membranes prepared from transfected cells contained specific BK binding sites with a K_D of 0.13+/-.09 nM as determined by saturation binding analysis (Fig. 3). In 3 separate experiments the level of expressed receptor ranged from 210 to 450 fmole/mg protein. Scatchard analysis (Fig. 3, inset) of the saturation binding data suggests that there is a single high affinity BK

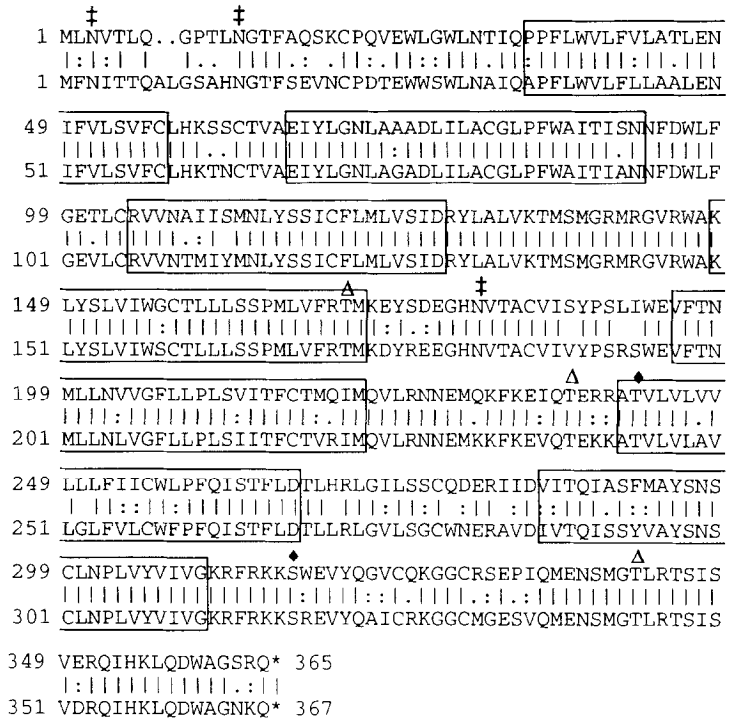

Fig. 2. Comparison of human and rat BK-2 receptor amino acid sequence. Amino acid sequence of human (top) and rat (bottom) BK-2 receptors were compared using the GCG program GAP. The boxed regions correspond to the predicted transmembrane spanning domains. **Symbols**: (‡) glycosylation site, (Δ) consensus site for protein kinase C, (♦) consensus site for cAMP dependent protein kinase.

binding site (K_D=0.13 nM). At higher ligand concentrations, > 3 nM BK, (data not shown) there is evidence for a second low affinity site that is not well defined by saturation binding analysis (K_D > 10 nM). The low affinity site may arise from BK receptors that are not coupled to G-proteins. The ability of at least a portion of the BK receptor expressed in COS-7 cells to couple to a G-protein is suggested by inhibition of 30% of specific [^{3}H] BK binding by 100 μM GppNHp . Control membranes prepared from COS-7 cells transfected with pcDNA I-Neo alone did not contain any detectable BK binding sites.

Competition binding studies indicate that the cloned BK receptor binds BK analogs with the specificity of BK>lys-BK>met-lys-BK (Fig. 4). In contrast, peptides reported to be specific for the BK-1 receptor have a very low affinity for this cloned receptor. At a concentration of 10μM the BK-1 agonist Des-Arg^{9}BK and the BK-1 antagonist Des-Arg^9,Leu^{8}BK inhibit BK binding by 18% and 11%, respectively (data not shown). No competition for BK binding was seen with the peptides angiotensin I and II, neurotensin, oxytocin, and endothelin (data not shown). These results indicate that the receptor cloned and described in this report has the pharmacological properties expected for a BK-2 receptor.

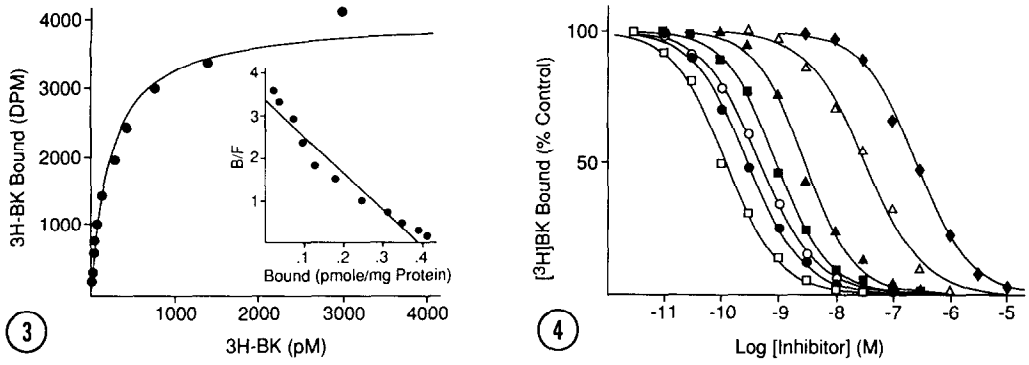

Fig. 3. Saturation binding of human BK-2 receptor expressed in COS-7 cells. Binding was done as described in Materials and Methods with [^{3}H] BK. The BK specific binding (y-axis) is shown with increasing concentrations of [^{3}H] BK (x-axis). The data were analyzed using the EBDA program. Inset shows Scatchard plot of these data.

Fig. 4. Competition binding of human BK-2 receptor expressed in COS-7 cells. BK compounds were titrated against 100 pM [^{3}H]BK. **Symbols:** open squares, D-Arg^0[Hyp^3, Thi^5, D-Tic^7, Oic^8]BK; closed circles, BK; open circle, Lys-BK; closed square, Met-Lys-BK; closed triangle, [Tyr^8]BK; open triangle, D-Arg^0[Hyp^{2,3}, Thi^{5,8}, D-Phe^7]BK; and closed diamond [Thi^{5,8}, D-Phe^7]BK.

The ability of the human BK-2 receptor to interact with BK-2 selective antagonists was analyzed (Fig. 4). Competition binding studies indicate that Hoe 140 (2), D-Arg^0[Hyp^3,Thi^5, D-Tic^7, Oic^8]BK, is a potent inhibitor with an IC_{50} for the cloned human receptor of 65 pM. Interestingly, the IC_{50} of Hoe 140 previously reported from binding studies on guinea pig ileum membranes is 1.07 nM (2). The reason for the apparent higher affinity for Hoe 140 for the human receptor remains to be determined.

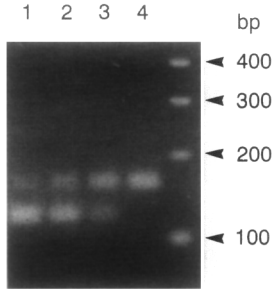

Fig. 5. Competitive PCR of human BK-2 receptor. Representative titration of rat competitor with human cDNA from kidney. PCR was performed according to Materials and Methods. The PCR products were digested with AccI which cuts the rat 155 nucleotide PCR product to generate a 115 nucleotide and a 40 nucleotide product but does not cut the human 155 nucleotide PCR product. **Lanes:** (1-3) AccI digest of PCR products from human kidney cDNA in the presence of decreasing concentrations of rat competitor, (4) AccI digest of PCR products from human kidney cDNA with no rat competitor; (5) DNA size markers.

Table 1. Tissue Distribution of Human BK-2 receptor. Summary of results of competitive PCR (see Materials and Methods) are summarized, each (+) indicates an estimated 5-fold increase in the level of human PCR product from equivalent levels of cDNA from each tissue.

Tissue	Relative level of mRNA
brain	+
heart	+
kidney	+++
lung	++
pancreas	+
testis	+
uterus	++

[^{3}H]BK binding to the human BK-2 receptor was also displaced by the BK-2 antagonists D-Arg^0-[Hyp^{2,3}, Thi^{5,8}D-Phe^7]BK, (IC_{50}=27 nM) and [Thi^{5,8}, D-Phe^7]BK, (IC_{50}=180 nM) (Fig. 4).

The expression of the cloned BK-2 receptor in various human tissues was analyzed by competitive PCR (19,21,22). Random primed cDNA was made from human mRNA prepared from brain, heart, lung, kidney, pancreas, testis and uterus. This cDNA was used to obtain a 155 nucleotide BK receptor derived PCR product in the presence or absence of known amounts of a competitor, the 155 nucleotide PCR product obtained from rat using the same primers. Human specific products can be distinguished from rat competitor by using a restriction site present in the rat but not the human (Fig. 5). The analysis suggests that the highest levels of BK-2 receptor are found in kidney, uterus, and lung with somewhat lower levels of expression in testis, pancreas, brain, and heart (Table 1). These results are similar to the tissue distribution of the rat BK-2 receptor previously reported (1). The isolation of the human receptor will assist in the discovery of therapeutic compounds that act through the BK-2 receptor.

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