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Cloning, sequencing and functional expression of a guinea pig lung bradykinin B_2 receptor

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Abstract

Kinin receptors are classified as B_1 and B_2 based upon agonist and antagonist potencies and cloning and expression studies. Using sequences from human and rat bradykinin B_2 receptors, polymerase chain reaction (PCR) was utilized to isolate cDNA from guinea pig lung. The receptor obtained is predicted to have 372 amino acids and shares $>80\%$ sequence homology with human, rat, rabbit and mouse B_2 receptors. In competition binding experiments in Chinese hamster ovary (CHO-K1) cells in which the guinea pig cDNA was expressed, [^{3}H]bradykinin was displaced by kinin receptor ligands with an order of potency consistent with a B_2 subtype. In CHO cells expressing the guinea pig receptor, bradykinin caused a concentration $^{45}Ca^{2+}$ efflux. A B_1 receptor agonist, desArg^9-bradykinin, also caused $^{45}Ca^{2+}$ efflux but with a potency several orders of magnitude lower than bradykinin. Curiously, several B_1 and B_2 receptor antagonists induced $^{45}Ca^{2+}$ efflux, indicating that this receptor may be coupled differently in CHO cells than in native tissues. © 1998 Elsevier Science B.V.

Keywords: Bradykinin; Bradykinin receptor; (Guinea pig); Bradykinin B_2 receptor; Cloning; Functional expression

1. Introduction

Receptors for bradykinin and related kinins are divided into B_1 and B_2 subtypes based on agonist and antagonist potency ratios in various tissues (Farmer and Burch, 1992; Hall and Morton, 1997). Cloning and sequencing studies have confirmed the existence of bradykinin B_2 receptors in rats (McEachern et al., 1991), humans (Eggerickx et al., 1992; Hess et al., 1992; Powell et al., 1993), mice (McIntyre et al., 1993; Hess et al., 1994; Borkowski et al., 1995) and rabbits (Bachvarov et al., 1995). Bradykinin B_1 receptors from the mouse (McIntyre et al., 1993; Hess et al., 1996; Pesquero et al., 1996), rabbit (MacNeil et al., 1995), and human (Menke et al., 1994; Webb et al., 1994; Bachvarov et al., 1996; Chai et al., 1996) have also been cloned, and their distinct sequence and pharmacology from B_2 receptors confirmed. The deduced amino acid sequences from the four species' B_2 receptors show extensive similarity ($>80\%$) and they all belong to the G protein-coupled, seven transmembrane domained receptor superfamily. Recently, an avian kinin receptor was cloned. It shows 31% and 49% sequence identity, respectively, to human B_1 and B_2 receptors and is a G protein-coupled receptor (Schroeder et al., 1997).

The genomic organization of the bradykinin B_2 receptor has been studied for the human, rat and mouse genes. The 5′ untranslated regions are interrupted by introns (Ma et al., 1994; Pesquero et al., 1994) and the intron/exon boundaries are highly conserved. There is also evidence for alternative splicing of the 5′ untranslated region of the rat B_2 receptor gene (Pesquero et al., 1994). Numerous pharmacological studies in guinea pigs indicate that B_2 receptors are expressed ubiquitously in tissues from this species (Farmer and Burch, 1992; Hall and Morton, 1997). Although there is no evidence for guinea pig B_1 receptors, pharmacological studies in airway tissues from this species have suggested another subtype of bradykinin receptor, termed 'B_3' (Farmer et al., 1989b, 1991; Pyne and Pyne, 1993; Farmer and DeSiato, 1994). The existence of the B_3 receptor, however, is controversial (Trifilieff et al., 1991; Field et al., 1992; Da Silva et al., 1995; Scherrer et al., 1995). Evidence for bradykinin B_3 receptors, as well as the 'unusual' nature of guinea pig B_2 receptors compared with

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those from other species, was reviewed extensively by Hall and Morton (1997). To date, there are no reports on the cloning or sequencing of any guinea pig kinin receptors. In the present paper, we report the isolation, sequencing and functional expression of a guinea pig lung bradykinin receptor.

2. Materials and methods

2.1. Isolation and characterization of a guinea pig lung bradykinin receptor

RNA was isolated from whole lung tissue using guanidinium isothiocyanate as described elsewhere (Chirgwin et al., 1979). RACE-PCR (rapid amplification of cDNA ends-polymerase chain reaction) (Frohman et al., 1988; Frohman and Martin, 1990) was utilized to isolate both 5′ and 3′ ends of the guinea pig lung bradykinin receptor cDNA. To isolate the 3′ end of the cDNA, synthesis was carried out with superscript reverse transcriptase (Gibco BRL, Paisley, Scotland) using 5 μg total RNA, and the RACE 58-nucleotide primer, 5′d(AAGGATCCGTCGACATCGATAATACGACTCACTATAAGGGA$(T)_{17}$). Dilutions of the cDNA were used as templates in primary PCR reactions with an amplimer derived from the third transmembrane segment of human B_2 receptor cDNA (Powell et al., 1993), 5′d(TGTTTCCTGATGCTGGTGAGCATC), and an amplimer derived from the 5′ end of the RACE primer above, 5′d(AAGGATCCGTCGACATCGATAAT). Following denaturation at 100°C for 5 min, PCR (Saiki et al., 1988) was performed in a Techne PHC-1 thermal cycler for 33 cycles (93°C for 2 min, 60°C for 2 min and 72°C for 3 min) using Amplitaq (Perkin-Elmer, Norwalk, CT). One percent of the products were used for secondary PCR with an amplimer derived from transmembrane segment-IV of the rat B_2 receptor, 5′d(CTGGTGATCTGGAGCTGTACGCTGCT), and a nested RACE amplimer, 5′d(GATAATACGACTCACTATAAGGGA) using conditions as described above. A product of 760 base pairs hybridized to full-length rat and human B_2 bradykinin receptor cDNA probes. This product was isolated from two independent PCR reactions, purified and directly sequenced. Each of the sequences were identical.

To isolate the 5′ end of the cDNA, 5 μg of total guinea pig lung RNA was reverse transcribed using a primer, 5′d(CACATACACCAGCGGGTTGAGGCAGC), designed from the 760 base pair 3′ product at transmembrane segment-VII. The cDNA was tailed at its 3′ end with dATP and terminal transferase (Gibco BRL), purified on a Microcon 100 dialysis unit (Amicon, Beverly, MA), and second strand cDNA was synthesized with Amplitaq by priming with the RACE 58-residue primer. Primary PCR was performed for 35 cycles, as described above, using the RACE outer amplimer and an amplimer derived from transmembrane segment-V, 5′d(ATGCTGAGGGGGAGCAGGAAGCCCAC). A total of 0.1% of the PCR products were subjected to secondary PCR using the nested RACE amplimer and an amplimer spanning the junction of the second extracellular loop and transmembrane segment-V, 5′d(GGACATTGGTGAACACCTGCCAG), under identical conditions to the primary reaction. A product of 700 base pairs was isolated from two independent PCR reactions, purified and directly sequenced, and each of the sequences were identical.

Full length cDNA was obtained from 5 μg of guinea pig lung total RNA by reverse transcription using oligo d(T)15. Primary PCR was performed for 33 cycles with amplimers from the 5′ and 3′ untranslated regions, 5′d(AAGCACCTGCACCCACCGAAGCCTCC) and 5′d(CCTGCTGGACCCATGCTGAAGAGC), respectively. Secondary PCR was carried out under the same conditions using 0.1% of the primary reaction products and site-tagged amplimers. The 5′ amplimer, 5′d(GCGCGAATTC GCCGCCACCATGTTCAACATCACCTCGCA AGTGTCA), contained a Kozak consensus sequence (Kozak, 1989) (underlined) immediately upstream of the initiator ATG. The 3′ amplimer, 5′d(GCGCGGCCGCTGCTCCCTGCTGGACCCATGCTGAAG), is positioned 58 base pairs downstream from the stop codon. These contained *Eco*RI and *Not*I restriction sites at their 5′ ends, respectively. This product was cloned unidirectionally into pcDNA3 (Invitrogen, San Diego, CA). Eight clones were sequenced and found to be an identical match to the sequences from the RACE-PCR products.

2.2. Expression of the guinea pig bradykinin receptor in Chinese hamster ovary (CHO) cells

CHO-K1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 50 U ml^{-1} penicillin, 50 mg ml^{-1} streptomycin, 2 mM L-glutamine and essential amino acids (0.1 mM). The cells were plated at a density of 1.9×10^6 per 175 cm^2 flask 12 h before transfecting with 50 mg of *Pvu*I-linearized guinea pig lung bradykinin receptor/pcDNA plasmid using a $CaPO_4$ transfection kit (5 Prime > 3 Prime, Boulder, CO) according to the manufacturer's protocol. Following 15% glycerol shock for 90 s, the cells were grown in nonselective media for 48 h, trypsinized, diluted 1 to 15, and G418 (Gibco BRL) added to a final concentration of 1 mg ml^{-1}. Twenty individual clones were isolated 10 to 14 days later and whole cell [^{3}H]bradykinin ligand-binding assays were performed to determine the highest specific binding. Transfectants with the highest specific binding were expanded and used for further studies.

2.3. Pharmacology of guinea pig bradykinin receptors in CHO cells

In preliminary experiments in whole cells, two transfected CHO cell clones (Nos. 16 and 20) exhibited the

highest specific binding (88 and 91% of total binding, respectively) for [^{3}H]bradykinin. Clone 20 was used for all subsequent pharmacological analyses. Membranes were prepared from CHO cells transfected with guinea pig lung bradykinin receptor cDNA and competition binding experiments were performed as described elsewhere (Farmer et al., 1989a, Farmer et al., 1989b). Ligand was 1 nM [^{3}H]bradykinin (New England Nuclear, Boston, MA), and nonspecific binding was determined in the presence of 1 mM unlabeled bradykinin. Competing ligands were bradykinin and desArg^9-bradykinin (Bachem, Torrance, CA), DArg-[Hyp^3,DPhe^7]bradykinin (NPC 567) and DArg-[Hyp^3,Thi^5,DTic^7,Tic^8]bradykinin (NPC 16731) (SciosNova, Baltimore, MD), and DArg-[Hyp^3,Thi^5,DTic^7,Oic^8]bradykinin (Hoe 140, synthesized in-house). The nonpeptide B_2 receptor antagonist, Win 64338 (phosphonium, [[4-[[2-[[bis(cyclohexylamino) methylene]amino]-3-(2-naphthalenyl) 1-oxopropyl]amino]-phenyl]-methyl]-tributyl, chloride HCl), was a gift from Sterling-Winthrop Pharmaceuticals, (Collegeville, PA).

2.4. $^{45}Ca^{2+}$ efflux experiments

Bradykinin-induced efflux of $^{45}Ca^{2+}$ was performed, utilizing procedures described in detail elsewhere (Farmer et al., 1989b, 1991), in CHO cells from clone 20 transfected with guinea pig lung bradykinin receptor cDNA and also in nontransfected CHO cells. Assay medium was DMEM supplemented with 20 mM HEPES, 10 mM captopril, 0.1% bovine serum albumin, and 100 mg ml^{-1} bacitracin. Briefly, cells were incubated with 2.5 mCi $^{45}Ca^{2+}$ in 1 ml assay media for 4 h in 12-well plates. After this time, the medium was aspirated and wells washed five times with 2 ml of assay medium. Basal efflux of radioactivity was measured for 3 min and efflux of $^{45}Ca^{2+}$, in response to bradykinin receptor ligands (10^{-13}–10^{-4} M, depending upon ligand), was measured over the following 6 min. At the end of the efflux period, cells were solubilized with 1 ml Triton X-100, and radioactivity remaining in the cells, as well as the efflux, was determined by liquid scintillation counting. Fractional $^{45}Ca^{2+}$ efflux at each

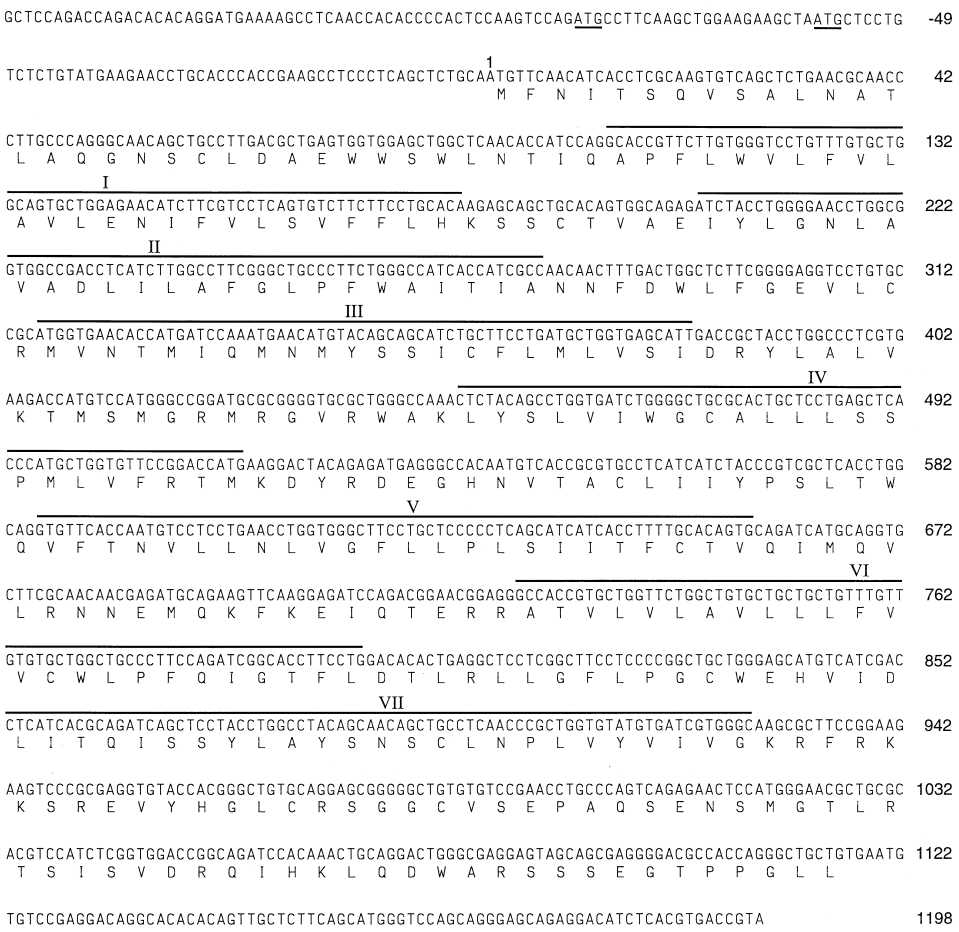

Fig. 1. Nucleotide sequence of the guinea pig B_2 receptor cDNA. The DNA sequence is shown together with the deduced amino acid sequence. The nucleotide residues are numbered beginning with the A of the initiator methionine numbered as 1. Nucleotides 5′ of nucleotide 1 are indicated by negative numbers. The positions of upstream in-frame ATG's are shown underlined. Positions of the putative transmembrane domains are indicated by Roman numerals above the DNA sequence. The sequences have been deposited in GenBank (accession No. AJ003243).

interval was determined by division of radioactivity lost during a given 3-min period by the amount of radioactivity remaining associated with the cells. Efflux of $^{45}Ca^{2+}$ in response to various agents was expressed as a percent of the maximum efflux induced by 10 μM bradykinin. Concentration–response curves to kinin analog-induced $^{45}Ca^{2+}$ efflux were obtained, the curves were fitted and EC_{50} values determined using Prism (GraphPad Software, San Diego). All data are expressed as the mean ± S.E. mean, and numbers in parentheses denote the number of experiments.

3. Results

3.1. Isolation and characterization of a guinea pig lung bradykinin receptor

The 5′ and 3′ fragments of cloned guinea pig lung bradykinin receptor were isolated by RACE-PCR. Direct sequence analysis revealed that the 5′ product was from (−)137 to 601, and the 3′ product was from position 457 to 1198 (Fig. 1), with an overlap of 144 base pairs. A cDNA containing the whole coding sequence, corresponding to positions 1 to 1176 and containing the whole coding sequence, was isolated by reverse transcription (RT)-PCR. Sequences obtained from several independent full-length clones agreed with that of the directly sequenced RACE-PCR products. Guinea pig lung bradykinin receptor cDNA was detectable by PCR in both lung and bladder tissues (data not shown).

The protein sequence of the guinea pig lung bradykinin receptor is 372 amino acids in length and has a predicted molecular mass of 42 200 Da, in the absence of any added carbohydrate. This protein shares high sequence homology with the human (81.5%), rat (82.6%), mouse (81.5%) and rabbit (80.7%) B_2 receptors (Fig. 2). The B_2 receptors from these species possess three potential N-glycosylation sites with two in the N-terminal domain and one in the

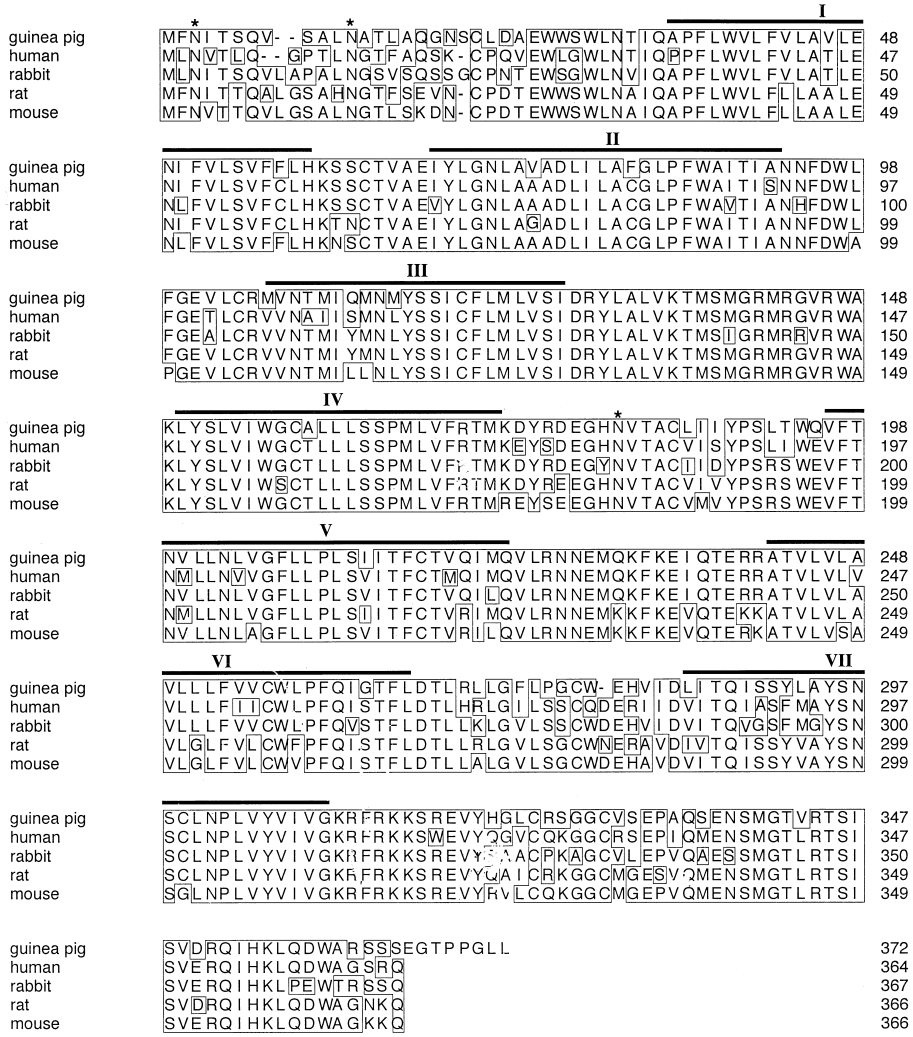

Fig. 2. Alignment of amino acid sequences of guinea pig, human, rabbit, rat and mouse B_2 receptors. Identical residues aligned in more than one species are shown boxed. Positions of the putative transmembrane domains are indicated above the amino acid sequence. Potential glycosylation sites are denoted by asterisks. Note the extra eight amino acid residues at the C-terminus of the guinea pig receptor sequence.

second extracellular loop. The guinea pig B_2 receptor is predicted to have an extra eight amino acids at the C-terminus which are not present in human, rat, mouse or rabbit sequences (Fig. 2).

3.2. Expression and pharmacology of guinea pig lung bradykinin receptors in CHO cells

As noted in Section 2, CHO cells were transfected with guinea pig lung bradykinin receptor cDNA and cell cultures exhibiting the highest levels of expression (Clone 20), as determined from whole cell binding of [^{3}H]bradykinin, were utilized for further studies. Control, nontransfected CHO cells exhibited no significant binding of [^{3}H]bradykinin.

In transfected cells, binding of [^{3}H]bradykinin exhibited a high degree of specificity ($>90\%$), and was saturable (B_{max} 1689 fmol/mg tissue). In competition binding experiments, bradykinin was displaced in a concentration-dependent manner by several agonists and antagonists of the B_2 receptor, whereas desArg^9-bradykinin, a B_1 receptor ligand, was without effect (Table 1).

Bradykinin caused concentration-dependent efflux of $^{45}Ca^{2+}$ from CHO cells transfected with guinea pig lung bradykinin receptor cDNA, yielding a pD_2 value of 10.32 ± 0.12 (Fig. 3). The B_1 receptor agonists, desArg^9-bradykinin (which was inactive in binding; Table 1), elicited $^{45}Ca^{2+}$ efflux, albeit with a potency several orders of magnitude lower than bradykinin (Fig. 3). B_2 receptor antagonists, Hoe 140 and NPC 16731, induced Ca^{2+} efflux with pD_2 values, respectively, of 8.95 ± 0.69 and 9.02 ± 0.51. A weak peptide B_2 receptor antagonist, NPC 567, a B_1 receptor antagonist, desArg^9-[Leu^8]bradykinin, as well as a nonpeptide antagonist, Win 64338, also were impotent and inefficacious agonists in transfected CHO cells.

In order to ascertain the specificity of these responses, we examined the effects of several of the kinin receptor ligands in CHO cells that had not been transfected with guinea pig lung bradykinin receptor cDNA. Bradykinin and a unrelated peptide, neurokinin A (each at 10 μM), as well as NPC 567 (10 μM) and Hoe 140 (10 nM) did not elicit efflux of $^{45}Ca^{2+}$ in these cells.

Table 1
Competition binding $-\log K_i$ values for displacement of [^{3}H]bradykinin binding by kinin receptor ligands in CHO cells transfected with cDNA for bradykinin receptors cloned from guinea pig lungs

Ligand	$-\log K_i$ * (nM)
Bradykinin	9.25 ± 0.04
Hoe 140	9.21 ± 0.09
NPC 16731	8.96 ± 0.09
Win 64338	7.57 ± 0.10
NPC 567	7.54 ± 0.07
desArg^9-bradykinin	$>30\,000$

Specific binding was 90%. K_d for bradykinin was 3.77 nM, and B_{max} was 1689 fmol mg^{-1} tissue. Values are reported as mean ± S.E.M. of four experiments.

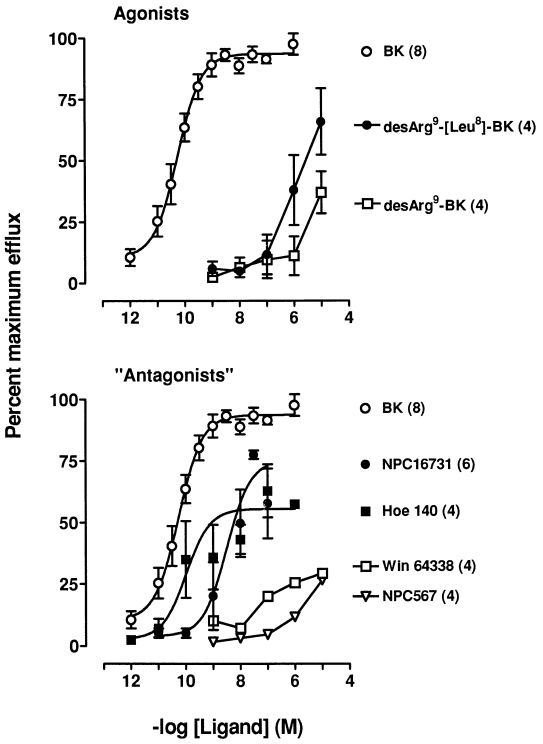

Fig. 3. Bradykinin receptor agonist- and antagonist-induced fractional efflux of $^{45}Ca^{2+}$ from CHO-K1 cells transfected with cloned guinea pig lung bradykinin receptor cDNA. See Section 2 for experimental details. Data are presented as mean ± S.E.M., and the numbers of observations are shown in parentheses.

4. Discussion

We report for the first time the cloning, sequencing and functional expression of a guinea pig lung bradykinin receptor. This receptor shares $>80\%$ sequence homology with kinin B_2 receptors from human, rat, mouse and rabbit. Furthermore, in binding the rank order of potency of competing ligands in the cloned guinea pig receptor is consistent with it being a B_2 subtype (Farmer and Burch, 1992; Hall and Morton, 1997). As noted earlier, there are many reports that guinea pig bradykinin B_2 receptors exhibit pharmacological properties, e.g., lower apparent affinities for several ligands, suggesting that there are species differences with this receptor subtype (see review by Hall and Morton, 1997). To date, however, there is no published evidence for more than one B_2 receptor gene within a single species. As with bradykinin B_2 receptors from other species (Hess, 1997), the guinea pig receptor is a seven transmembrane-spanning protein that possesses three potential N-glycosylation sites: two in the N-terminus and one in the second extracellular loop. Similarly, this receptor contains sites for potential phosphorylation by

protein kinases C (Thr^{171} and Thr^{238}) and A (Ser^{316}). The guinea pig receptor, unlike that from all other species examined to date, has an extra eight amino acids at the C-terminus. Furthermore, the C-terminal sequence in guinea pig lung bradykinin receptor contains three serines and one threonine which may also be potential sites for phosphorylation.

A recent publication (AbdAlla et al., 1996a) demonstrated that, for human and rat B_2 receptors, there is an in-frame ATG codon which are upstream of the previously reported translation start site, and that the first AUG is used as a translation start site. It was also postulated that, in the mouse B_2 receptor, the first in-frame AUG is used as a start site for translation. These proposed start sites would extend the human, mouse and rat B_2 receptors by 27, 26 and 30 amino acids, respectively, and the extra sequence was annotated as ED1A (with ED1B being the original predicted extracellular domain) (AbdAlla et al., 1996b). It has been shown that the long and short versions of the human B_2 bradykinin receptor have almost identical ligand binding and that both are coupled efficiently to signaling pathways (AbdAlla et al., 1996b). The heterologous expression work described herein was performed using the guinea pig equivalent of the ED1B form of the receptor. However, the guinea pig B_2 sequence (Fig. 1) contains two upstream ATG codons shown underlined at positions (−)81 and (−)57. The ATG at position (−)81 is analogous to the newly assigned translation start site for the human, rat and mouse receptors (AbdAlla et al., 1996a), and there is amino acid sequence homology in this region (ED1A) with the human and rat B_2 receptors. The methionine at position 168 (McEachern et al., 1991) conformed best to a consensus translation start sequence (Kozak, 1989) and was presumed to be the first amino acid of the primary translation product, although a construct which started upstream of the methionine at position 78 was actually used for expression in Xenopus oocytes. All other studies of recombinant B_2 receptors from human (Eggerickx et al., 1992; Hess et al., 1992; McIntyre et al., 1993; Powell et al., 1993), rat (McIntyre et al., 1993), mouse (McIntyre et al., 1993; Hess et al., 1994) and rabbit (Bachvarov et al., 1995) have used the initiation methionine shown in Fig. 2. In addition, RT-PCR of rat B_2 receptor (Pesquero et al., 1994) detected two different B_2 mRNAs containing or lacking exon 3 in all rat tissues tested, and of which the shorter RNA represents the abundant species which would also correspond to the rat B_2 receptor shown in Fig. 2. Further investigation of the alternative upstream initiation methionine residues and possible effects on the pharmacological properties of these receptors will be interesting. In addition, many of the respective properties of various subtypes of kinin receptors may reflect species differences and/or the characteristics of G protein coupling in different cell types.

In functional studies, the cloned guinea pig lung bradykinin receptor exhibited unusual behavior in that all bradykinin receptor ligands tested—even agents that are potent B_2 receptor antagonists at normally constitutive mammalian B_2 receptors—stimulated $^{45}Ca^{2+}$ efflux. Efflux of $^{45}Ca^{2+}$ was mediated specifically via the cloned guinea pig receptor as all agents tested had no effect in nontransfected CHO cells. Also, an unrelated receptor agonist, neurokinin A, was inactive at the cloned bradykinin receptor. Thus, although competition binding experiments indicate that the guinea pig cloned receptor is of the B_2 subtype, this was not confirmed in a functional assay. Thus, even though bradykinin was a potent stimulant of $^{45}Ca^{2+}$ efflux ($EC_{50} < 10^{-10}$ M), and was several orders of magnitude more potent than a B_1 receptor agonist, the B_1 and B_2 receptor antagonists tested each behaved as agonists (see Fig. 3). Leeb-Lundberg et al. (1994) found that, in rat uterus smooth muscle, Hoe 140 behaves as an inverse agonist but this is unlikely to explain the stimulatory actions of this agent or the other antagonists in the present study. There are previous reports that some 'first generation' bradykinin receptor antagonists such as NPC 567 can act as partial agonists in fresh tissues (Farmer and Burch, 1992; Hall and Morton, 1997) and in cloned bradykinin receptors (Sawutz et al., 1992; McIntyre et al., 1993). In addition, it has been noted that Hoe 140, one of the most potent known peptidic B_2 receptor antagonists, exerts spasmogenic activity in guinea pig isolated trachea (Trifilieff et al., 1992). Interestingly, Hoe 140 is a full agonist at the ornithokinin (avian) receptor in stimulating phosphatidylinositol hydrolysis and intracellular free Ca^{2+} in chicken embryo fibroblasts and in CHO cells transfected with the ornithokinin receptor (Schroeder et al., 1997). Bradykinin is inactive in these systems. To our knowledge, Win 64338, a nonpeptide antagonist, has not been reported previously to behave as an agonist, albeit such an action was very weak in the present study. We have found that NPC 16731 is a full agonist (pD_2 8.45) for inducing phosphatidylinositol metabolism, but has no effect on $^{45}Ca^{2+}$ efflux in CHO cells transfected with cDNA encoding the human B_2 receptor (Farmer, S.G. and Prosser, J., unpublished observations). The agonist response to this 'antagonist' was blocked potently (pA_2 9.32) by Hoe 140. In contrast to the cloned guinea pig receptor, however, neither Win 64338 nor Hoe 140 exhibited agonist-like effects in CHO cells expressing the human B_2 receptor (Farmer, S.G. and Prosser, J., unpublished). It would appear then, that guinea pig and human bradykinin receptors expressed in CHO cells respond to some ligands, normally considered to be antagonists, with agonist-like activity, perhaps due to an aberrant coupling mechanism. The reasons underlying this phenomenon are unknown, but deserve further study.

The guinea pig B_2 receptor was isolated from total lung RNA by RACE-PCR using primers derived from both the human and rat B_2 receptor sequences. Many pharmacological studies have confirmed the presence of guinea pig B_2 receptors in gut, blood vessels, nerves, and most other

tissues including the lungs (Farmer and Burch, 1992; Hall and Morton, 1997). It is not surprising, therefore, that we have cloned a B_2 receptor from this species. Future studies utilizing low stringency screening of guinea pig cDNA libraries may determine whether the putative 'B_3 receptor' can be detected at the molecular level.

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