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Molecular characterisation of cloned bradykinin B_1 receptors from rat and human

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Abstract

This report describes the characterisation of cloned rat and human bradykinin B_1 receptors in African green monkey kidney fibroblast (Cos-7) cells. A ligand binding assay with [^{3}H]des-Arg^{10}-kallidin was used to compare their pharmacology with respect to known bradykinin B_1 and B_2 receptor ligands. In addition, the pharmacology of T-kinin and its' derivative des-Arg^{11}-T-kinin was investigated. The cloned rat receptor had a similar pharmacology to that of the recently described mouse receptor and differs from that described for the human receptor. The rat receptor had a higher affinity for des-Arg^{11}-T-kinin than the human receptor. These differences in pharmacological properties may relate to the presence of T-kinin, bradykinin and their des-Arg derivatives as the major physiological peptides in rat and the predominance of kallidin and its derivatives in human. We confirm that the rat bradykinin B_1 receptor gene is organised in a two exon structure and differs from the human gene which has a three exon structure and we further examine the inducible expression of this gene in a wide range of tissues using Northern blotting.

Keywords: Bradykinin B_1 receptor; Bradykinin; T-kinin; G-protein coupled receptor; Gene; (Rat)

1. Introduction

Kinins are pharmacologically active peptides that are formed by the action of kallikrein enzymes on either high or low molecular weight kininogens in humans, and T-kininogen or low molecular weight kininogen in rats. These enzymes release a number of potent vasoactive peptides including bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) and kallidin (Lys-bradykinin) in humans and bradykinin and T-kinin in rats (Ile-Ser-bradykinin). The kinins play an important role in pathophysiological conditions involving inflammation and pain (for review see Marceau et al., 1998) and have been shown to regulate mitogenesis and proliferation in a variety of cell types (Dixon and Denis, 1997; Marceau et al., 1998).

Kinins exert their actions by binding to and activating two subtypes of G-protein coupled receptors, which are generally coupled to G_q/G_{11}, G-protein alpha subunits (Jones et al., 1995; DeWeerd and Leeb-Lundberg, 1997). The differences in the kinin precursors and their products in human and rats imply that there may be structural differences in the kinin receptors that have arisen through divergent evolution. There are two distinct kinin receptors subtypes, B_1 and B_2. The bradykinin B_2 receptor is constitutively expressed in a wide range of cell types, whereas the bradykinin B_1 receptor is expressed in a narrower range of cell types only after tissue injury or inflammation. It can also be upregulated specifically by cytokines such as interleukin-1β and tumor necrosis factor α. The bradykinin B_1 receptor was first distinguished from the bradykinin B_2 receptor by its' sensitivity to the agonist des-Arg^9 bradykinin (Regoli et al., 1977). The rat and human B_2 bradykinin receptors have been cloned and compared (McEachern et al., 1991; Hess et al., 1992; Eggerickx et al., 1992; McIntyre et al., 1993) and bradykinin B_1 receptors have been cloned from human, rabbit and mouse (Menke et al., 1994; MacNeil et al., 1995; Hess et al., 1996;

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Pesquero et al., 1996). Studies on these receptors led to the finding that the agonist selectivity of the mouse bradykinin B_1 receptor is different from the human and rabbit receptors (Hess et al., 1996). This is consistent with there being divergent evolution of function among the bradykinin B_1 receptors from different species. More recently, while this paper was in preparation, the rat bradykinin B_1 receptor gene was cloned and the rat bradykinin B_1 receptor pharmacology has been partially characterised (Ni et al., 1998). In this paper we describe the cloning of the rat bradykinin B_1 receptor gene and cDNA from rat bladder. Our data confirm and extend the recent findings of Ni et al. (1998) with respect to gene structure, except that we find a different start site of transcription. We have characterised the pharmacology of this cloned rat bradykinin B_1 receptor and compared it with data from a cloned human receptor expressed in mammalian cells and with data from the native receptor in the literature.

We have further extended the findings of Ni et al. (1998) by studying the influence that bacterial lipopolysaccharide has on expression of the rat gene using Northern blots rather than RT-PCR, in a wide range of tissues.

2. Materials and methods

2.1. Cell culture

WI38 human foetal lung fibroblast cells, Cos 7 African green monkey kidney fibroblast cells (Flow laboratories) were grown in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% foetal calf serum.

2.2. RNA purification and Xenopus oocyte recordings

Female Wistar rats (approximately 200 g) were given an intraperotoneal injection of oestradiol benzoate (0.5 mg/kg) followed, 18 h later, by an intravenous injection of lipopolysaccharide (3 mg/kg). The animals were sacrificed 4 h after the injection of LPS. Lung, aorta, heart, duodenum, bladder, uterus, brain, spinal cord, dorsal root ganglion, thymus and spleen were removed from naive and treated animals, chopped and frozen immediately in liquid nitrogen. Blood and peritoneal macrophages were isolated as described by Bockmann and Paegelow (1995). The tissues and cells were stored at −70°C until the RNA was extracted. RNA was extracted and Poly A^+ RNA purified from tissues and cells as previously described (Webb et al., 1994). Poly A^+ RNA from WI38 cells was size fractionated by sucrose density centrifugation and the presence of bradykinin B_1 receptor mRNA in RNA samples was measured by injection into *Xenopus* oocytes and two electrode voltage clamp recordings as previously described (Webb et al., 1994).

2.3. cDNA library construction

A human WI38 cDNA library was constructed in λ ZAP Express (Stratagene) according to the manufacturers instructions with slight modifications, using poly A^+ RNA that was enriched for bradykinin B_1 receptor recordings as previously described (Webb et al., 1994). Forty pools of 10,000 clones and thirty pools of 20,000 clones were prepared. A rat bladder library was made in the same manner and screened by sequence homology as described below.

2.4. Expression cloning the human bradykinin B_1 receptor cDNA and expression in mammalian cells

Recombinant phage DNA for in vitro transcriptions was purified from each amplified pool as described (Ausubel and Frederick, 1994). DNA was prepared and the in vitro transcription and the *Xenopus* oocyte electrophysiological assay was carried out as previously described (Webb et al., 1994). One positive pool of 10,000 clones was detected and it was further divided and the sub-pools were tested until we had isolated a single clone. The plasmid containing the human bradykinin B_1 receptor insert was excised from λ ZAP express and grown at 37°C in LB broth (Gibco/BRL) with 25 μg/ml Kanamycin (Sigma). Plasmid DNA was purified using a Wizard Megaprep kit (Promega).

Limited DNA sequencing was carried out using the Sequenase II kit (USB) and the final plasmid insert was completely sequenced by Lark Sequencing Technologies (Houston, USA). This sequence has the EMBL accession number AJ238044. The cDNA was cloned as a *Pst*I to *Not*I fragment into pcDNA3 for expression in mammalian cells. Analysis of DNA sequences and predicted protein sequence was carried out using the GCG suite of programs from the University of Wisconsin and Lasergene Software package (DNAstar).

2.5. Cloning the rat bradykinin B_1 receptor gene

A cosmid clone encoding the rat bradykinin B_1 receptor was isolated from a Sprague Dawley rat DNA library in SuperCos 1 (Stratagene) by hybridisation with a 750 bp *Xba*I–*Eco*RI fragment from the human bradykinin B_1 receptor cDNA. A single cosmid containing the rat bradykinin B_1 receptor gene was cloned.

An approximately 2 kb *Bam*HI fragment of the cosmid was cloned into pBKCMV (Stratagene) and a 1.3 kb *Bam*HI–*Eco*RI fragment was cloned into pcDNA3 for expression in mammalian cells. The sequence of this 1.3 kb fragment was determined by manual sequencing with Sequenase II as described above.

2.6. Cloning the rat bradykinin B_1 receptor cDNA

A λ ZAP express library was constructed from Poly A^+ RNA that had been extracted from rat bladders that had been incubated overnight in Kreb's balanced salt solution to allow maximum expression of bradykinin B_1 receptor (Marceau et al., 1980). The cDNA library was screened with a 864 bp *Pst*I fragment of the rat genomic DNA coding region. A single clone was isolated and sequenced and was found to contain a deletion in the region encoding the predicted transmembrane domains 6 and 7. The following PCR primers were synthesised (5′AGGTCACCATCAAAAACACAGG3′ and 5′CTTCT-TGGCTCCCTGCTGTT3′) from the 5′ and 3′ untranslated regions of this clone and used for RT-PCR from rat bladder cDNA. The product of the PCR was of the size predicted for an undeleted clone. The 1.25 kb PCR product was cloned into pCR2 (Invitrogen) and sequenced. The sequence corresponding to the full length cDNA has been submitted to the EMBL database (Accession number AJ132230).

2.7. Primer extension analysis

Primer extension analysis was carried out using 5 μg of poly A^+ RNA isolated from normal and 4 h LPS treated rat uterus according to the method of Ausubel and Frederick (1994), except that 200 units of Superscript II (Life Sciences, UK) were used for 1 h at 42°C and then for 15 min at 50°C to overcome any secondary structure in the 5′ end of the RNA, using an oligonucleotide primer 5′-GCTGGAGCTCCAACAAGACCTCGGACGCCA-3′. The completed reaction was resuspended in 2 μl TE and 2 μl RNA gel loading buffer (Sigma), denatured and run on a 6% polyacrylamide gel (Biorad) with a parallel sequencing reaction using a universal primer on pUC18. The gel was dried under vacuum and exposed to the phosphorimager overnight.

2.8. Rapid amplification of cDNA ends, RACE

5′ RACE was carried out using a modified procedure of Frohman et al. (1988). First strand cDNA was synthesised using Gibco Superscript II reverse transcriptase on 100 ng of poly A^+ from control and 4 h LPS treated rat uterus tissue. The cDNA was tailed using terminal transferase (Boehringer Mannheim). The tailed cDNA was subsequently amplified using a T_{17} adapter primer 5′-GACTC-GAGTCGACATCGA$(dT)_{17}$-3′ and a bradykinin B_1 specific primer 5′-GAGCCTGTAGCGGTCCTG-3′. 1 μl of the first round PCR product was then used for a second round of amplification using the adapter primer 5′-GACTCGAGTCGACATCG-3′ and a bradykinin B_1 gene specific primer 5′-CTATGGTTAAGCGCTGCTGCCT-3′. The 5′ RACE products were cloned directly into the PCR 2.1 vector (Invitrogen) and sequenced.

2.9. Northern blotting

Northern analysis was performed according to the methods described in (Ausubel and Frederick, 1994). Briefly 5 μg of each RNA was run on a formaldehyde denaturing gel and blotted to Nylon (Gene screen PLUS, DuPont) overnight in 3 M sodium chloride and 0.3 M sodium citrate (20 × SSC), rinsed in 10 × SSC, dried at room temperature and baked at 80°C for 2 h. The membranes were hybridised with the rat bradykinin B_1 receptor probe (as described for cDNA library screening) for 18 h and were then washed at room temperature with 2 × SSC for 15 min, followed by 0.2 × SSC at 60°C with constant agitation until the background was low. The blots were stripped and reprobed with a β-actin probe to check that the loadings were equivalent for quantitative analysis.

2.10. Transfection

Cos-7 cells were grown in 175 cm^2 flasks and transfections were carried out using the Promega ProFection calcium phosphate transfection kit according to the manufacturers' instructions using 150 μg/ml rat bradykinin B_1 receptor cDNA and 100 μg/ml human bradykinin B_1 receptor cDNA. On the day following transfection the precipitate was removed, fresh media added and the cells were then grown for a further two days.

2.11. Membrane preparation

Cos-7 cells were harvested by incubating at 37°C for 5 min with 1 mM EDTA made up in calcium- and magnesium-free Hank's Balanced Salts Solution containing 10 mM HEPES pH 7.4, which detached the cells. The cells were pelleted by centrifugation for 5 min at 300 × *g* and the cell pellet washed three times in the EDTA solution by resuspension and centrifugation. The cells were then resuspended in homogenisation buffer (50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 140 μg/ml bacitracin, 50 μg/ml chymotrypsin, 4 μg/ml leupeptin and 4 μg/ml trypsin inhibitor) and homogenised at 10,000 rpm for 30 s with a Polytron homogeniser. The suspension was centrifuged at 40,000 × *g* for 30 min in a Sorvall SS34 rotor. The pellet was then re-homogenised and centrifuged twice more. The final pellet was resuspended at a concentration of 4 mg protein/ml in 50 mM Tris/HCl pH 7.4 plus 10% glycerol and stored at −70°C. Protein concentrations were determined by the method of Bradford (1976) using a Biorad Kit.

2.12. Binding experiments

Binding assays were carried out in 96 well plates at 4°C, in triplicate in a buffer containing 10 mM TES (*N*-tris[hydroxymethyl]methyl-2-aminoethane sulphonic acid) pH 7.4, 1 mM EDTA, 0.1% bovine serum albumin, 1 μM MERGEPTA, 1 μM enalapril, 10 μM thiorphan (total assay volume 500 μl). For saturation studies, membranes (approximately 50 μg/well) were incubated with a range of concentrations of [^{3}H]des-Arg^{10}-kallidin. For each concentration of radioligand, non-specific binding was determined with 3 μM des-Arg^{10}-kallidin. All incubations were terminated by rapid filtration through GF/B filters presoaked in 0.6% polyethyleneimine on a Packard Filtermate. The filters were washed 6 times with ice cold 50 mM Tris/HCl pH 7.4. Following addition of 50 μl Microscint-40 per well, the filters were counted on a Packard Topcount. The K_D and B_{max} values were calculated in Origin (Microcal Software, USA) using a hyperbolic fit.

For displacement studies, the membranes (approximately 50 μg/well) were incubated with either 1.5 nM [^{3}H]des-Arg^{10}-kallidin for membranes expressing the rat

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        1                                                  50
Ratb1   MAS.EVLLEL QPSNRSLQAP ANITSCESAL EDWDLLYRVL PGFVITICFF
Murb1   MAS.QASLKL QPSNQSQQAP PNITSCEGAP EAWDLLCRVL PGFVITVCFF
Rabb1   MAS.QGPLEL QPSNQSQLAP PNATSCSGAP DAWDLLHRLL PTFIIAIFTL
Humb1   MASSWPPLEL QSSNQSQLFP QNATACDNAP EAWDLLHRVL PTFIISICFF
                                                       TM1
        51                                                 100
Ratb1   GLLGNLLVLS FFLLPWRQWW WQQRQRQQRL TIAEIYLANL AASDLVFVLG
Murb1   GLLGNLLVLS FFLLPWRRWW ...QQRRQRL TIAEIYLANL AASDLVFVLG
Rabb1   GLLGNSFVLS VFLLARR... ........RL SVAEIYLANL AASDLVFVLG
Humb1   GLLGNLFVLL VFLLPRR... ........QL NVAEIYLANL AASDLVFVLG
                                                   TM2
        101                                                150
Ratb1   LPFWAENIGN RFNWPFGTDL CRVVSGVIKA NLFVSIFLVV AISQDRYRLL
Murb1   LPFWAENVGN RFNWPFGSDL CRVVSGVIKA NLFISIFLVV AISQDRYRLL
Rabb1   LPFWAENVRN QFDWPFGAAL CRIVNGVIKA NLFISIFLVV AISQDRYSVL
Humb1   LPFWAENIWN QFNWPFGALL CRVINGVIKA NLFISIFLVV AISQDRYRVL
                                                TM3
        151                                                200
Ratb1   VYPMTSWGYR RRRQAQATCL LIWVAGGLLS IPTFLLRSVK VVPDLNVSAC
Murb1   VYPMTSWGNR RRRQAQVTCL LIWVAGGLLS TPTFLLRSVK VVPDLNISAC
Rabb1   VHPMASRRGR RRRQAQATCA LIWLAGGLLS TPTFVLRSVR AVPELNVSAC
Humb1   VHPMASRRQQ RRRQARVTCV LIWVVGGLLS IPTFLLRSIQ AVPDLNITAC
                                            TM4
        201                                                250
Ratb1   ILLFPHEAWH FARMVELNVL GFLLPVTAII FFNYHILASL RGQKEASRTR
Murb1   ILLFPHEAWH FVRMVELNVL GFLLPLAAIL YFNFHILASL RGQKEASRTR
Rabb1   ILLLPHEAWH WLRMVELNLL GFLLPLAAIL FFNCHILASL RRRGERVPSR
Humb1   ILLLPHEAWH FARIVELNIL GFLLPLAAIV FFNYHILASL RTREEVSRTR
                                      TM5
        251                                                300
Ratb1   CGGPKGSKTT GLILTLVASF LVCWCPYHFF AFLDFLVQVR VIQDCSWKEI
Murb1   CGGPKDSKTM GLILTLVASF LVCWAPYHFF AFLDFLVQVR VIQDCFWKEL
Rabb1   CGGPRDSKST ALILTLVASF LVCWAPYHFF AFLECLWQVH AIGGCFWEEF
Humb1   CGGRKDSKTT ALILTLVVAF LVCWAPYHFF AFLEFLFQVQ AVRGCFWEDF
                                        TM6
        301                                                350
Ratb1   TDLGLQLANF FAFVNSCLNP LIYVFAGRLL KTRVLGTL*. ..........
Murb1   TDLGLQLANF FAFVNSCLNP LIYVFAGRLF KTRVLGTL*. ..........
Rabb1   TDLGLQLSNF SAFVNSCLNP VIYVFVGRLF RTKVWELCQQ CSPRSLAPVS
Humb1   IDLGLQLANF FAFTNSSLNP VIYVFVGRLF RTKVWELYKQ CTPKSLAPIS
                  TM7

        351          365
Ratb1   .......... .....
Murb1   .......... .....
Rabb1   SSRRKEMLWG FWRN*
Humb1   SSHRKEIFQL FWRN*
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Fig. 1. Bradykinin B_1 receptor protein alignment. Predicted protein sequences from the rat, murine (10), rabbit (9) and human (8 and this study). Bradykinin B_1 receptors were aligned using the GCG PILEUP program. Predicted transmembrane domains are underlined and contentious amino acids are marked in italics.

receptor or 0.15 nM [^{3}H]des-Arg^{10}-kallidin for membranes expressing the human receptor, together with a range of concentrations of displacing ligand. Non-specific binding was determined with 3 μM des-Arg^{10}-kallidin. K_I values were calculated from the IC_{50} value (concentration causing 50% displacement of specific binding) using the Cheng–Prusoff approximation (Cheng and Prusoff, 1973).

2.13. Materials

Des-Arg^{10}-kallidin, HOE (D-Arg[Hyp^3,Thi^5,dTic^7,Oic^8] bradykinin), des-Arg^{10}-HOE, des-Arg^9-Leu^9 bradykinin and kallidin were from Pennisula labs. Sar^0-D-Phe^{8}des-Arg^9-bradykinin and T-kinin were from Nova Biochem. Lys-Lys[Hyp^3,Igl^5,D-Igl^7,Oic^8,des-Arg^9]-bradykinin and des-Arg^{11} T-kinin were from Phoenix and the Fujisawa compound, FR173657 ((*E*)-3-(6-acetamido-3-pyridyl)-*N*-[*N*-(2, 4-dichloro-3-[(2-methyl-8-quinolinyl) oxymethyl] phenyl]-*N*-methylaminacarbonylmethyl] acrylamide; Asano et al., 1997) was kindly synthesised and supplied by Dr. David Xu (Novartis, Summit USA).

3. Results

The rat bradykinin B_1 receptor cDNA and gene were isolated and the receptor sequence and gene structure were compared with other bradykinin receptors. It has the same predicted amino acid sequence as the genomic DNA recently cloned by Ni et al. (1998). We have further characterised the rat gene with respect to gene structure, tissue specific expression and induction and pharmacology. We have directly compared the pharmacological properties of cloned rat and human bradykinin B_1 receptors following expression in Cos-7 cells. The human bradykinin B_1 receptor cDNA was expression cloned from a WI38 cDNA library and had the same predicted amino acid sequence as the cDNA cloned by Menke et al. (1994).

3.1. Isolation of human and rat bradykinin B_1 receptor clones

A rat cosmid library from Sprague Dawley rat genomic DNA was screened as described above and a single positive clone was isolated. The putative protein coding domain in this clone appeared to be present on a single exon and a 1.3 kb *Bam*HI to *Eco*RI fragment, which contained the entire coding domain, was sub-cloned and sequenced.

A rat bradykinin B_1 receptor cDNA clone was isolated from a rat bladder library in λ ZAP express. This clone contained 103 bp of upstream untranslated sequence and 159 bp of 3′ untranslated sequence. The predicted coding domains from genomic DNA and cDNA derived clones showed that the putative protein coding domain was on a single exon and was contained in the *Bam*HI to *Eco*RI fragment that had been cloned. This fragment was sub-cloned into pcDNA3 for expression studies. The sequence of the rat cDNA was determined and found to be the same as that found by Ni et al. (1998). The amino acid sequence of the receptor was predicted and compared with the predicted bradykinin B_1 receptor sequences from human, rabbit and mouse (Fig. 1). The most striking differences between the predicted protein sequence of rat and both the rabbit and human bradykinin B_1 receptor are the fact that the rat receptor protein is twenty six amino acids shorter than the human and rabbit sequences at the C-terminus and that there an extra eleven amino acids in the predicted first intracellular loop of the rat receptor. In addition, the rat sequence lacks a consensus site for palmitoylation found in both the human and rabbit receptors. Like the rat, the predicted mouse receptor sequence also has a slightly longer first intracellular loop and shortened tail. When analysed with the GAP program, the rat and human pro-

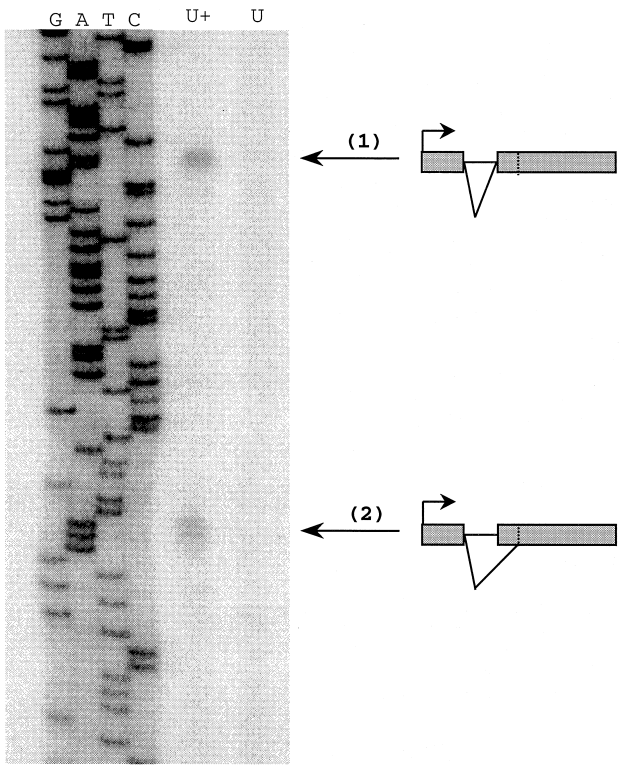

Fig. 2. Determination of the transcription start site by primer extension analysis. 5 μg of poly A$^+$ RNA isolated from control (U) and LPS treated (U$^+$) rat uterus were hybridised with an oligonucleotide primer 5′GCTGGAGCTCCAACAAGACCTCGGACGCCA 3′ and extended with reverse transcriptase as described in Section 2. Extension products were size fractionated on a denaturing polyacrylamide gel alongside a Sanger dideoxynucleotide sequencing ladder (G,A,T,C) primed on PUC18 with the universal primer 5′GTTTTCCCAGTCACGACGTTGTA3′. The initiations are shown by arrows. (1) corresponds to two initiation sites located 87 and 88 bp from the 3′ end of exon 1. (2) corresponds to two start sites 41 bp (the distance of the alternative splice donor) smaller than the initiation sites shown in (1). These sites at (1) and (2) correspond to a start site shown at the first and second of the three G residues 87 and 88 bp from the 3′ end of exon 1 assuming that they correspond to alternative splice variants. The start sites are shown diagrammatically alongside the initiation sites and confirmed in 5′ RACE.

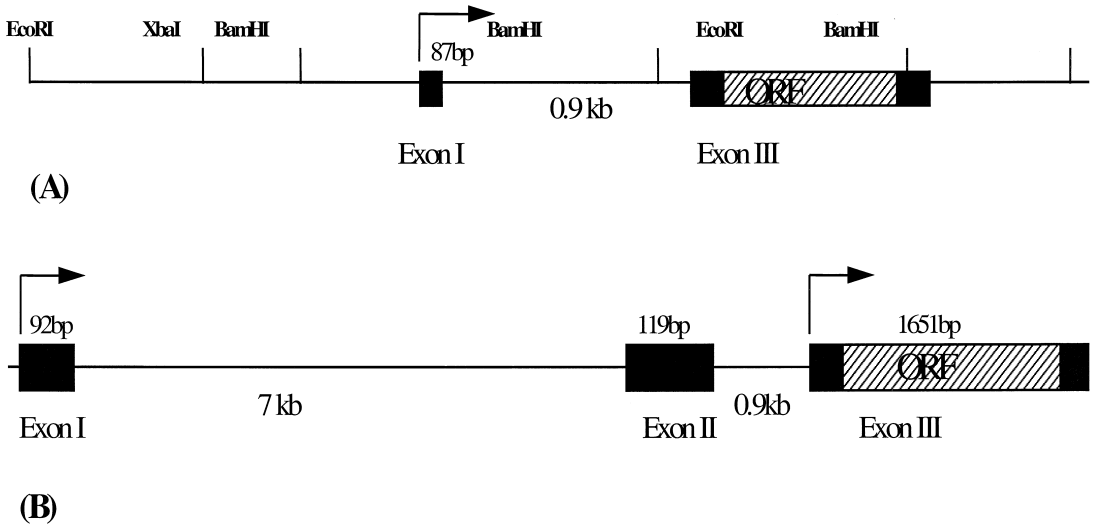

Fig. 3. Structural organisation of the rat and human B_1 bradykinin receptor genes. (A) Structural organisation of the rat bradykinin B_1 receptor gene. Locations of exons and introns and their sizes are shown. Exons are indicated by solid boxes, the coding region is shown by a hatched box inside exon II. Relevant restriction endonucleases utilised for genomic mapping are indicated. (B) Structural organisation of the human B_1 receptor gene (17,18). Locations of exons and introns and their sizes are shown. Exons are indicated by solid boxes, the coding region is shown by a hatched box inside exon III.

teins have 73.1% identity while the rat and murine proteins have 89.9% identity. The rat and rabbit proteins have 71.6% identity.

3.2. Gene structure

We have compared the sequence of genomic and cDNA clones from a bladder library and from 5′ RACE analysis (Frohman et al., 1988) of uterus RNA. Analysis of the rat bradykinin B_1 receptor gene transcription start sites using primer extension analysis (Fig. 2) and 5′ RACE analysis shows a start site located at the first and second of three G residues 20 bp downstream of the start site identified by Ni et al. (1998). Our results also show the existence of an alternative splice acceptor at the start of exon 2 as reported by Ni et al. (1998). The use of the alternative splice acceptor sites results in a 41 bp difference between the mRNA transcripts. We confirm the two exon structure of the rat bradykinin B_1 receptor gene (Fig. 3). Sequencing of the putative 5′ regulatory sequence showed that the sequence was the same as that reported by Ni et al. (1998) up to position −411 bp (relative to our reported start site) at which point the sequences diverge (Accession no. AJ237644). Alignment of our 87 bp of sequence from the point of divergence showed 77.5% homology with the human bradykinin B_1 promoter. We have yet to determine whether this region can function as a promoter and confer LPS inducibility of the rat bradykinin B_1 receptor gene. Like the human gene, the entire open reading frame of the rat bradykinin B_1 receptor is encoded within a single exon. The structure of the rat bradykinin B_1 receptor gene and comparison with the human bradykinin B_1 receptor gene is illustrated in Fig. 1. The rat bradykinin B_1 receptor gene consists of two exons of 87–88 bp (depending on which

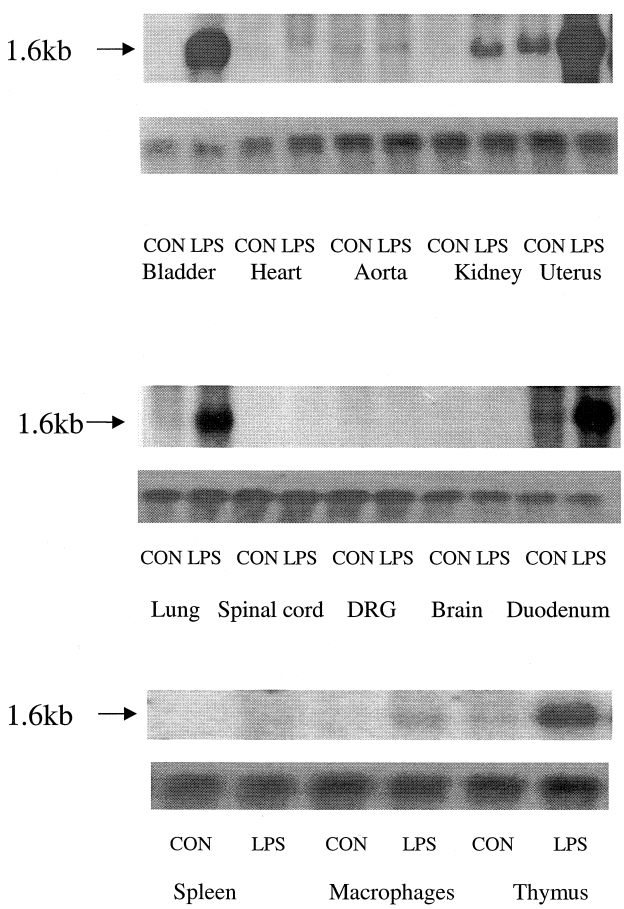

Fig. 4. Northern blot analysis. Northern blot analysis of rat B_1 bradykinin receptor mRNA extracted from tissues taken from rats after treatment with LPS or no treatment. Blots were probed with ^{32}P-labelled probes from rat B_1 bradykinin receptor (upper panels) stripped and re-probed with β-actin (lower panels). We have shown the 1.6 kb band from one of three experiments. CON = control and LPS = Lipopolysaccharide treated as described in Section 2.

start site is used) and 1224 bp respectively, separated by an intron of 1126 bp. This differs from the reported human bradykinin B_1 receptor gene structure, which has three exons, separated by two introns (Bachvarov et al., 1996; Yang and Polgar, 1996). Alignment of the 5′ non-coding region of the rat and human bradykinin B_1 receptor sequence shows 65.4% identity between exon 1 of the rat with exon 1 of the human. In comparison, alignment of exon 1 of the rat with exon 2 of the human shows only 41.4% identity. This suggests that exon 1 of the rat bradykinin B_1 receptor gene is equivalent to exon 1 of the human bradykinin B_1 receptor gene. Interestingly, we find no evidence from primer extension or 5′ RACE studies for the existence of a transcription start site at the beginning of the protein coding exon, as is seen in the human bradykinin B_1 receptor gene.

3.3. Gene expression studies

Northern blot analysis of rat tissues using the rat bradykinin B_1 receptor gene as a probe revealed a distinct pattern of expression in tissues.

Treatment of rats with bacterial LPS resulted in a five to fifteen fold increase in bradykinin B_1 receptor mRNA in bladder, lung, duodenum, kidney, uterus, thymus and heart and a less than five fold increase in macrophages, aorta and spleen and we could not detect significant message in spinal cord, dorsal root ganglia or brain (Fig. 4). Two extra transcripts of 4 and 8 kb that are induced along with the expected band of 1.6 kb were detected in Northern blots of tissues with abundant induced expression. These longer transcripts are due to read-through of the RNA polymerase into 3′ flanking regions, since a genomic probe located 3′ of the putative polyA$^+$ addition site only hybridised to the 4 and 8 kb transcripts (data not shown).

3.4. Pharmacological comparison

The kinin system in the rat is different from the human system in two respects. Firstly kininogen gives rise only to bradykinin in the rat and not to kallidin. Secondly, the rat expresses T-kininogen, a protein that does not occur in the human. T-kininogen, is susceptible to kininogenase and gives rise to T-kinin (Ile-Ser-bradykinin), a ligand that is unique to small rodents (e.g., rat and mouse). It seems likely, therefore, that the pharmacology of the rat receptor will be significantly different from that of human B_1 receptor. In particular, the pharmacology of T-kinin and its putative kininase I metabolite des-Arg^{11}-T-kinin have not been studied in either the rat or the human bradykinin B_1 receptor.

The pharmacological properties of the rat receptor were examined by investigating the ability of a range of kinin agonists and antagonists to inhibit the binding of [^{3}H]des-Arg^{10}-Kallidin. For comparison the pharmacology of the human B_1 receptor was investigated in parallel. Initially, ligand saturation experiments were performed with [^{3}H]-des-Arg^{10}-Kallidin on membranes from Cos-7 cells expressing the rat and the human B_1 bradykinin receptors. The cloned rat receptor had a K_D for [^{3}H]des-Arg^{10}-kallidin of 1.01 ± 0.21 nM ($n = 3$) (B_{max} 0.276 ± 0.012 fmol/μg, $n = 3$). This was lower than the affinity of the cloned human receptor for [^{3}H]des-Arg^{10}-Kallidin determined in the present study (K_D value of 0.064 ± 0.021

Table 1
Displacement of [^{3}H]des-Arg^{10}-kallidin binding from membranes prepared from Cos-7 cells expressing the rat or human bradykinin B_1 receptors

Ligand	Human B_1 K_I (nM)	Rat B_1 K_I (nM)
Bradykinin and bradykinin-derived ligands		
Bradykinin	10,732 ± 3986	6199 ± 920
des-Arg^9-bradykinin	1620 ± 153	15 ± 5
des-Arg^9-Leu^8-bradykinin	116 ± 32	31 ± 9
Sar^0-D-Phe^{8}des-Arg^9-bradykinin	1919 ± 87	59 ± 19
T-kinin and T-kinin-derived ligands		
T-kinin	5898 ± 233	8282 ± 1839
des-Arg^{11}-T-kinin	1492 ± 355	46 ± 14
Kallidin and kallidin-derived ligands		
Kallidin	15 ± 3	285 ± 38
des-Arg^{10}-kallidin	0.19 ± 0.05	1.6 ± 0.2
Lys-Lys[Hyp^3,Igl^5,D-Igl^7, Oic^8, des-Arg^9]-bradykinin	1.58 ± 0.36	3.0 ± 1.0
Other ligands		
HOE140	154 ± 48	573 ± 169
des-Arg^{10}-HOE140	4.9 ± 1.3	35 ± 7
FR 173657 (non-peptide B_2 antagonist)	> 30,000	> 30,000

Membranes (approximately 50 μg protein/well) were incubated with 0.15 nM (human) or 1.5 nM (rat) [^{3}H]des-Arg^{10}-kallidin and a range of concentrations of displacer for 1 h at 4°C. Non-specific binding was determined with 3 μM des-Arg^{10}-kallidin. K_I values were calculated from the IC_{50} value (Cheng and Prusoff, 1973).

nM ($n = 3$), B_{max} 0.092 ± 0.005 fmol/μg ($n = 3$)) and in a previous study by Menke et al. (1994) (K_D value 0.4 nM).

The results from the displacement studies are summarised in Table 1. The K_I value for des-Arg^{10}-Kallidin at the cloned rat receptor was 1.6 ± 0.2 nM ($n = 3$) (see displacement curve in Fig. 5) which was in good agreement with the K_D value obtained in saturation experiments carried out with [^{3}H]-des-Arg^{10}-Kallidin (see above) and with the value of 2.42 nM reported by Ni et al. (1998) for displacement of [^{125}I]Sar-TyrεAhx-Lys-des-Arg9-Bk from the rat B_1 receptor cloned from a vascular smooth muscle cell line. The K_I value for des-Arg^{10}-Kallidin at the human B_1 receptor (0.19 ± 0.05 nM, $n = 3$) was also in agreement with its K_D value in the saturation experiments.

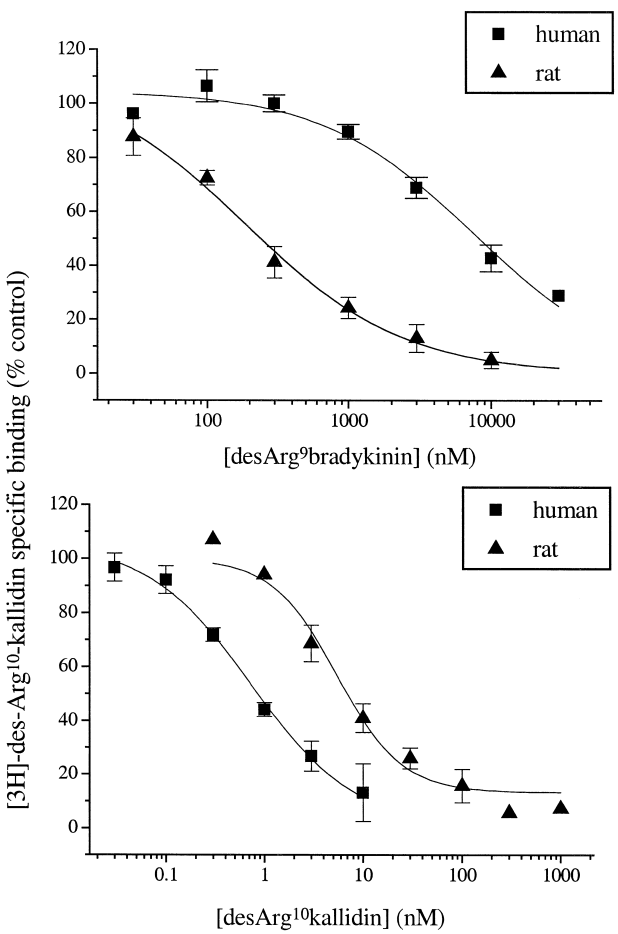

Fig. 5. Inhibition of [^{3}H]des-Arg^{10} kallidin binding to the rat and human bradykinin B_1 receptors by des-Arg^9-bradykinin des-Arg^{10}-Kallidin. [^{3}H]des-Arg^{10} Kallidin (0.15 nM for the human receptor and 1.5 nM for the rat receptor) was incubated with membranes from Cos-7 cells (approximately 50 μg protein/assay) expressing either the rat or the human bradykinin B_1 receptor for 1 h at 4°C with increasing concentrations of the displacing ligand. Non-specific binding was determined with 3 μM des-Arg^{10}-kallidin. The data shown are the mean ± SEM of 3 to 4 separate experiments performed in triplicate.

The bradykinin B_2 receptor ligands, bradykinin and T-kinin had extremely low potency for both the rat and the human bradykinin B_1 receptors. Kallidin, on the other hand, which is also a B_2 ligand, was relatively active at the human bradykinin B_1 receptor (K_I value 15 ± 3 nM, $n = 3$) and even at the rat receptor it had some activity (K_I value 285 ± 38 nM, $n = 3$). The displacement curves for the bradykinin metabolite, des-Arg^9-bradykinin are also shown in Fig. 5. In contrast to des-Arg^{10}-kallidin, which had higher affinity for the human bradykinin B_1 receptor than for the rat B_1 receptor, des-Arg^9-bradykinin had considerably higher affinity for the rat receptor (K_I value 15 ± 5 nM, $n = 3$) than the human B_1 receptor (K_I value 1620 ± 153 nM, $n = 3$). Similarly, the bradykinin-derived antagonists des-Arg^9-Leu^8-bradykinin and Sar^0-D-Phe^{8}des-Arg^9-bradykinin had higher affinities for the rat receptor (K_I values 31 ± 9 nM ($n = 3$) and 59 ± 19 nM ($n = 3$), respectively) than the human receptor (K_I values 116 ± 32 nM ($n = 3$) and 1919 ± 87 nM ($n = 3$), respectively). Interestingly, des-Arg^{11}-T-kinin, the putative kininase I metabolite of T-kinin, also had significantly higher affinity for the rat bradykinin B_1 receptor (K_I value 46 ± 14 nM, $n = 3$) than for the human receptor (K_I value 1492 ± 355 nM, $n = 3$). The non-peptide bradykinin B_2 receptor antagonist from Fujisawa (FR173657) was completely inactive at both the human and the rat bradykinin B_1 receptors, although the peptide B_2 receptor antagonist HOE140 had low affinity for both receptors.

4. Discussion

We have described the cloning and molecular characterisation of the rat bradykinin B_1 receptor. The sequence is identical to that described recently by Ni et al. (1998). Both the structure and pharmacology of the rat receptor show a high degree of similarity to the mouse bradykinin B_1 receptor described by Hess et al. (1996) and Pesquero et al. (1996). In contrast, both rodent receptors show marked differences from the human and rabbit receptor sequences. Significant divergence of the bradykinin B_1 and B_2 receptors has been noted previously (Menke et al., 1994). Despite their limited homology to each other, bradykinin B_1 and B_2 receptors are obviously functionally related in that they both bind derivatives of kallidin and bradykinin. These receptors are most likely derived by divergent evolution from the same ancestral gene (Schroeder et al., 1997). The structural and functional differences seen between bradykinin B_1 receptors from different species represents further divergent evolution within this subgroup of receptors.

The exon/intron structure of the rat bradykinin B_1 receptor gene differs from that seen in the human receptor gene, with two exons instead of three. However, the coding domain of the rat receptor is found on one exon, as

is the case for the human bradykinin B_1 receptor (Bachvarov et al., 1996; Yang and Polgar, 1996). We have noted the existence of a previously undescribed transcription start site which is about 20 bases downstream of that described by Ni et al. (1998) and this indicates that there are subtle differences in transcription of this gene in different cell types. We have confirmed the existence of two splice variants in the rat receptor mRNA (Ni et al., 1998). It is not known if similar splice variants exist in the human bradykinin B_1 receptor mRNA and it is also unclear if there is any functional significance of the different mRNA forms. It is possible that they alter mRNA stability or the efficiency of translation.

The observed differences between the predicted protein sequences of the rat, mouse, human and rabbit bradykinin B_1 receptors is interesting. The large insertion of basic amino acids in the first cytoplasmic loop of the rat protein together with the shortened C-terminal cytoplasmic tail, may be reflected in differences in the coupling of the rat and human receptors to heterotrimeric G-proteins.

The rat bradykinin B_1 receptor mRNA is regulated by LPS to varying degrees in different tissues. In uterus, lung, bladder and duodenum, the RNA is very strongly upregulated from low basal levels, while in kidney and aorta, it is readily detectable before induction and it does not go up significantly. In other tissues, such as heart and thymus it is regulated but the final levels are not very high. We were unable to detect the mRNA in brain, spinal cord or DRG. The lack of significant mRNA signal from DRG is in agreement with our previous findings that the bradykinin B_1 receptor is not expressed on DRG neurones (Davis et al., 1996). Thus there appears to be a great deal of heterogeneity of responses to LPS which may reflect the heterogeneity of the signalling pathways in different tissues and the way in which the promoter of the gene responds to them. In tissues where there is strong upregulation of the mRNA, the bradykinin B_1 receptor may play a key role in pathophysiological mechanisms.

The pharmacology of the bradykinin B_1 receptor cloned from rat was similar to the receptor described by Ni et al. (1998) and to that of the native rat bradykinin B_1 receptor. It was also similar to that of the cloned mouse B_1 receptor (Hess et al., 1996). Competition binding studies, using [^{3}H]des-Arg^{10}-kallidin as the radioligand, gave a K_I value for des-Arg^9-bradykinin, a natural ligand for the B_1 receptor in the rat of 15 ± 5 nM. This value was somewhat higher than the value of 2.03 nM obtained by Ni et al. (1998) for the displacement of [^{125}I]Sar-TyrεAhx-Lys-des-Arg^9-Bk by des-Arg^9-bradykinin from the cloned rat B_1 receptor, but lay within the range of EC_{50} values obtained at the native rat receptor, (0.8 nM in rat gastric mucosa (Brown et al., 1992) to 46 nM in rat portal vein rings (Campos and Calixto, 1994)). Similarly, the K_I value for the selective bradykinin B_1 receptor antagonist des-Arg^{9}Leu^8-bradykinin at the cloned rat receptor was 31 nM, which lay within the range of activities obtained at the native rat receptor: these range from a K_I value of 0.5 nM in rat mesangial cells (Bascands et al., 1993) to a pA2 of 6.7 in rat portal vein rings (Campos and Calixto, 1994). The K_I value for des-Arg^{10}-kallidin at the cloned rat B_1 receptor in this study (1.6 ± 0.2, $n = 3$) was in good agreement with the value obtained by Ni et al. (1998) for displacement of [^{125}I]Sar-TyrεAhx-Lys-des-Arg^9-Bk (2.42 nM) from the rat B_1 receptor cloned from A10 cells and also with K_I value for des-Arg^{10}-kallidin at the mouse receptor (Hess et al., 1996). Fewer studies have been carried out with des-Arg^{10}-kallidin at the native rat receptor, but in the ileum, the PD_2 for this agonist has been reported as 7.67 (Meini et al., 1996). It is, perhaps surprising, that des-Arg^{10}-kallidin is so active at the B_1 receptor in rats and mice when this peptide is not formed in these species. In fact in the present study des-Arg^{10}-kallidin was somewhat more potent that des-Arg^9-bradykinin, at the rat receptor, and Ni et al. (1998) reported that these two ligands had equal affinity for the rat receptor. Despite this, however, it is significant that des-Arg^{10}-kallidin was about ten times more potent at the human than at the rat B_1 receptor and that its affinity for the human B_1 receptor was over 5000-fold greater than for des-Arg^9-bradykinin.

In addition to the lack of kallidin in the rat, a major difference between the human and rat kinin systems is the presence of T-kininogen in rat which gives rise to T-kinin. The affinity of bradykinin B_1 receptors for T-kinin and its potential kininase I derivative des-Arg^{11}T-kinin had not previously been studied. T-kinin itself had very low affinity for both the human and the rat bradykinin B_1 receptor. This was not surprising as it is likely to be an agonist at the bradykinin B_2 receptor. Preliminary data (not shown) indicated that T-kinin was 200-times more active at the rat B_2 receptor than at the rat B_1 receptor and that it was about 20-times more potent at the rat B_2 bradykinin receptor than at the human B_2 receptor. Des-Arg^{11}-T-kinin, however, had relatively high affinity for the rat B_1 receptor (K_I value 46 ± 14 nM) although its affinity for the human B_1 receptor, which would not be expected to come into contact with this ligand was considerably lower (30-fold).

It would appear, therefore, that because of the differences in the kinin systems between human and rodents, the human receptor has evolved greater sensitivity to kallidin and des-Arg^{10} kallidin, whereas the rodent bradykinin B_1 receptor has evolved sensitivity towards bradykinin and T-kinin derivatives. However, despite the fact that kallidin is not formed in the rodent, its bradykinin B_1 receptors are capable of responding to des-Arg^{10}-kallidin in a relatively sensitive manner.

The region of the receptor responsible for these affinity differences is not clear. The greatest differences between the rodent and the human receptors is in the tail and intracellular loop regions, however these regions are unlikely to be involved in peptide binding. The sequence differences, which account for the different pharmacologies are likely to be more subtle. The affinity and selectiv-

ity of peptide agonists for bradykinin B_1 and B_2 receptors is greatly influenced by basic residues at the N- and C-termini of the peptides and some of the determinants in the sequence of B_1 and B_2 receptors may interact with those terminal regions. Determinants in the sixth transmembrane domain of the bradykinin receptors are known to affect the binding of des-Arg^{10} kallidin (Leeb et al., 1997), although the authors did not identify the interacting residues. By comparing the predicted protein sequences of the four known bradykinin B_1 receptors in transmembrane domain six and the third extracellular loop and by classifying rabbit and human receptors, which have similar pharmacological properties (MacNeil et al., 1995), together and rat and mouse receptors together (this paper, Hess et al., 1996; Ni et al., 1998) it is possible to see five residues that are the same in the rat and mouse and either the same or similar in the human and rabbit. Using rat receptor numbering, these are glycine at position 260 for alanine in human and rabbit, aspartate at 283 for glutamate, arginine at 289 for either histidine in human or glutamine in rabbit, aspartate at 293 for glycine and lysine at 297 for glutamate. The only other candidate in the rest of the receptor would be arginine at 110 for glutamine in the first extracellular loop between transmembrane domains two and three. Since the affinity of des-Arg kinin derivatives for the rat bradykinin B_1 receptor is greater than for the human receptor, it appears likely that the C-terminus of these peptides interact with an amino acid that has a similar charge to arginine in the rat and mouse but not in the human or rabbit. This could be arginine 289 or lysine 297. Mutating bradykinin B_1 receptors and comparing their pharmacology in an heterologous expression system could test the involvement of these residues.

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