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Review

The kinin B_2 receptor gene structure, product processing and expression in adult and fetal rats: evidence for gene evolution

C.E. França[1], C.F. Vicari[1], A.M. Piza[1], E.A. Geroldo[1], M.L. Beçak[2], W. Beçak[2], R.C. Stocco[2] and C.J. Lindsey[1]

[1]Departamento de Biofísica, Universidade Federal de São Paulo, São Paulo, SP, Brasil
[2]Laboratório de Genética, Instituto Butantan, São Paulo, SP, Brasil

Corresponding author: C.J. Lindsey
E-mail: c.lindsey@unifesp.br

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ABSTRACT. We examined the structure of the rat kinin B_2 receptor gene (KB_2r) and encoding messenger RNA (mRNA) processing. Differently from the closely related mouse and rabbit genes that have three exons and two introns, the rat gene purportedly consists of four exons and three introns. There are two purported gene products; one of them contains an upstream ~180-bp open reading frame region ("exon-X") potentially expressed as a result of alternative processing. To examine the processing of rat KB_2r mRNA, cDNA amplicons were generated using primer pairs directed towards 5' or 3' exon or intron flanking regions. Analyses of intron/exon primary cDNA amplicons showed that introns 1 to 3 are removed sequentially and that "exon-X" removal follows that of intron-3. No evidence was found for "exon-X" expression in polyadenylated (mature) mRNA of adult Wistar, Wistar Kyoto, spontaneously hypertensive or Sprague-Dawley rat tissues. Nor was "exon-X" detected in tissues subject to inflammatory stimulus expressing B_1 kinin receptor mRNA or in 1- to 21-day-old rat embryos or fetuses. The lack of evidence for the expression of "exon-X" in mature mRNA indicates

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that the structure of the rat gene is similar to that of the mouse, rabbit and human genes, all consisting of three exons and two introns. The "exon-X" fragment may result from interstitial gene duplication, be a fragment of the ancestral gene, or most likely heterologous transposon insertion of an exon-like fragment into intron-2 of the KB_2r gene.

Key words: Alternative processing; Rat embryos; Kinin B_2 receptor gene

INTRODUCTION

Bradykinin, a potent neuropeptide and a vasoactive peptide, has a wide spectrum of biological activities (Couture and Lindsey, 2000; Leeb-Lundberg et al., 2005). Under physiological conditions, kinin activity is mediated by the interaction of the B_2 kinin receptors (Leeb-Lundberg et al., 2005). These are present in organ and arterial smooth muscle cells and in rat and human brain nuclei, such as the paratrigeminal nucleus and the nucleus of the solitary tract (Lindsey et al., 1997; de Souza Buck et al., 2002). Stimulation of arterial B_2 kinin receptors causes resistance vessel vasodilatation and hypotension (Sharma, 2006). On the other hand, stimulation of central kinin receptors leads to blood pressure increases (Lindsey et al., 1997), mediated by central sympathetic mechanisms (Caous et al., 2004). Thus, among other important functions, such as blood pressure regulation, kinins are characterized as mediators of pain and inflammatory responses, both in the central and in the peripheral nervous systems (Leeb-Lundberg et al., 2005); they are also important mediators in the cascade of the febrile response (Pessini et al., 2006). Additionally, bradykinin apparently plays a role in the rat reproductive tissues via the kinin B_2 receptor (Holland et al., 2001). As vascular agents, they promote vascular permeability and vasodilatation (Leeb-Lundberg et al., 2005), which are important mechanisms related to the anti-hypertensive action of kinase II inhibitors (Lindsey et al., 1987).

Two receptor types, both of the protein G receptor family, are known to mediate kinin effects in different tissues throughout the body (Leeb-Lundberg et al., 2005). The B_1 receptor, normally not present in healthy tissues, has its expression promoted by inflammatory responses caused by injury, infection, or endogenous and exogenous pyrogens (Lungu et al., 2007). The transcription nuclear factor κB is involved in inducible expression of the human and rat B_1 receptor gene (Ni et al., 1998; Medeiros et al., 2004). On the other hand, the B_2 kinin receptor is constitutively expressed in most body tissues and is commonly involved in most of the biological activity of kinins in healthy tissues (Leeb-Lundberg et al., 2005).

In the rat, the coding sequence of B_2 kinin receptors, deduced from cDNA (McEachern et al., 1991), and the cloning and sequencing of the gene (Pesquero et al., 1994; Wang et al., 1994) accompanied molecular characterization of the human (Eggerickx et al., 1992; Powell et al., 1993; Park et al., 1994; Ma et al., 1994a; Kammerer et al., 1995), murine (McIntyre et al., 1993; Ma et al., 1994b), guinea pig (Farmer et al., 1998), rabbit (Bachvarov et al., 1995), dog (Hess et al., 2001), chicken (Schroeder et al., 1997), frog (Suzuki et al., 2007), and zebrafish (Bromée et al., 2005) B_2 receptor gene. The B_1 receptor gene has also been cloned and sequenced from humans (Bachvarov et al., 1996), rabbits (MacNeil et al., 1995), mice (McIntyre et al., 1993) and rats (Ni et al., 1998; Jones et al., 1999). Genomic Southern blot analysis of human, rabbit and rat genomes revealed that a single-copy gene encodes the kinin B_2 receptor (Kammerer et al., 1995; Bachvarov et al., 1995). This also holds true for the human B_1 receptor (Bachvarov et al., 1996).

Several reports indicate that the gene of these two receptors contains three exons separated by two introns and an intronless coding region located on the third exon (McIntyre et al., 1993; Ma et al., 1994a,b; Wang et al., 1994; Bachvarov et al., 1995). However, Pesquero et al. (1994), based on the findings of others (McEachern et al., 1991), included an "extra" exon in the gene, predicting a four exon-three intron gene structure for the rat (*Ratus norvegicus*) kinin B_2 receptor gene. Alternative splicing of B_2 receptor mRNA would result in rat "exon-3", herein named "exon-X". This exon, not predicted for mice or for the human kinin B_2 receptor gene, was suggested based on evidence from amplification of reverse-transcriptase products from total RNA (Pesquero et al., 1994). Results obtained with domain-specific anti-peptide antibodies, amino acid sequence analysis and *in vitro* transcription and translation suggested a first translation initiation codon in the human and rat "exon-X" (AbdAlla et al., 1996) rather than in exon-4, as previously thought. This would imply a four-exon structure for the rat and human kinin B_2 receptor gene. Translation of gene products initiating either in rat or human exon-3 or exon-4 would putatively lead to expression of B_2 receptor subtypes. However, translation products containing the extended N-terminus have never been reported in rats or humans (Couture and Lindsey, 2000).

Alternative processing has been suggested for a number of receptor proteins, including rat kinin B_1 (Ni et al., 1998), the NK1 tachykinin (substance P) receptor (Page, 2006), the endothelin B receptor (Suzuki et al., 2004) and the glutamate-receptor-coupled ion channel (Kumar et al., 2009). However, experimental evidence shows that the single type of mature, polyadenylated mRNA does not contain the proposed "exon-X". Furthermore, given that the rat B_2 gene is highly related to the mouse and guinea pig genes, a different gene structure and alternative processing of the rat B_2 receptor mRNA is unlikely.

For a better understanding of the processing of rat kinin B_2 receptor gene products and to gain insight concerning gene structure, we analyzed mature and immature RNA transcripts in adult hypertensive and normotensive rat strains, rat embryos and fetuses, as well as in animals expressing the B_1 kinin receptor induced by pyrogen treatment. The 211 bp of the purported "exon-X" kinin B_2 gene and predicted protein sequences were respectively compared to available data base sequences for a clearer understanding of the structure of this particular gene as to further our knowledge on gene structure and evolution.

MATERIAL AND METHODS

Tissue specimens

Tissues were collected from five adult (three months old) Wistar, Wistar Kyoto, spontaneously hypertensive rats (SHR; Okamoto et al., 1964), and Sprague-Dawley male and female rats. Under CO_2 anesthesia, animals were quickly dissected for removing the aorta, mesenteric arteries, liver, uterus, lungs, ovaries, hypothalamus, lower brainstem, solitary tract, and paratrigeminal nuclei. Approximately 50 mg of each tissue sample was rapidly frozen in liquid nitrogen and then stored in phenol solution (Trizol, Invitrogen Corporation, Carlsbad, CA, USA) at -80°C for not over 24 h before processing.

Embryo collection

Five female Wistar rats per group were treated with 150 IU/kg pregnant mare serum

gonadotropin (Sigma Chemical Company, St. Louis, MO, USA) and after 46 h with 75 IU/kg human chorionic gonadotropin (Sigma Chemical Company) to provoke super ovulation. Animals were mated and 24 h later vaginal smears were collected for pregnancy verification. From days 1 to 5, 50 embryos were collected from oviducts of 10 rats with 80 µL hyaluronidase (Sigma Chemical Company) to detach embryo cells from granulosa cells; the embryos were washed with M2 and stored in M16 medium (Sigma Chemical Company) and stored in phenol solution at -80°C. Six- to 21-day fetuses were directly collected from the rat uterus, chopped into small pieces, homogenized in a phenol solution, rapidly frozen in liquid nitrogen and stored at -80°C.

LPS treatment

Sixteen animals were injected intraperitoneally with the vehicle alone or 50 µg/kg *Escherichia coli* bacterial endotoxin lipopolysaccharide (LPS, Sigma Chemical Company) 4 h before sacrifice. Body temperature was monitored every 15 min to determine if there was a febrile response.

RNA extraction

Embryos or 50 mg samples of tissues or fetus were homogenized in 1, 2 or 3 mL phenol. Following addition of chloroform, total RNA was precipitated from the aqueous phase with isopropanol and washed with 75% ethanol.

Reverse transcriptase and amplification reactions

Five-microliter aliquots of total RNA, suspended in RNAse-free water, were submitted to reverse transcriptase (Mulv, Invitrogen Corporation) and c-DNA amplification (ThermoScript RT-PCR system, Invitrogen Corporation) with oligo dT primers for mature mRNA or with random primers for primary mRNA. c-DNA amplification was carried out with selective primers directed towards regions close to the intron/exon boundaries. Amplification products were revealed on 1.5% agarose gels (Gibco, New York, USA). Selected samples were reamplified and the reaction products examined on silver-stained 12.5% polyacrylamide gels (PhastSystem, Pharmacia Biotech, Uppsala, Sweden) and stained with ethidium bromide (United Biochemical Corp., Cleveland, OH, USA). Uterine samples were cycle-sequenced using an Alf automatic sequencer (Pharmacia Biotech).

Statistical analysis

All data are reported as means ± SEM and were analyzed by ANOVA followed by the Student *t*-test for unpaired analysis. Results with $P < 0.001$ were considered to be significant.

RESULTS

Processing of the kinin B_2 receptor gene

In order to investigate the sequence of intron removal, primary mRNA cDNA was

amplified with primers directed to 3' boundaries of introns 1, 2, or 3 and to the 3' regions of the downstream exons. Table 1 shows the primer location on the kinin B_1 and B_2 receptors as well as on the reference β-actin protein-encoding genes; it also shows the expected size of the respective amplification products.

Table 1. Location of primer pairs, nucleotide sequences and amplification product sizes expected for cDNA of primary or ripe mRNA from Wistar rat kinin B_2, kinin B_1 receptor and ß-actin proteins.

Protein	Location/nucleotide		Nucleotide sequence	Expected product sizes (kb/bp)	
				Primary mRNA	Ripe mRNA
Kinin B_2 receptor					
fKB_2-E1a	Ex1-Ex3	74 to 95	5'GAC TCC CTA CAA CAC AGA ACC G 3'	~3.5, 1.7, 0.9, 0.7	702
rKB_2-E3a		986 to 966	5' CCG TGA CGT TGT GCC CCT CTT 3'		
fKB_2-E2	Ex2-Ex3	160 to 177	5'AAG CGA CCC GTG CTC CTG 3'	~2.9, 1.2, 0.38, 0.17	169
rKB_2-E3		539 to 518	5' CCT GGA TGG CAT TGA GCC AGC T 3'		
fKB_2-EX	ExX-Ex3	294 to 313	5'CGC TGT GCC TCC TCC CGT GC 3'	~1.1, 0.25	-
rKB_2-E3		539 to 518	5' CCT GGA TGG CAT TGA GCC AGC T 3'		
fKB_2-In2	In2-Ex3	-120 to -140	5' GCA TCT TCA TAA TCA TGC TT 3'	~1.1	-
rKB_2-EX		539 to 518	5' CCT GGA TGG CAT TGA GCC AGC 3'		
fKB_2-In1	In1-ExX	-250 to -271	5' GAG CAT CCA GTG CTC ATA ATG 3'	~2	-
rKB_2-E3b		353 to 332	5' TTG TGT CCA TGC AGC AGA GCA C 3'		
Kinin B_1 receptor					
fKB_1-E1	Ex1-Ex2	143 to 162	5' CTG TGG ATG GCG TCC GAG GT 3'	-	~806 bp
rKB_1-E3		939 to 923	5' GCT ACC AGT GTG AGG AT 3'		
β-actin					
fBAc-E1	Ex2-Ex4	92 to 108	5' ATA TCG CTG CGC TCG TC 3'	-	597 bp
rBAc-E2		688 to 670	5' TGG TGG TGA AGC TGT AGC C 3'		

Contrary to exon/exon primer pairs that generated multiple fragments, each intron/exon primer pair produced only one amplification product. Thus, intron-1, -2 and -3/exon-3 primer pairs generated 0.17-, 1.1- and 3-kb fragments, respectively, each containing all downstream introns and exons of the gene (Figure 1A). This may be taken as unequivocal evidence that introns 1, 2 and 3 are removed in sequential order. Similarly, the intron-2/exon-3 primer pair produced a 1.2-kb fragment containing "exon-X", intron-3 and the 5' region of exon-3 (Figure 1A).

On the other hand, amplification of primary mRNA with primers directed towards 5' regions of exons and 3' regions of downstream exons produced multiple fragments, corresponding to the different stages of mRNA processing. The exon-1/exon-3 primer pair produced four fragments: a 702-bp product containing exon-2 and the 3' and 5' areas of exons 1 and 3; a 913-bp fragment containing exon-2 and exon-X; a 1.1-kb fragment containing exon-2, exon-X and intron-3, and a 3-kb product containing, in addition to the latter, 1.8 kb of intron-2. The ~26-kb fragment containing the ~23-kb sequence of intron-1, if amplified to any extent, would be unresolvable on high-density gels.

The exon-2/exon-3 primer pair produced four products, with sizes of 0.17, 0.38, 1.3, and 3.1 kb, all homologous to the fragments described above. The exon-X/exon-3 primer pair generated two products sized 0.23 and ~1.1 kb, one of which contained the 0.8-kb sequence of intron-3, making "exon-X" the last fragment to be removed (Table 1, Figure 1).

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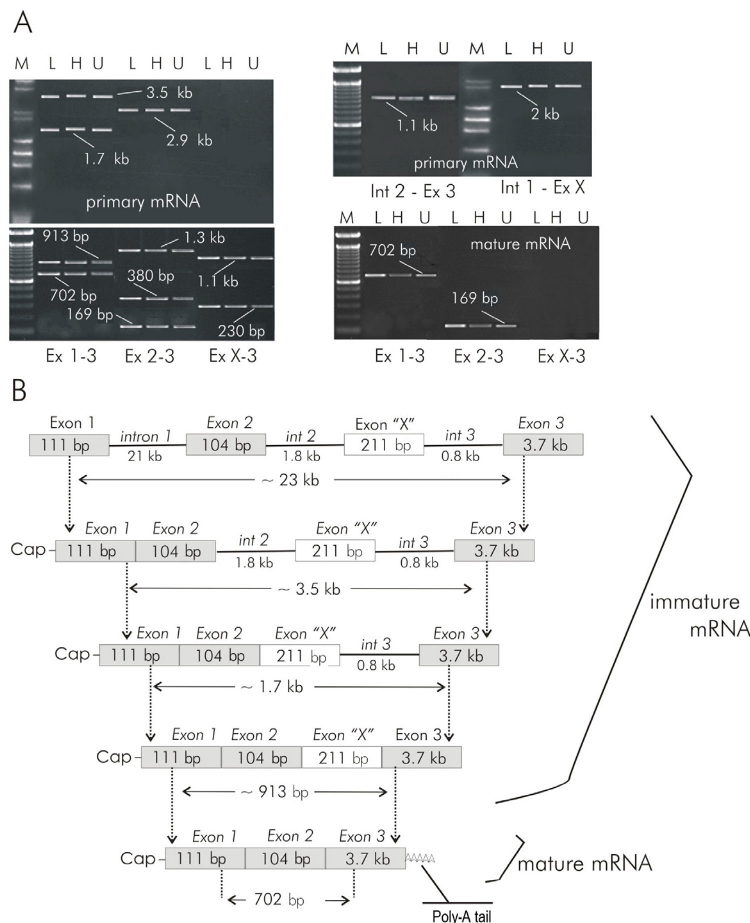

Figure 1. The processing of the rat B_2 kinin receptor protein. **A.** Reverse transcriptase amplifications of primary and mature mRNA representative of Wistar, Wistar Kyoto, Sprague-Dawley, and spontaneously hypertensive rat tissues. To the left, agarose gels showing exon-1/exon-3, exon-2/exon-3 and "exon-X"/exon-3 primary mRNA amplicons. The top right gel shows primary mRNA amplicons of intron-1/exon-2 and intron-2/ "exon-X" primer pairs. At the bottom right, the mature mRNA amplification products of exon/exon primer pairs. **B.** Representation of the processing of the kinin B_2 gene, deduced from the analyses of exon/exon and intron/exon amplification products shown in *A*. Note the sequential removal of introns 1, 2, and 3, followed by that of "exon-X", representing a three-exon gene structure in the form of mature mRNA genomic transcripts. M = molecular weight marker; L = liver; H = hypothalamus; U = uterus; Cap = 5´capping of primary mRNA; Poly-A tail = polyadenylation of 3' mature mRNA.

Exon composition of kinin B_2 receptor-encoding mature mRNA

The amplification of reverse transcribed cDNA from polyadenylated mRNA (RT/

PCR) generated only one gene product, independent of the primer pair, with the exception of the primer pairs with one constituent directed towards exon-X. Thus, the exon-1/exon-3 pair produced a 702-kb fragment and the exon-2/exon-3 primer pair produced a 170-kb product.

Amplification of mature, polyadenylated mRNA of brain and visceral tissues, such as paratrigeminal nuclei, nuclei of the solitary tract, uterus, ovaries, stomach, liver, kidneys, and lungs of Sprague-Dawley, Wistar and Wistar Kyoto rat strains, with exon-1/exon-3 primer pairs only produced the 702-bp fragment containing 5' exon-1, exon-2 and 3' exon-3. The same held true for the amplification of mature mRNA from SHR (data not shown). Ten-microliter aliquots uterus amplification reactions were re-amplified and resolved on silver-stained 12.5% polyacrylamide gels. In spite of the high sensitivity of the procedures, no trace of the 211-bp exon was detected. The "exon-X"/exon-3 pair failed to amplify the B_2 kinin receptor mature mRNA in any of the tissues (Figure 1).

Cycle sequencing of primary and mature mRNA cDNA transcripts only identified the "exon-X" 211-bp nucleotide sequence in the primary mRNA transcripts (Figure 2).

Figure 2. Genomic sequence showing contiguous exon-2 3' and exon-3 5' regions (displayed in grey boxes) obtained by cycle-sequencing ripe kinin B_2 receptor-encoding mRNA. In the white box, the genomic sequence of the 211-bp "exon-X" is interposed between exons 2 and 3. The latter was obtained by sequencing the 913-bp primary mRNA amplicon produced by the same exon-2/exon-3 primer pair used for immature mRNA amplification.

Exon composition in pyrogen-treated rats

Mature mRNA was also examined in rats treated with an exogenous pyrogen, a bacterial LPS. Fifty micrograms LPS administered directly into the rat peritoneum produced fever and expression of B_1 kinin receptor mRNA, without altering the expression of the kinin B_2 receptor or the β-actin protein mRNA (Figure 3A). The exon-1/exon-3 primer pair yielded only one 702-bp product, both in naive and LPS-treated animals. Figure 3B shows the expression levels of B_1 and B_2 mature receptor mRNA in the different tissues of the Wistar rat.

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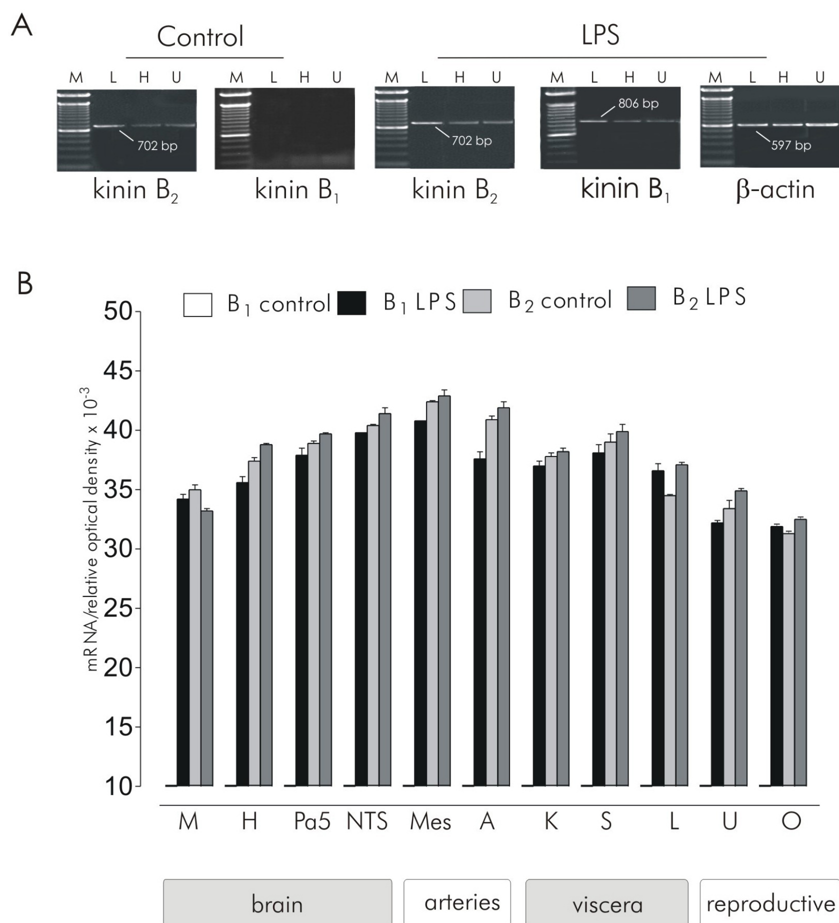

Figure 3. Expression of B_1 kinin receptor encoding mRNA in tissues of pyrogen (bacterial lipopolysaccharide, LPS)-treated (50 µg/kg, *ip*) rats. **A.** Amplification products of B_1 and B_2 kinin receptor mRNA and of the β-actin protein in ripe mRNA. **B.** Relative expression of B_1 and B_2 mRNA in central nervous system structures, such as the medulla (M); hypothalamus (H); paratrigeminal nucleus (Pa5); nucleus of the solitary tract (NTS); the mesenteric artery (Mes); aorta (A); kidney (K); stomach (S); lung (L); uterus (U); ovary (O).

B_2 kinin receptor expression during ontogenetic development

Primary and mature mRNA was obtained from rat embryonic and fetal tissues at 1 to 21 gestational days. cDNA amplification showed that the 0.6-kb product of the β-actin protein and the 702-bp product of the B_2 kinin receptor were expressed in embryonic cells from day 1 through 21, both from primary and mature mRNA. In primary RNA, the 913-bp product containing "exon-X" was apparent from day 5 onwards. The 702-bp fragment (not containing "exon-X") was the only amplification product of the exon-1/exon-3 primer pair from mature

mRNA cDNA (Figure 4A). Reamplification of 10-µL samples of the PCR amplification reactions, resolved on silver-stained polyacrylamide gels, failed to identify the 913-bp product containing the 211-bp sequence. Expression of the 702-bp fragment increased relative to that of the reference β-actin protein from gestational days 8 to 14 (Figure 4B).

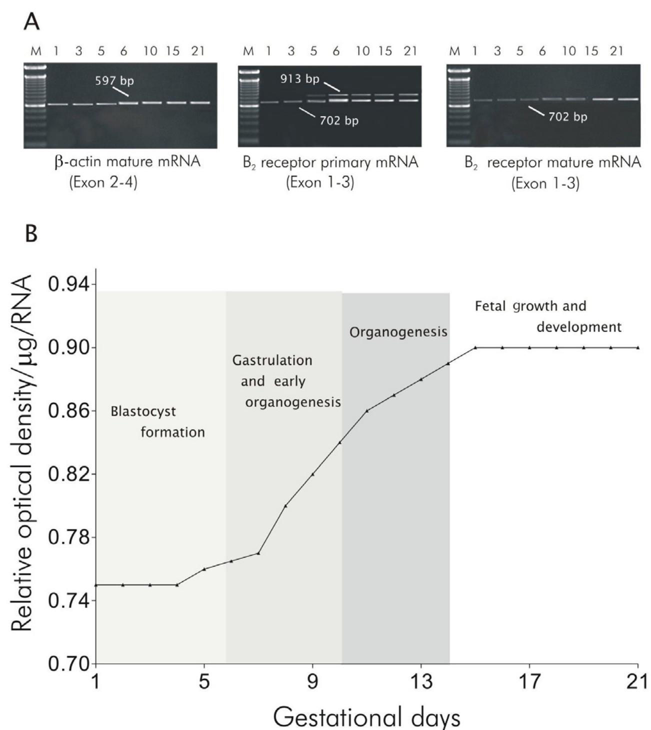

Figure 4. Kinin B_2 receptor encoding primary and mature mRNA expressiom in rat embryos and fetuses. **A.** Exon-1-3 amplicons mature kinin B_2 receptor mRNA showing the single 702-bp bands at different days of ontogenetic development (center). To the left, amplification of mature mRNA β-actin reference protein and to the right exon-1/exon-3 B_2 receptor primary mRNA amplicons showing both the 702- and the 211-bp bands, the latter containing "exon-X". M = molecular weight marker. **B.** Expression of kinin B_2 receptor mature mRNA relative to that of β-actin mature mRNA at the different embryogenic phases over the 21-day gestational period.

Data base matching of gene products and predicted proteins

The rat "exon-X" 211-bp sequence does not share any homology with the recently described kinin B_2 receptor gene sequence predicted from the rat mature mRNA (Genbank, *Rattus norvegicus* accession number NM_173100.1), horse (*Equus caballus*, XM_001489272.1), domestic cow (*Bos taurus*, XM_583508.3), mouse (*Mus musculus*, NM_009747.2), rabbit (*Oryctolagus cuniculus*, U33334), guinea pig (*Cavia porcelus*, AJ003243.1), dog (*Canis lupus familiaris*, NM_001003095.1), human (*Homo sapiens*, NM_000623), chicken (*Gallus gallus*, NM_001031553.1), and frog (*Xenopus tropicalis*, BC155000.1). "Exon-X" displayed full homology to the kinin B_2 receptor genes de-

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scribed by McEachern et al. (1991) and Pesquero et al. (1994) (a P25023). However, when compared to the entire rat or mouse kinin B_2 receptor gene sequences, significant homology of exon-X was found with intron regions (Table 2). Within the 211-bp sequence from Srague-Dawley or Wistar rat, 210 bp showed 96% homology to the 46554-46770 region of the *Rattus norvegicus* genome (NW_047762.2) and 172 bp showed 76% homology to the *Mus musculus* genome (31444-31669, NT_166318.1). In both cases, the alignments occurred in intron regions between exons 2 and 3 of the respective kinin B_2 receptor genes. On the other hand, 18 bases of the rat "exon-X" 211-bp sequence showed 90% homology alignment to the exon-3, 3' untranslated region (UTR, 47101-47120), of the *Homo sapiens* kinin B_2 receptor gene (NT_026437.12).

Table 2. Sprague-Dawley rat kinin B_2 receptor gene alignment with the rat, mouse and human genes.

Sprague-Dawley rat	*Rattus norvegicus* (39.7 kb) chromosome 6	*Mus musculus* (37.4 kb) chromosome 12	*Homo sapiens* (49.4 kb) chromosome 14
Genbank: P25023	Genbank: NW 047762.2	Genbank: NT_166318.1	Genbank: NT_026437.12
Exon-1 (111 bp)	Not available	96/114 (84%): 3703-3815	12/12 (100%): 1583-1594
Exon-2 (104 bp)	104/104 (100%): 44784-44887	82/98 (83%): 28764-28861	30/37 (81%): 37287-37321
Exon-X (211 bp)	210/217 (96%): 46554-46770	172/226 (76%): 31444-31669	18/20 (90%): 47101-47120
Exon-X (Wistar rat) (211 bp)	210/217 (96%): 46554-46770	Idem	Idem
Exon-3 (3711 bp)	3711-3720 (99%): 47531-51242	1683/1896 (88%): 32140-34000 685/854 (80%): 34988-35801	939/1146 (81%): 40583-41723
ORF Exon-3 (1101 bp)	1101/1101 (100%): 47538-48639	1013/1101 (92%): 32147-33247	888/1067 (83%): 40583-41649
3'UTR Exon-3 (2603 bp)	2601/2604 (99%): 48639-51242	662/786 (84%): 33248-34000 685/854 (80%): 34988-35801	39/47 (82%): 43763-43809

[a]Reference gene (Rattus norvegicus) segment to align with species genome-derived kinin B2 receptor genes. The numbers in parentheses represent homology of the respective alignments. [b]Regions of species kinin B2 receptor genes to which the reference gene aligned to. ORF = open-reading frame; UTR = untranslated region.

Mouse exon-1 displayed 84% homology to 96 bases of rat exon-1, while the human gene showed 100% homology to 12 bases of the 111-bp rat exon-1. Eighty-two bases of the 104-bp rat exon-2 showed 83% homology to the mouse gene, while 30 bases of the same exon displayed 81% homology with the human gene exon-2. We also found that 1.35 kb of the rat 3' UTR (exon-3) had 82% homology to the mouse 3' UTR, while 39 bases of the latter displayed 82% homology to human kinin B_2 receptor gene 3' UTR. However, a 662-bp segment of the rat 3'UTR aligned to the mouse gene region, immediately following the 3' open-reading frame (ORF), while a contiguous 685 segment aligned 988 bp downstream (Table 2).

The rat exon-3 ORF showed high compatibility to the mouse (92%) and human (83%) kinin B_2 receptor encoding sequences. As in the rat, the mouse and human ORFs have a continuous 1.1-kb sequence, all in 5'exon-3 (Figure 5B). However, when the rat ORF was aligned to mouse or human kinin B_2 receptor gene sequences predicted from mRNA (GenBank NM_009747.2 and NM_000623, respectively), ~70 bp of the 3' region showed homology to mouse and human exon-2, revealing an error in the deduced gene structures.

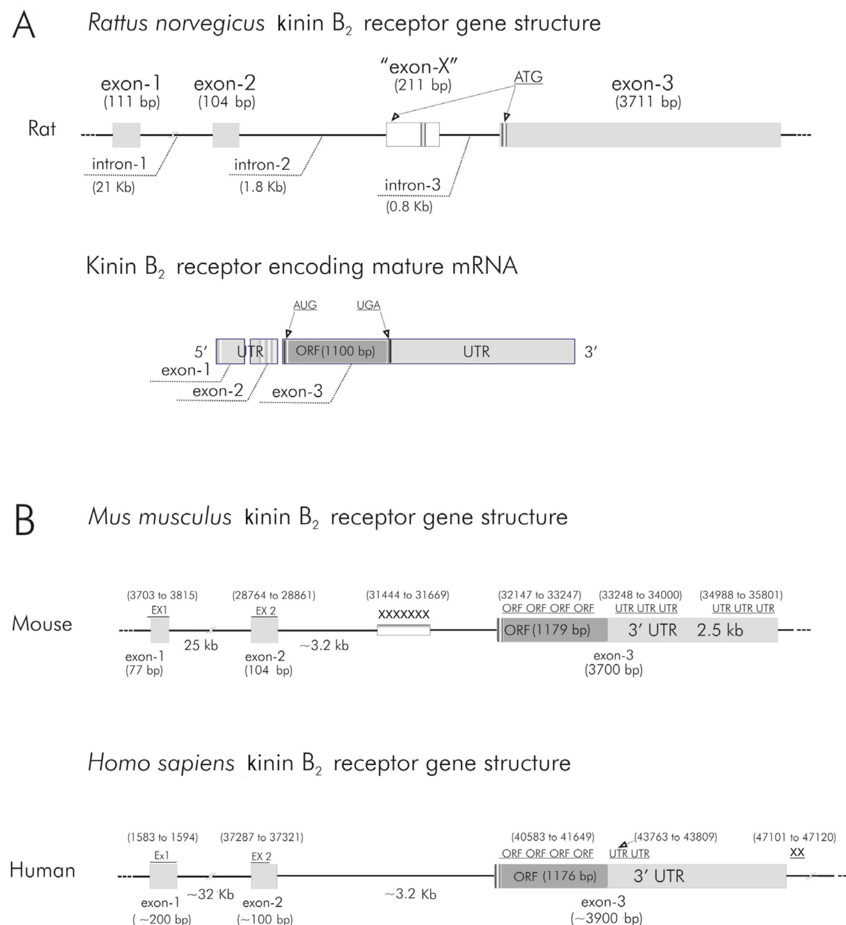

Figure 5. A. Gene structure of the kinin B_2 receptor gene showing three exons, the introns and the putative "exon-X", which apparently is not expressed in mature mRNA of the rat or other species. The first AUG translation initiation codon in exon-3 is depicted in the mature mRNA (dark vertical bar) alongside the 1100-bp open reading frame (ORF), which is followed by a UGA stop codon. The 3' and 5' untranslated regions (UTR) in exons 1 and 2 or exon-3, as well as the non-functional upstream AUG initiation codons (uAUGs, light vertical bars) in exons 1 and 2 are depicted in their approximate positions. **B.** Alignment of the Sprague-Dawley rat kinin B_2 receptor exons with the rat (*Rattus norvegicus*) chromosome 6, the mouse (*Mus musculus*) chromosome 12 and human (*Homo sapiens*) chromosome 14 genes.

The predicted N-terminal ~27 residue related to the 211-bp rat kinin B_2 receptor gene "exon-X" did not match published amino acid sequences (Genbank, accession number P25023). Curiously, the predicted alternative N-terminus of human, rat and mouse proteins (AbdAlla et al., 1996) displayed negligible homology with each other. Figure 5A shows the rat kinin B_2 receptor gene structure and Figure 5B shows the respective encoding mature mRNA and exon alignments to the mouse and human genome.

DISCUSSION

We describe new details of the processing of the B_2 kinin receptor-encoding mRNA; the introns are sequentially removed, since amplification of cDNA from primary mRNA with intron/exon primer pairs produced only one product containing all downstream introns and exons. During processing, "exon-X" is the last fragment to be removed. This occurs after removal of intron-3, suggesting that "exon-X" may have been incorporated to intron-2 of murines or phylogenetic ancestrals. Notwithstanding the evolutionary significance of this fragment, our finding that the 211 bp is not present in mature mRNA led us to conclude that the rat B_2 receptor gene structure is similar to that of mice, humans, and probably other mammalian species (Figure 5). Moreover, this interpretation of the rat gene coincides with a recently published consensus sequence of the N-terminus rat kinin B_2 receptor gene (Genbank accession No. NM_173100). In the latter sequence, deduced from the murine and human kinin B_2 receptor published by McIntyre et al. (1993), in which the 211-bp sequence of "exon-X" is not portrayed. This updated N-terminal rat kinin B_2 receptor sequence is presently used as a reference for the rat gene (Martins et al., 2008; Sivritas et al., 2008; Hansen et al., 2009; Tang et al., 2009). We also now have a better understanding of the mouse and human kinin B_2 receptor genes. As in the rat, the mouse and human ORFs consist of a continuous 1.1-kb sequence, all contained in 5'exon-3 (Figure 5). In contrast, earlier models of the mouse and human gene structures predicted from mRNA (Ma et al., 1994b; Hess et al., 1992) showed 0.7 kb of 3'ORF regions of mouse and human exon, 2 with the reminder of the ORF region in exon-3. Investigation of the murine and human genomes permitted identification of the mistake in gene structure prediction; consequently, the concept of the kinin B_2 receptor or gene-encoding region being in one intronless exon is also sustained for the mouse and human genes.

When we examine rat kinin B_2 receptor gene product processing, it is not yet clear whether the observed fragment removal pattern is common to the processing of other receptor-protein gene products of the same family. The fact that the "exon-X" nucleotide sequence was not found in polyadenylated mature mRNA in a wide range of tissues pertaining from various rat strains, both in unstimulated tissues or during an acute inflammatory process, which promotes expression of B_1 kinin receptor mRNA, practically rules out the possibility of alternative processing of the B_2 receptor gene that would lead to expression of an "exon-X"-containing mature gene product in adult animals. Furthermore, the lack of evidence for sustained expression of kinin B_2 receptor encoding gene products during early and late embryonic development is further evidence that the "exon-X"-containing gene product is not a constitutively or inductively expressed gene sequence in the mature or developing rat.

During the initial cloning of the rat kinin B_2 receptor gene by McEachern et al. (1991), the "exon-X" gene was included as part of the gene-encoding sequence. This lead to a hypothesis of alternative processing of the B_2 receptor protein gene. According to this hypothesis, alternative gene splicing would produce two mature mRNAs, one of them containing the 211-bp sequence of "exon-X", the earlier initially dubbed "exon-3" (Pesquero et al., 1994). The latter configuration suggested a four-exon, three-intron gene structure for the rat gene. Others (AbdAlla et al., 1996), based on *in vitro* studies, reported that the "exon-X" transcripts, containing an AUG initiation codon anteceding an ORF in the purported "exon-X", would add about 27 amino acids to the N-terminal end of the human and rat kinin receptor proteins. The reasons for "exon-X" to be mistakenly identified as a constituent of the mature B_2 kinin

receptor mRNA (Pesquero et al., 1994) are hinged on two non-converging aspects apart from the misleading published information (McEachern et al., 1991). First, the methodology used to isolate the poly-adenylated mRNA (poly-T columns) was not sufficiently stringent and thus permitted substantial amplification of primary mRNA transcripts containing "exon-X" genetic material. Second, the late removal of "exon-X", after that of intron-3, generating an apparent four-exon amplification product (albeit unsuspectingly amplified from primary mRNA), resulting in a false perception of an anticipated four-exon, three-intron gene structure. Here, we amplified mature mRNA from reverse transcripts using poly-T primers as transcription initiators. The stringency of this approach is attested by the fact that fragments containing intron sequences were never amplified from polyadenylated mRNA preparations.

However, these findings clearly demonstrate a functional three-exon gene structure for the rat kinin B_2 receptor gene, notwithstanding the purported *in vitro* expression of translated "exon-X" ORF N-terminal residues. In fact, the transcribed sequence that putatively encodes the rat "exon-X" ORF is not identifiable in the mammalian, avian, amphibian nor teleost fish genes encoding the kinin B_2 receptor proteins. The fact that mature mRNA containing the "exon-X" sequence was not detected in embryonic rat mRNA may not be relevant to this hypothesis, since it could be expressed in minute amounts, in specific tissues and during a very short time period, possibly hours or less, as may be the case for the ETB endothelin receptor gene during ontogenetic development of the gut (Suzuki et al., 2004). Neither does this mean that the potentially extended kinin B_2 receptor protein would necessarily be devoid of function if were expressed during early development. The lack of expression of the ETB receptor in the gut during a short period of organogenesis leads to a condition analogous to that of muscularis inflammation and the loss of Cajal interstitial cells, leading to intestinal obstruction and premature death of sub-adult endothelin-ETB receptor cells (Suzuki et al., 2004). The expression of the kinin B_2 receptor encoding mRNA sharply increases in proportion to that of β-actin protein during early organogenesis and levels off during fetal growth, demonstrating the significance of this kinin B_2 receptor for the multiple physiological functions played by kinins in the mature organism.

"Exon-X"-containing mature mRNA was not found in a wide range of tissues of adult Wistar, Sprague-Dawley and SHR animals, including brain structures known to express high densities of kinin receptors, such as the nucleus of the solitary tract, the paratrigeminal nucleus. Neither was the "exon-X"-containing mature mRNA found in the hypothalamus, which expresses components of the kinin-kallikrein system (Couture and Lindsey, 2000). The visceral tissues that were examined included those of arteries, lungs, kidneys, liver, stomach, ovaries, and uterus; the latter tissue was used for molecular cloning of the B_2 kinin receptor gene.

Thus, the structure of the rat gene may be considered akin to that of the mouse and human kinin B_2 receptor genes; all include a three-exon structure, as depicted in Figure 5. The latter displays the three-exon gene structure of the kinin B_2 receptor gene, based on our interpretation here and the finding of a single mature mRNA product. The lack of success in finding homology of the predicted ~30 residue N-terminal of the human or rat kinin receptor (AbdAlla et al., 1996) with the predicted kinin proteins of the frog (Suzuki et al., 2007), zebra and puffer fish (Bromée et al., 2005, 2006), as well as avian and mammal proteins, suggest that the "exon-X"-encoded protein N-terminus is not expressed in these animal classes. However, the 211-bp sequence of exon-X still has significant homology with the mouse intron region between exons 2 and 3. Comparing rat and mouse exons 1, 2 and "X" gives interspe-

cies homology ratings of 84, 83 and 76%, respectively; it appears that the latter gene fragment is under lower conservational pressure (Kumar and Hedges, 1998; Blair Hedges and Kumar, 2003) than exons 1 and 2. The same holds true when compared to the 1.3-kb sequence of the rat 2.6-kb 3' UTR. Half of the 1.3-kb sequence aligned with position 33248-34000 and the other half with position 34988-35801 of the mouse kinin B_2 receptor gene. The homology of these alignments was 84 and 80%, respectively, suggesting that the 3' UTR of the gene are under greater selective pressure (Blair Hedges and Kumar, 2003) than exon-X. Curiously, 18 bases of the 211-bp sequence showed 90% homology with the human 3' UTR at position 47101-47120. The significance, if any, of the latter sequence homology, which covers only 8.5% of "exon-X", is not clear.

Further investigation will be required to understand the evolutionary implications of "exon-X". It is possible that this fragment is the result of an early interstitial gene duplication (Prowald et al., 2005), resulting in the 211-bp insertion, with subsequent loss of the duplicate sequence. "Exon-X" could also be an ancestral form of the gene. Considering that "exon-X" is removed late during processing after the downstream intron-3 and that it has an AUG initiation codon that in *in vitro* systems prevails over the *in vivo* functional exon-3 translation-initiation codon, it is possible that the current gene structure reflects earlier forms of this protein, probably expressed in phylogenic ancestrals of amphibians and teleosts. However, a more compelling hypothesis for the origin of this 211-bp fragment is that it arose from a homologous, orthologous or heterologous transposon inserted before the 3' flanking regions of kinin B_2 receptor intron-2. This exon-like fragment, containing an AUG close to its 5' extremity, would create an impression of a four-exon, three-intron gene structure. Transposable elements (Nekrutenco and Li, 2001) together with polyploidy duplication (Beçak and Kobashi, 2004) are one of the main mechanisms for promotion of diversity and genetic material gain in vertebrates. Approximately 90% of the transposons in a phylogenetically recent and rapidly evolving species, such as *Homo sapiens*, were inserted into introns (Nekrutenco and Li, 2001) and were recruited into encoding regions as novel exons.

REFERENCES

AbdAlla S, Godovac-Zimmermann J, Braun A, Roscher AA, et al. (1996). Structure of the bradykinin B2 receptors' amino terminus. *Biochemistry* 35: 7514-7519.

Bachvarov DR, Saint-Jacques E, Larrivee JF, Levesque L, et al. (1995). Cloning and pharmacological characterization of the rabbit bradykinin B2 receptor. *J. Pharmacol. Exp. Ther.* 275: 1623-1630.

Bachvarov DR, Hess JF, Menke JG, Larrivee JF, et al. (1996). Structure and genomic organization of the human B1 receptor gene for kinins (BDKRB1). *Genomics* 33: 374-381.

Becak ML and Kobashi LS (2004). Evolution by polyploidy and gene regulation in *Anura*. *Genet. Mol. Res.* 3: 195-212.

Blair Hedges S and Kumar S (2003). Genomic clocks and evolutionary timescales. *Trends Genet.* 19: 200-206.

Bromée T, Conlon JM and Larhammar D (2005). Bradykinin receptors in the zebrafish (*Danio rerio*). *Ann. N. Y. Acad. Sci.* 1040: 246-248.

Bromée T, Venkatesh B, Brenner S, Postlethwait JH, et al. (2006). Uneven evolutionary rates of bradykinin B1 and B2 receptors in vertebrate lineages. *Gene* 373: 100-108.

Caous CA, Balan A and Lindsey CJ (2004). Bradykinin microinjection in the paratrigeminal nucleus triggers neuronal discharge in the rat rostroventrolateral reticular nucleus. *Can. J. Physiol. Pharmacol.* 82: 485-492.

Couture R and Lindsey CJ (2000). Brain Kallikrein-Kinin System: from Receptors to Neuronal Pathways and Physiological Functions. In: Handbook of Chemical Neuroanatomy: Peptide Receptors Part I (Quirion R, Björklund A and Hökfelt T, eds.), Chapter 7. Elsevier Science Ltd., Amsterdam, 16: 241-300.

de Sousa Buck H, Ongali B, Thibault G, Lindsey CJ, et al. (2002). Autoradiographic detection of kinin receptors in the human medulla of control, hypertensive, and diabetic donors. *Can. J. Physiol. Pharmacol.* 80: 249-257.

Eggerickx D, Raspe E, Bertrand D, Vassart G, et al. (1992). Molecular cloning, functional expression and pharmacological characterization of a human bradykinin B2 receptor gene. *Biochem. Biophys. Res. Commun.* 187: 1306-1313.

Farmer SG, Powell SJ, Wilkins DE and Graham A (1998). Cloning, sequencing and functional expression of a guinea pig lung bradykinin B2 receptor. *Eur. J. Pharmacol.* 346: 291-298.

Hansen JL, Hansen JT, Speerschneider T, Lyngso C, et al. (2009). Lack of evidence for AT1R/B2R heterodimerization in COS-7, HEK293, and NIH3T3 cells: how common is the AT1R/B2R heterodimer? *J. Biol. Chem.* 284: 1831-1839.

Hess JF, Borkowski JA, Young GS, Strader CD, et al. (1992). Cloning and pharmacological characterization of a human bradykinin (BK-2) receptor. *Biochem. Biophys. Res. Commun.* 184: 260-268.

Hess JF, Hey PJ, Chen TB, O'Brien J, et al. (2001). Molecular cloning and pharmacological characterization of the canine B1 and B2 bradykinin receptors. *Biol. Chem.* 382: 123-129.

Holland AM, Findlay JK and Clements JA (2001). Kallikrein gene expression in the gonadotrophin-stimulated rat ovary. *J. Endocrinol.* 170: 243-250.

Jones C, Phillips E, Davis C, Arbuckle J, et al. (1999). Molecular characterisation of cloned bradykinin B1 receptors from rat and human. *Eur. J. Pharmacol.* 374: 423-433.

Kammerer S, Braun A, Arnold N and Roscher AA (1995). The human bradykinin B2 receptor gene: full length cDNA, genomic organization and identification of the regulatory region. *Biochem. Biophys. Res. Commun.* 211: 226-233.

Kumar J, Schuck P, Jin R and Mayer ML (2009). The N-terminal domain of GluR6-subtype glutamate receptor ion channels. *Nat. Struct. Mol. Biol.* 16: 631-638.

Kumar S and Hedges SB (1998). A molecular timescale for vertebrate evolution. *Nature* 392: 917-920.

Leeb-Lundberg LM, Marceau F, Muller-Esterl W, Pettibone DJ, et al. (2005). International union of pharmacology. XLV. Classification of the kinin receptor family: from molecular mechanisms to pathophysiological consequences. *Pharmacol. Rev.* 57: 27-77.

Lindsey CJ, Bendhack LM and Paiva AC (1987). Effects of teprotide, captopril and enalaprilat on arterial wall kininase and angiotensin converting activity. *J. Hypertens.* (Suppl 5): S71-S76.

Lindsey CJ, Buck HS, Fior-Chadi DR and Lapa RC (1997). Pressor effect mediated by bradykinin in the paratrigeminal nucleus of the rat. *J. Physiol.* 502 (Pt 1): 119-129.

Lungu C, Dias JP, Franca CE, Ongali B, et al. (2007). Involvement of kinin B1 receptor and oxidative stress in sensory abnormalities and arterial hypertension in an experimental rat model of insulin resistance. *Neuropeptides* 41: 375-387.

Ma JX, Wang DZ, Ward DC, Chen L, et al. (1994a). Structure and chromosomal localization of the gene (BDKRB2) encoding human bradykinin B2 receptor. *Genomics* 23: 362-369.

Ma JX, Wang DZ, Chao L and Chao J (1994b). Cloning, sequence analysis and expression of the gene encoding the mouse bradykinin B2 receptor. *Gene* 149: 283-288.

MacNeil T, Bierilo KK, Menke JG and Hess JF (1995). Cloning and pharmacological characterization of a rabbit bradykinin B1 receptor. *Biochim. Biophys. Acta* 1264: 223-228.

Martins AH, Alves JM, Trujillo CA, Schwindt TT, et al. (2008). Kinin-B2 receptor expression and activity during differentiation of embryonic rat neurospheres. *Cytometry A* 73: 361-368.

McEachern AE, Shelton ER, Bhakta S, Obernolte R, et al. (1991). Expression cloning of a rat B2 bradykinin receptor. *Proc. Natl. Acad. Sci. U. S. A.* 88: 7724-7728.

McIntyre P, Phillips E, Skidmore E, Brown M, et al. (1993). Cloned murine bradykinin receptor exhibits a mixed B1 and B2 pharmacological selectivity. *Mol. Pharmacol.* 44: 346-355.

Medeiros R, Cabrini DA, Ferreira J, Fernandes ES, et al. (2004). Bradykinin B1 receptor expression induced by tissue damage in the rat portal vein: a critical role for mitogen-activated protein kinase and nuclear factor-kappaB signaling pathways. *Circ. Res.* 94: 1375-1382.

Nekrutenko A and Li WH (2001). Transposable elements are found in a large number of human protein-coding genes. *Trends Genet.* 17: 619-621.

Ni A, Chai KX, Chao L and Chao J (1998). Molecular cloning and expression of rat bradykinin B1 receptor. *Biochim. Biophys. Acta* 1442: 177-185.

Okamoto K, Aoki K, Nosaka S and Fukushima M (1964). Cardiovascular diseases in the spontaneously hypertensive rat. *Jpn. Circ. J.* 28: 943-952.

Page NM (2006). Characterization of the gene structures, precursor processing and pharmacology of the endokinin peptides. *Vascul. Pharmacol.* 45: 200-208.

Park J, Freedman R, Bach C, Yee C, et al. (1994). Bradykinin-B2 receptors in humans and rats: cDNA structures, gene structures, possible alternative splicing, and homology searching for subtypes. *Braz. J. Med. Biol. Res.* 27: 1707-1724.

Pesquero JB, Lindsey CJ, Zeh K, Paiva AC, et al. (1994). Molecular structure and expression of rat bradykinin B2 receptor gene. Evidence for alternative splicing. *J. Biol. Chem.* 269: 26920-26925.

Pessini AC, Santos DR, Arantes EC and Souza GE (2006). Mediators involved in the febrile response induced by *Tityus*

serrulatus scorpion venom in rats. *Toxicon* 48: 556-566.
Powell SJ, Slynn G, Thomas C, Hopkins B, et al. (1993). Human bradykinin B2 receptor: nucleotide sequence analysis and assignment to chromosome 14. *Genomics* 15: 435-438.
Prowald A, Wemmert S, Biehl C, Storck S, et al. (2005). Interstitial loss and gain of sequences on chromosome 22 in meningiomas with normal karyotype. *Int. J. Oncol.* 26: 385-393.
Sivritas SH, Ploth DW and Fitzgibbon WR (2008). Blockade of renal medullary bradykinin B2 receptors increases tubular sodium reabsorption in rats fed a normal-salt diet. *Am. J. Physiol. Renal Physiol.* 295: F811-F817.
Suzuki H, Iwamuro S, Ohnuma A, Coquet L, et al. (2007). Expression of genes encoding antimicrobial and bradykinin-related peptides in skin of the stream brown frog *Rana sakuraii*. *Peptides* 28: 505-514.
Suzuki T, Won KJ, Horiguchi K, Kinoshita K, et al. (2004). Muscularis inflammation and the loss of interstitial cells of Cajal in the endothelin ETB receptor null rat. *Am. J. Physiol. Gastrointest. Liver Physiol.* 287: G638-G646.
Tang M, Cui M, Dong Q, Ren HM, et al. (2009). The bradykinin B2 receptor mediates hypoxia/reoxygenation induced neuronal cell apoptosis through the ERK1/2 pathway. *Neurosci. Lett.* 450: 40-44.
Wang DZ, Ma JX, Chao L and Chao J (1994). Molecular cloning and sequence analysis of rat bradykinin B2 receptor gene. *Biochim. Biophys. Acta* 1219: 171-174.