Cloning, structural characterization and functional expression of a zebrafish bradykinin B2-related receptor

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The actions of bradykinin (BK) in mammals are mediated through the activation of the B1 and B2 BK receptors. The only BK receptor that has been cloned from a non-mammalian species is a B2-like receptor from the chicken (termed the ornithokinin receptor). Pharmacological studies have demonstrated the presence of BK receptors in tissues of teleost fishes, such as trout and cod, but the ligand-binding properties of these receptors differ appreciably from those of the mammalian and chicken receptors. We report here the cloning of a B2-like receptor in zebrafish that shares 35% identity with human B2 and 30% identity with human B1. Phylogenetic analyses confirm a closer relationship with B2 than B1. The receptor gene was mapped to linkage group 17, which is syntenic to the human

B2–B1 gene region. After functional expression of the zebrafish B2 receptor in mammalian cells, nanomolar concentrations of trout BK ([Arg⁰,Trp⁵,Leu⁸]-BK) and the derivative [des-Arg⁰,Trp⁵,Leu⁸]-BK (where 'des' indicates a missing amino acid) induced a significant transient rise in intracellular free Ca²⁺. The B1-selective analogue [Arg⁰,Trp⁵,Leu⁸,des-Arg⁹]-BK was inactive at nanomolar concentrations. Taken together, these results strongly support the gene's identity as a piscine orthologue of the mammalian B2 receptor.

Key words: Ca²⁺, chromosome, G-protein-coupled receptor, evolution, teleost, trout.

INTRODUCTION

The actions of bradykinin (BK) in mammals are mediated through interaction with two well-characterized BK receptors, termed B1 and B2, that are differentiated pharmacologically by the rank orders of potencies of selected agonists and antagonists (reviewed in [1,2]). The primary structure of the B2 receptor has been deduced from the nucleotide sequences of cloned cDNAs and/or genomic fragments from human, rat, mouse, guinea-pig and rabbit and the amino acid sequence of the B1 receptor is known from human, mouse and rabbit (reviewed in [3,4]). The amino acid sequence of the human B1 receptor is 36% identical to that of the human B2 receptor, indicating that the receptors probably arose by duplication of an ancestral BK receptor gene. The great sequence divergence suggests either that the duplication happened long ago or that the receptor genes have diverged rapidly (or both).

Despite intensive study of BK and its receptors in mammals, the kallikrein/kinin system in non-mammalian vertebrates has received relatively little attention. BK-related peptides have been isolated from the plasma of a range of birds, reptiles and fish (reviewed in [5]), but the only non-mammalian BK receptor to be characterized structurally is that encoded by an intronless gene that was cloned from a genomic chicken DNA library using a PCR-based strategy [6]. This receptor, termed the ornithokinin receptor, shows 49% sequence identity to the human B2 receptor and 31% to the human B1 receptor. For comparison, the human and rat B2 receptors are 81% identical. The ornitho-

kinin receptor expressed in transfected Chinese hamster ovary K1 cells responded to ornithokinin ([Thr⁶Leu⁸]-BK), but not to BK, with increased intracellular inositol phosphate accumulation and a transient rise in intracellular free Ca²⁺ concentration.

A BK-related peptide ([Arg⁰,Trp⁵,Leu⁸]-BK) has been isolated from kallikrein-treated plasma of the trout [7], cod [8] and eel [9] but a fish BK receptor has not yet been characterized structurally. However, [Arg⁰,Trp⁵,Leu⁸]-BK produces a dose-dependent contraction of smooth muscle from trout [10] and cod [11] gastrointestinal tracts and pharmacological studies using a range of analogues of the peptide have demonstrated that the ligand-binding properties of the BK receptor(s) mediating these effects are appreciably different from those of the mammalian B1 and B2 receptors. As an initial step towards characterizing fish BK receptors and the evolution of the BK receptor family, we report here the cloning and characterization of a zebrafish B2-like receptor.

EXPERIMENTAL

Isolation and characterization of a zebrafish BK receptor

Degenerate PCR primers were designed from sequence alignments of all known B2 sequences. Primers were B2F (5'-TGYKSIYTICITTYTGCGC-3') and B2R (5'-GGRTTIAR-GCMRCTRTT-3'), which are directed to transmembrane (TM) regions 3 and 7 of the mammalian B2 and chicken ornithokinin receptors. The primers were used to obtain a PCR product from

Abbreviations used: BK, bradykinin; B1, BK receptor B1; B2, BK receptor B2; *bdkrb2*, zebrafish BK receptor B2 gene; TM, transmembrane; LB, Luria–Bertani; BAC, bacterial artificial chromosome; RT-PCR, reverse transcriptase-PCR.

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genomic zebrafish DNA. Touchdown PCR was performed using Stoffel *Taq* Polymerase (Perkin Elmer, Stockholm, Sweden). The following PCR conditions were applied: 95 °C for 30 s, followed by 20 cycles of 15 s at 95 °C, 30 s at 55 °C and 45 s at 72 °C. In the first 20 cycles the annealing temperature was automatically decreased by 0.5 °C for each cycle. After this, another 20 cycles of 95 °C for 15 s, 50 °C for 30 s and 72 °C at 50 s followed. At the end samples were held at 72 °C for 5 min. A 20 µl reaction mixture contained 2 units of Stoffel *Taq* polymerase, 1 × Stoffel PCR reaction buffer (Perkin Elmer), 0.2 mM dNTPs (Amersham Bioscience, Uppsala, Sweden), 3.0 MgCl₂, 100 ng of genomic zebrafish DNA, 2 µM forward primer and 2 µM reverse primer. A PCR product of 640 bp was isolated, reamplified and cloned into the pCR®2.1 TOPO vector followed by heat-shock transformations into DH5α cells using the TOPO™ TA Cloning® kit (Invitrogen, Groningen, The Netherlands) according to protocols provided by the manufacturer. Transformants were inoculated on Luria–Bertani (LB) agar plates supplemented with 40 µl of X-galactose (20 µg/ml), 40 µl of ampicillin (25 µg/ml) and 20 µl of isopropyl β-D-thiogalactoside (0.1 µg/ml). White colonies representing plasmids containing insert were chosen for plasmid preparation and sequencing. The selected colonies were grown in 10 ml of LB medium complemented with ampicillin (25 µg/ml).

Plasmid DNA was prepared using the Wizard® Plus SV miniprep DNA-purification system (Promega). LB medium and LB agar plates were prepared according to standard protocols [12]. Sequences were determined using the BigDye™ Terminator Cycle Sequencing Ready Reaction kit (ABI Prism™; Perkin Elmer, Foster City, CA, U.S.A.) with AmpliTaq® DNA polymerase, on an ABI Prism 310 Genetic Analyser. M13 forward and reverse vector primers were used. Sequences were found to display high sequence identity to the mammalian B2 receptor. The 640 bp PCR product was used as a probe to screen a genomic zebrafish bacterial artificial chromosome (BAC) library (Genome Systems) according to the protocol provided by the manufacturer, in order to isolate a full-length clone. The zebrafish BAC library consists of nylon filters on which individual BAC zebrafish clones have been spotted at a very high density. The library was screened with ³²P-labelled probe (Megaprime™ DNA labelling system; Amersham Bioscience). Hybridizations were performed for 16 h at 65 °C in 50 % formamide and 6 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate). Washes were done at 65 °C in 0.5 × SSC and 0.1 % SDS. Positive BAC clones were sequenced by primer walking to obtain the full-length sequence of the coding region.

Receptor sequence alignments and tree construction

The full-length amino acid sequences were aligned using Clustalw1.7 [13]. Sequences were aligned and edited in Megalign (DNASTAR, Madison, WI, U.S.A.). The tree was constructed using the neighbour-joining algorithm.

Cloning into expression vector

PCR primers were designed matching the beginning of the 5' and 3' coding regions. The forward primer contained a recognition site (shown here in bold) for the restriction enzyme *Hind*III (cephind.F, 5'-GACATCA**AGCTT**CAATACAGTCTAATA-ACAAAAG-3'). The reverse primer had the restriction site for *Bam*HI and excluded the stop codon of the receptor gene sequence (cepXho.R, 5'-AAGT**CTCGAGG**TCTTTTTCGTTCA-GAACCACA-3'). The PCR reactions were run with proofread-

ing PfuTurbo™ DNA Polymerase (Stratagene). PCR product was purified using a QIAquick PCR Purification Kit (Qiagen) and cut with *Hind*III and *Bam*HI (Amersham Bioscience). The 1.1 kb fragment was purified on a 1 % agarose/Tris/borate/EDTA gel using the QIAquick Gel Extraction Kit and ligated into an expression vector. The expression vector was a modified pCEP4 plasmid (Invitrogen), with an intron from pCI-neo (Promega) located 5' to the cloning site. The cloned PCR product in the expression vector was fully sequenced and found to be identical with the genomic sequence.

Transfection protocol

For transient expression the plasmid was transfected into HEK-293-EBNA-1 cells with FuGENE™6 Transfection Reagent (Boehringer Mannheim, Biberach an der Riss, Germany), diluted in Optimem medium (Gibco BRL, Stockholm, Sweden) according to manufacturer's recommendations. Transfected cells were grown in Dulbecco's modified Eagle's medium/Nut Mix F-12 without L-glutamine (Gibco BRL) containing 10 % fetal calf serum (Biotech Line AS, Denmark), 2.4 mM L-glutamine (Gibco BRL), 2.5 mg/ml G-418 (Gibco BRL), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco BRL).

Peptides

Trout BK ([Arg⁰,Trp⁵,Leu⁸]-BK), mammalian BK and the derivatives [des-Arg⁰,Trp⁵,Leu⁸]-BK (where 'des' indicates a missing amino acid) and [Arg⁰,Trp⁵,Leu⁸,des-Arg⁹]-BK were synthesized and purified as described in [10].

Fura 2 measurement of intracellular calcium release

HEK-293-EBNA-1 cells with a semi-stable expression of the cloned zebrafish B2 gene (*bdkrb2*) were assayed for intracellular calcium release upon stimulation with agonist as described previously [14]. For single-cell measurements, the cells were grown on circular glass coverslips (diameter, 25 mm) and loaded with 4 µM fura 2 acetoxymethyl ester at 37 °C in TBM (Tes-buffered medium: 137 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1.2 mM MgCl₂, 0.44 mM KH₂PO₄, 4.2 mM NaHCO₃, 10 mM glucose, 0.5 mM probenecid and 20 mM Tes, adjusted to pH 7.4 with NaOH) for 20 min. Coverslips were placed in a perfusion chamber (0.2 ml volume) and perfused with a rate of 0.5 ml/min. Additions of the ligands were made by perfusion. Fluorescence emissions from fura 2-loaded single cells were analysed using the Intracellular Imaging InCyt2 fluorescence imaging system (Cincinnati, OH, U.S.A.). The cells were excited by alternating wavelengths of 340 and 380 nm with narrow-band excitation filters. The fluorescence was measured through a 430 nm dichroic mirror and a 510 nm barrier filter with a Cohu CCD camera.

For population measurements, the cells were cultured on circular plastic culture dishes (inner diameter, 90 mm; Nunc, Roskilde, Denmark). The cells were harvested using PBS containing 0.2 g/l EDTA, loaded with fura 2 acetoxymethyl ester (4 µM, 20 min, 37 °C) in TBM, washed once with Ca²⁺-free TBM and stored on ice as pellets (medium removed). For the measurement of intracellular free calcium, one pellet was resuspended in TBM at 37 °C. The fluorescence was monitored in a stirred quartz microcuvette in the thermostat-controlled cell holder of either a Hitachi F-2000 or F-4000 fluorescence spectrophotometer at 340 nm (excitation) and 505 nm (emission).

		TM1	TM2	
human B2	MFSPWKISMFLSVREDSVPTTASFSADMLNVTLOGPT--LNGTFAQSK--	CPQVEWLGMNTIOPPFLLVFLVLTLENIFVLSVFQ	LKSSCTVAETIYLGNAAD	103
guinea pig B2	-----F I S VSA-- A L GNS LDA WS	A A PFLVFLV V F	V	77
mouse B2	-----F T VLGSA LSKDN	DT WS A A L A L F N		78
rat B2	-----F I T ALGSAH SEVN	DT WS A A L A L TN	G	78
rabbit B2	-----I S VLAPA SVS SG	NT S V A L L V		79
dog B2	...A RP...FH P...L TE F S S DLMPT LPSP--	VYP WN I A L L I	L	104
ornithokinin	...ITITT NVTQLYNVTATQETITSPAEFHNGSVHOMDEYK--	INPDTWK Q F G I I I G I A I LYF R S A M F		98
zebrafish B2	-----MOPEEF SSVTLHPP ASVTPAYLDFFNSTEWRLVFS I YIFIVCLMGI G S L L QGRWS P		L	82
human B1	-----MAS WPPLE QSSN SOLFPO A A QDN--AP AWDL	HRVL T IISICFFGL G L L L PRROLN	A S	83
	TM2	TM3	TM4	
human B2	LILA-CGLPFWAITL SNNFDWLFGETLCRRVNAITSMNLYSSICFLMLYSIDRYLAL	VKTMSMGRMRGV	RWAKLYSLVIWGCTLLSSPMLVFR	209
guinea pig B2	...-F...A...V...V...M TM Q M		D R	183
mouse B2	...-F...A...V...V...TM Y		R E	184
rat B2	...-F...A...V...V...TM Y		D RE	184
rabbit B2	...-F...A...V...V...TM Y		D R Y	185
dog B2	...-S...A...V...V...TMY		R Y	210
ornithokinin	M V S N G Q P L F KA T NY S Y T	L RTAC WN I M A IC TM	NLOYFKE--Y	202
zebrafish B2	FTSLC MN L Y M Y FM K MS MV M T YM VM NV	L RARWL RK Y ICVIL LFG GMGA TAAM	KLIDIPAHQ--SV	187
human B1	VFV-L EN W Q N P A I J GV KA F I FLVVAI Q RV	HP ASR QORR Q RVTCLV VVGG I TELL	SIOAVP LN--I	187
	TM5	TM6	TM7	
human B2	ADVISYPSLTIWEVFTNMLNNGVGLPLSVITCTMD	MOVLRRN-EMOKFKE	TGT-ERRATVLVLVLLLF IICWLPFOISTFLDTL	314
guinea pig B2	L I T Q V L I V		RL F PG W HV L	287
mouse B2	V RS V L VR L K V K A G VL V		L V G W HAV	289
rat B2	V RS L VR L K V K A G VL F		L V G W N AV IV	289
rabbit B2	I D RS V L VR L K V K A G VL V		L K V W HV	290
dog B2	I RT V F T V		L D G RH HL V F	315
ornithokinin	SLD TPY HPAN C A MI CI Y T	IKA QS -KS L L K L A V	LI VCY AGNLK L D N IV	306
zebrafish B2	MLN NRN RIAHLQ I A FL CFN V A KRR-RDSGYW DGN-D K I CA TF FF G HFL L CD KV DEKEWAHFLNIGN			292
human B1	ILL HEA HFARIVE IL AA V FNYH LAS TRE VSRTRCGGRKDSKT A I TLVVA LV A YHFA EF FOVQAVRG F W DF LGL			294
	TM7			
human B2	QIASFMAYSNSCLNPLVYIVIGKFRFKKSWEVYOGVCKGGRSEPIOMENSMGLRTSISVERQIHKLQDWAGSRQ			391
guinea pig B2	S YL R H L RS V A S D R SSEGTPPGLL			372
mouse B2	S YV G R RVL MG V KK			366
rat B2	S YV A L R MG SV D NK			366
rabbit B2	VG G R RAA P A VL V A S PE TR S			367
dog B2	YV R FR L VL SNK D V PE ENS			393
ornithokinin	M IYC F IL H Q AV LFKDLIPDRK SVKI QD S HLRK SVLPLSQ			381
zebrafish B2	FSVYL F VL S NY R VSSI KRR-- QSSDATTL RTALT T T NQIKPVVLNEKD			360
human B1	L N F FT S VI F RL T V L K QTP SLAPISSSHRKEIFOLFWRN			353

Figure 1 A comparison of the deduced amino acid sequences of BK receptors from species of different taxa

The human B2 sequence serves as a master sequence. In the other sequences only positions that differ from the human B2 are shown, whereas dots indicate identities and dashes represent gaps introduced to optimize alignment. The TM regions inferred from bovine rhodopsin [21] are in grey boxes. Potential glycosylation sites are underlined. Extracellular cysteines are marked with boxes, as are C-terminal cysteines expected to anchor the receptor to the cell membrane via palmitate. GenBank accession numbers are: rat B2, OORTB2; rabbit B2, Q28642; ornithokinin receptor, CAA05099; mouse B2, I49519; human B1, XP_007275.21; human B2, XP_007276.1; guinea-pig B2, BRB2-CAVPO; dog B2, AF334948.1; zebrafish B2, AF497802.

Calibration of each experiment was performed by adding 60 µg/ml digitonin, which gives the maximum value of fluorescence, and 10 mM EGTA, which gives the minimum value of fluorescence. The leaked fura 2 was measured in separate experiments by adding 10 mM EGTA, which chelates Ca²⁺ bound to extracellular indicator. The corrected fluorescence values were used to calculate the intracellular Ca²⁺ concentration. The values are given as means ± S.E.M. The value *n* refers to the number of independent batches of cells used for measurements. Non-linear curve fitting was performed using SigmaPlot for Windows 4.00 (Jandel Scientific, Corte Madera, CA, U.S.A.).

Genetic mapping

For meiotic mapping of *bdkrb2*, we identified genetic polymorphisms segregating in the HS (heat-shock-generated) meiotic mapping panel using single-strand conformation polymorphism as described in [15]. Genomic DNAs from the mapping panel were amplified using primers for the 5' untranslated region of *bdkrb2* (forward, +46GGG TTT CGG ACG CAG CCT AT; reverse, -274CCC AAG GTA TAT TTA AAG TGC AAG TCT

GA), giving a 228 bp fragment. Original mapping data are available in Map Manager [16] format on-line at <http://www.neuro.uoregon.edu/postle/PostleLab.html>. Comparative mapping was accomplished as described in [15].

Reverse transcriptase-PCR (RT-PCR)

Zebrafish used for RNA purifications were received from Fyris Zoo (Uppsala, Sweden). Eight animals were anaesthetized in ice-cold water and then decapitated. Total RNA preparations from eye, brain, muscle and gastrointestinal tract were made according to RNeasy kit protocol (Qiagen) including RNase-free DNase treatment in order to eliminate genomic DNA contaminations as a possible template in RT-PCR reactions. cDNA was synthesized from 5 µg of total RNA according to the first-strand cDNA synthesis kit (Pharmacia) and gave a total volume of 35 µl from each tissue. To 35 µl of cDNA, 65 µl of water was added and samples were heated to 70 °C for 10 min to inactivate any accessory enzymes. Full-length primers for RT-PCR were the same as for cloning in the expression vector (see above). The primers were shown to generate products when run under

standard PCR conditions with *Taq* polymerase (Gibco BRL) on zebrafish genomic DNA as a template. For a negative control, RT-PCR primers were tested under standard PCR conditions with *Taq* polymerase (Gibco BRL) on the RNA preparations. No products were generated.

The products were analysed on standard 1.5 % agarose gels. The gels were photographed and blotted to a nylon filter. The filter was prehybridized in hybridization buffer containing 25 % formamide, 5 × Denhardt's solution, 6 × SSC, 10 % dextran sulphate and 0.1 % SDS. The probe was made from the cloned PCR-generated insert, labelled with ^{32}P (Amersham Bioscience) using the Megaprime kit according to the manufacturer's protocol (Amersham Bioscience). The filter was hybridized with labelled probe at 65 °C in hybridization solution overnight, and washed four times with 0.2 % SSC and 0.1 % SDS at 65 °C for 30 min. The filter was exposed to X-ray film for 20 min at -70 °C.

RESULTS

Isolation of a zebrafish B2 receptor clone

Degenerate primers based on sequence alignments of all known B2 sequences were used for PCR of zebrafish genomic DNA. A 640 bp PCR product was generated, cloned and sequenced, and used to screen a zebrafish genomic BAC library and a single positive clone was isolated. A hybridizing fragment was subcloned and sequenced. An uninterrupted open reading frame was found to encode a receptor protein of 360 amino acids. The zebrafish receptor sequence was compared with all known B1 and B2 sequences. An alignment is shown in Figure 1, with the human B1 sequence as a representative for the B1 receptors. The zebrafish receptor displays 35 % overall amino acid identity with mammalian B2 and the ornithokinin receptor and 30 % with the human B1 sequence as well as other mammalian B1 sequences. Comparison of the TM regions showed 40 % identity to the mammalian B2 and ornithokinin sequences and 38 % identity to human B1. The region encoding the N-terminal domain of the human B2 receptor contains an intron, but the zebrafish gene lacks the consensus splice acceptor site for an intron, which is why it is most likely that the methionine shown in Figure 1 is used for initiation of translation. This methionine is preceded by a termination codon in the same reading frame which rules out translation initiation further upstream. The authenticity of the ends of the coding region was confirmed by the RT-PCR experiments on RNA from three different tissues (see below). A cysteine pair presumably links extracellular loops one and two, in agreement with other B2 receptors. The possible absence of an attachment site for palmitate in the cytoplasmic tail and the possible existence of a third extracellular cysteine residue after TM6 is discussed further below. Two consensus sites for N-linked glycosylation are present in the N-terminal extracellular domain. A single amino acid insertion has occurred in the middle of TM2.

The phylogenetic tree shown in Figure 2 was calculated using the Clustalw1.7 software and tested with the bootstrap method. Full-length coding sequences were used. A tree calculated using the segment from TM1 to TM7 displayed the same topology (results not shown). The human angiotensin 2 receptor was used as an outgroup to root the tree. The tree provides further evidence that the cloned zebrafish receptor is an orthologue of the mammalian B2 receptor.

Functional expression of the zebrafish BK receptor gene

In order to be able to use HEK-293-EBNA-1 cells for functional characterization of the zebrafish BK receptor, we wanted to

Figure 2 Phylogenetic tree for the BK receptor family

The tree was calculated using the neighbour-joining method in the Clustalw1.7 software. Numbers at branch points are bootstrap values. The tree was constructed with 1000 replications giving bootstrap values representing the probability of a certain node in the tree. Branch lengths are proportional to distances between sequences. Human angiotensin 2 receptor was used as an outgroup to root the tree.

exclude any interference from putative endogenous BK receptors. We therefore exposed untransfected HEK-293-EBNA-1 cells to mammalian BK. This led to Ca^{2+} elevation of approx. 100 nM above the basal level, indicating the presence of an endogenous BK receptor. However, this receptor was not activated by trout BK and the analogues [des-Arg⁹]-BK and [des-Arg¹⁰]-BK since these compounds, even at 1.5 μM , did not evoke any Ca^{2+} elevation (results not shown). Therefore, EBNA cells appear to be suitable for investigation of the zebrafish BK receptor.

The coding region of the zebrafish B2 gene was transferred by PCR to a modified version of the expression vector pCEP4 for stable expression in the mammalian cell line HEK-293-EBNA-1 as described in the Experimental section. HEK-293-EBNA-1 cells transfected with this zebrafish receptor construct had a basal intracellular Ca^{2+} concentration of 81 ± 4 nM ($n = 3$). In these cells, trout BK elicited striking Ca^{2+} elevations. Essentially all the cells responded to the trout BK, although there were differences in the magnitude of the Ca^{2+} response between the cells (Figure 3a). The magnitude of the response was also dependent on the concentration of the trout BK in both single-cell and population measurements (Figure 3b). For a statistically reliable estimate of the potency of trout BK, the concentration-response relationship was evaluated in large population measurements in suspension. Trout BK activated the

Figure 3 Ca^{2+} responses upon activation of zebrafish BK receptors in HEK-293-EBNA-1 cells

(a) The responses to 300 nM trout BK in 10 individual cells on one coverslip are shown. The data are presented as ratio of the fluorescence intensity 340 nm/380 nm. The calibration bars indicate 1 ratio unit (ordinate) and 10 s (abscissa). (b) Representative traces of population measurements in suspension are shown. Responses to 10 and 300 nM trout BK are indicated. (c) Concentration-response curves for trout BK analogues are shown. The responses were normalized to the trout BK maximum (100%).

zebrafish BK receptor with an EC_{50} of 6.6 nM ($pEC_{50} = 8.18 \pm 0.06$, $n = 5$; where pEC_{50} is the negative logarithm of EC_{50} ; Figure 3c). The pharmacological profile of the receptor was investigated further using [des-Arg⁰]-BK and [des-Arg⁹]-BK. Both of these peptides were significantly less potent than trout BK (Figure 3c). [des-Arg⁰]-BK activated the receptor with an EC_{50} of 370 nM ($pEC_{50} = 6.43 \pm 0.08$, $n = 4$) and the maximum response to trout BK and [des-Arg⁰]-BK was not significantly different. The activity of [des-Arg⁹]-BK was so low that neither the maximum response nor the EC_{50} value could be estimated reliably, but the concentration-response-relationship appears to have shifted more than 10-fold to the right compared with [des-Arg⁰]-BK (Figure 3c).

Chromosomal localization of the receptor gene

The zebrafish *bdkrb2* gene was mapped to linkage group 17 (Figure 4) with high statistical significance, to marker z1490 [17]

with a log of odds (LOD) value of 9.9 and to *vg1* [18] with an LOD value of 10.2. Analysis of apparent orthologues [15,19] shows that at least 10 loci in linkage group 17, in addition to *bdkrb2*, share synteny across the length of human chromosome 14 (<http://www.ncbi.nlm.nih.gov/genome/guide/>). Interestingly, synteny for this chromosome segment have been conserved in zebrafish and human lineages, but have been disrupted by a translocation in the mouse after its divergence from the human lineage: five of these loci plus *Bdkrb2* are on mouse chromosome 12, and two are on mouse chromosome 14, whereas the locations of three loci are as yet unknown in mouse (http://www.informatics.jax.org/searches/marker_form.shtml). Although loci on this entire ancient chromosome segment may have maintained synteny since the divergence of human and zebrafish lineages, intrachromosomal rearrangements, including inversions, have altered locus order within the segment, as has been observed in other comparisons between human and zebrafish chromosomes [15,20].

Figure 4 Genetic mapping

Conserved synteny support the conclusion that zebrafish and human *BDKRB2* are orthologues. In addition to *bdkrb2*, at least 10 loci in linkage group 17 (LG17) have apparent orthologues on human chromosome 14 (Hsa14). This syntenic group is split into two chromosomes, 12 and 14 (Mmu12 and Mmu14), in mouse.

Figure 5 Reverse transcription

Total RNA was isolated from each tissue, converted to cDNA and a fraction used for RT-PCR with specific primers. Genomic DNA contamination was limited by treating total RNA preparations from zebrafish tissues with RNase-free DNaseI. PCR products were run on a standard agarose gel and stained with ethidium bromide (upper panel). Subsequently, the gel was blotted on to nylon membrane, hybridized to 32 P-labelled probes and exposed to film for 20 min at -70°C (lower panel). Omission of the cDNA-synthesis step abolished signal (results not shown). GI, gastrointestinal tract.

RT-PCR analysis of gene expression in zebrafish tissues

RT-PCR with primers flanking the complete coding region of the receptor was applied to zebrafish cDNA from eye, muscle, brain and gastrointestinal tract. Total RNA from each organ was run simultaneously without the cDNA-synthesis step as a negative control (results not shown). No contamination was detected. A prominent band was visible on the gel in the eye cDNA lane as well as a faint band for brain, but the assay was not designed to allow stringent quantification. A Southern blot of the same gel to a nylon filter probed with part of the zebrafish receptor clone, and exposed for 20 min, confirmed these results and also revealed faint bands in the lanes for muscle and gastrointestinal tract (Figure 5).

DISCUSSION

This study describes the cloning, functional expression, RT-PCR analysis and chromosomal mapping of the first piscine BK receptor. The deduced amino acid sequence of the zebrafish receptor is 360 amino acids in length and shows 35% overall identity to human B2 and the ornithokinin receptor, whereas it has 30% identity to human B1. In the TM regions the identity to human B2 and ornithokinin is 40% and to human B1 is 38% (Figure 1). In comparison, the ornithokinin receptor shows 49% overall sequence identity to the human B2 and 31% to the human B1 receptor [6]. Thus the B2 receptor appears to have evolved fairly rapidly, displaying only 80% identity between human, rabbit and rodents. In the neighbour-joining tree (Figure 2) the ornithokinin and zebrafish receptors form a cluster with the mammalian B2 receptors, with high bootstrap values, strongly supporting orthology between the zebrafish receptor, the ornithokinin receptor and the mammalian B2 receptor.

The zebrafish B2 receptor has the two extracellular cysteine residues in extracellular loops 2 and 3 found in all other BK receptors. However, zebrafish B2 lacks the cysteine residues in the N-terminus and the third extracellular loop of the other B2 receptors. A cysteine occurs immediately after TM6, giving the zebrafish B2 receptor an odd number of extracellular cysteines. This third cysteine residue is also present in the ornithokinin receptor and can theoretically be involved in homo- or heterodimer formation, but as it is located in immediate proximity to the TM region and is missing in mammalian B2, it is probably not involved in disulphide formation. All mammalian B2 receptors have two conserved cysteines in the C-terminus and the ornithokinin sequence has the second of these (albeit shifted one position). It is difficult to tell whether the cysteine residue at position 312 in zebrafish B2 may function as a site for palmitoylation. The new rhodopsin structure [21] suggests that TM7 may terminate before this position, thus allowing for palmitoylation. However, Cys-312 precedes the basic amino acid residues that are usually assumed to serve as the boundary of a segment embedded within the membrane. A few other receptors lack a C-terminal cysteine, for instance the mammalian gonadotropin-releasing hormone receptor [22] and the MC5 receptor [23].

BK receptors in mammals have been found to couple to different G-proteins depending on the cellular situation, signalling pathway and species analysed [24–27]. Functional coupling of the zebrafish B2 receptor was studied in transfected HEK-293-EBNA-1 cells with semi-stable expression of the *bdkrb2* gene but showed neither stimulation nor inhibition of cAMP production. Possibly, the zebrafish BK receptor requires some associated signal-transduction proteins that are not present in the human HEK-293-EBNA-1 cells used here or is otherwise incompatible with this type of signal transduction in these cells.

B2 receptors in many cell types are G_q -coupled and activate the phospholipase C or phospholipase A_2 pathway [28].

To determine the functional properties of the zebrafish B2 receptor, we also measured intracellular Ca^{2+} release in response to trout BK and selected derivatives. Untransfected HEK-293-EBNA-1 cells responded to mammalian BK with Ca^{2+} elevation, indicating the presence of an endogenous BK receptor. Therefore, this expression system does not seem to be suitable for testing any of the mammalian BK derivatives. Fortunately, the untransfected cells did not respond to trout BK.

The transfected HEK-293-EBNA-1 cells responded in a concentration-dependent manner to trout BK ($[Arg^0, Trp^5, Leu^8]$ -BK; Figure 3b) with an EC_{50} of 6.6 nM (Figure 3c). The N-terminally truncated analogue $[des-Arg^0]$ -BK stimulated the receptor with an EC_{50} of 370 nM, approx. 50 times less potent than trout BK. This indicates that the N-terminal arginine might be important for the ligand–receptor interaction. $[des-Arg^0]$ -BK, which may be regarded as a suitable ligand for binding to a B1-related receptor, was virtually inactive, even at concentrations as high as $1.5 \mu M$. This strengthens the classification of the zebrafish receptor as a piscine B2. The primary structure of zebrafish BK is not yet known but the amino acid sequence of BK has been fully conserved in three phylogenetically diverse teleost species (trout, cod and eel) [5]. Thus when exposed to this fish BK peptide, the cloned zebrafish BK receptor gives rise to a cellular response and appears, therefore, to be functional.

Chromosomal mapping showed that the zebrafish B2 gene is localized to linkage group 17, which is syntenic with *Homo sapiens* chromosome 14, where the human B1 and B2 genes are located. Thus this chromosome region has remained largely intact in the zebrafish and human lineages since they diverged approx. 400–420 million years ago ([29] and Figure 4). Thus far, a B1-like gene has not been reported in either zebrafish or chicken. However, the phylogenetic tree strongly suggests that the gene duplication took place prior to the split between ray-finned fishes and tetrapods. As the human B1 and B2 genes are located only 23 kb apart on chromosome 14q32.1–q32.2, we have used a human B1 probe to search for a zebrafish B1 gene in our B2 BAC clone. No signal was detected even after low-stringency hybridization. However, our BAC clone may not extend far enough in this direction.

The anatomical distribution of zebrafish B2 mRNA was investigated by RT-PCR. A signal was detected in all four organs investigated. The signal was strongest in eye and brain, whereas muscle and gastrointestinal tract gave very weak bands visible after Southern hybridization. However, it should be noted that the RT-PCR analysis was not designed for quantification. In mammals, the B2 receptor is constitutively expressed in a variety of tissues, whereas the B1 receptor is up-regulated in response to tissue inflammation. Immunoreactivity of B2 in the rat brain and expression of B2 as well as B1 receptor mRNA in the human brain has been demonstrated [30,31] but the functions of BK receptors in the brain are still not known. B2 receptor mRNA has been localized to rat retina and sclerocornea, suggesting a role in modulating light-induced electrical responses in the retina as well as other ocular functions [32]. Dog cornea epithelium has also been shown to respond to BK [33]. Figueroa et al. [34] detected the B2 receptor on the plasma membrane of striated skeletal muscle cells in rat, where it is involved in glucose uptake. Thus the B2 receptor is broadly expressed in both zebrafish and mammals, suggesting involvement in multiple physiological functions. These functions in teleost fish have yet to be elucidated fully but complex *in vivo* effects of $[Arg^0, Trp^5, Leu^8]$ -BK on cardiovascular function have been demonstrated in trout [35] and cod [8], and the peptide exerts an anti-dipsogenic effect in the

eel [9]. The present study suggests that a BK-related peptide may also be important in neurotransmission/neuromodulation in the CNS of teleosts as well as playing a role in ocular function.

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