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Pharmacological characterization of ligand–receptor interactions at the zebrafish bradykinin receptor

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1 Ligand interactions of a piscine bradykinin (BK) receptor expressed *in vitro* have been characterized for the first time by measuring inositol phosphate accumulation.

2 The ligands were analogues of zebrafish BK with serial substitutions by D-amino acids or alanine. Substitutions at residues Arg[1], Gly[4], Ser[6], Pro[7], Leu[8] and Arg[9] caused greatly reduced potency and maximum response. The Pro[3]→Ala analogue had higher potency but lower maximum response.

3 The peptide HOE140 was a weak partial agonist although it is an antagonist at the human B2 receptor and a potent agonist at chicken B2.

4 Thus, cloned zebrafish BK receptor reveals a ligand-interaction profile that is distinct from mammalian B1 and B2 receptors and from the previously characterized BK receptor in trout stomach, but similar to the receptor in cod intestine. These results increase our understanding of the evolution of BK receptors and the functions of the kallikrein–kinin system.
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Abbreviations: B1, Bradykinin receptor type 1; B2, Bradykinin receptor type 2; BK, Bradykinin; D-Tic, D-1,2,3,4-tetrahydro-3-isoquinolinecarbonyl; EBNA, Epstein–Barr nuclear antigen; EC, Effective concentration; HEK, human embryonic kidney; HOE140, D-Arg[0][Hyp[3], Thi[5], D-Tic[7], Oic[8]]-BK; Hyp, trans-4-hydroxy-L-prolyl; IP, inositol phosphate; Oic, L-(2α,3β,7aβ)-octahydro-1H-indole-2-carbonyl; PIP_2, phosphatidylinositol bisphosphate; Thi, 3-(2-thienyl)-L-alanyl

Introduction

The peptide hormone bradykinin (BK) is generated in the circulation from a large protein precursor, high-molecular-mass kininogen by the action of a serine protease, plasma kallikrein. Similarly, the N-terminally extended variant, kallidin ([Lys[0]]-BK) is released from low-molecular-mass kininogen by glandular kallikrein (Bhoola *et al*., 1992). Both BK and kallidin are naturally occuring substances and produce a range of biological effects in humans, including activation of pain sensory fibres, smooth muscle contraction and relaxation, hypotension, bronchoconstriction, inflammation and oedema (Regoli & Barabe, 1980). BK receptors belong to the superfamily of G-protein-coupled receptors, and based on their pharmacological profile they are classified into two subtypes, B1 and B2 (Regoli & Barabe, 1980; Regoli *et al*., 1998); Hess *et al*., 1994). The B1 receptor is upregulated in response to tissue inflammation and preferentially activated by the enzyme cleaveage products, des-Arg[9]-BK and des-Arg[9][Lys[0]]-BK (Marceau *et al*., 1998). The B2 receptor is constitutively expressed in most tissues of the body and preferentially binds kallidin and BK with subnanomolar affinities (Hall, 1997; Fathy *et al*., 2000). B1 binds with much lower affinity to kallidin ($K_D = 11.7$ nM) and even more poorly to BK ($K_D = 2.9\,\mu$M) (Fathy *et al*., 2000). B2 is inactive in response to des-Arg[9]-BK and des-Arg[9][Lys[0]]-BK (Regoli *et al*., 1996). The synthetic peptide analogue, HOE140 (D-Arg[0][Hyp[3], Thi[5], D-Tic[7], Oic[8]]-BK) is a potent antagonist at the B2 receptor and des-Arg[9][Leu[8]]-BK is an antagonist at the B1 receptor (Hock *et al*., 1991; Regoli *et al*., 1998). Both B1 and B2 receptors have been cloned and characterized in several mammals (reviewed in Mahabeer & Bhoola, 2000). B1 and B2 receptors have been shown to couple to $G\alpha_{i2/3}$ and $G\alpha_{q/11}$ (Austin *et al*., 1997; de weerd & Leeb-lundberg, 1997). In most cell types the B2 receptor couples to $G\alpha_{q/11}$ (Austin *et al*., 1997; Ricupero *et al*., 1997; Yang *et al*., 1999), resulting in activation of phospholipase C and increased turnover of phosphatidylinositol bisphosphate (PIP_2) leading to intracellular mobilization of Ca^{2+}. Until recently, the only nonmammalian kinin receptor to be cloned and characterized was a B2-like receptor in chicken that was designated the ornithokinin receptor (Schroeder *et al*., 1997) as it responds to the chicken BK, ornithokinin ([Thr[6], Leu[8]]-BK). No B1-like receptors have yet been identified in any nonmammalian species.

BK-like peptides are generated in the plasma of certain reptiles and phylogenetically ancient fish (lungfish, bowfin, gar and sturgeon) and they can also be extracted from the skins of amphibians (reviewed in Lazarus & Attila, 1993). In teleost fish, a BK-like peptide has been purified from trout (Conlon

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et al., 1996), cod (Platzack & Conlon, 1997) and eel (Takei *et al*., 2001), and identified in the zebrafish genome database (Bromée *et al*., unpublished). Although the primary structure of BK has been moderately conserved during the evolution of vertebrates, the amino-acid sequence is identical among the four fish species and contains two amino-acid substitutions ($Phe^5 \rightarrow Trp$ and $Phe^8 \rightarrow Leu$) and Arg^0 at the amino-terminus compared to human BK (Table 1). An additional Arg^0 has also been identified in BK from mouse (Takano *et al*., 1997).

We recently reported the cloning of the first piscine BK receptor in the zebrafish (Duner *et al*., 2002). On the basis of phylogenetic analyses and a preliminary investigation of pharmacological properties, this receptor was designated B2. In order to further characterize the ligand–receptor interactions, we describe here the functional coupling of the receptor to the phosphatidylinositol (PI) pathway in response to a large panel of BK-related peptides, including a complete series of alanine (Ala) substituted and D-amino-substituted zebrafish BK analogues. The data allow identification of those residues in zebrafish BK that are most important for ligand–receptor interactions. Properties of the zebrafish receptor are compared with those of previously reported BK receptors from other species.

Methods

Cell culture and transfection

For transient expression a modified plasmid pCEP4 episomal mammalian expression vector was transfected into a HEK 293-EBNA-1 cell line with FuGENE™6 Transfection Reagent (GibcoBRL, Stockholm, Sweden), Optimem medium (Gibco BRL) (Duner *et al*., 2002), according to the manufacturer's recommendations. Transfected cells were maintained in Dulbecco's minimal essential medium (GibcoBRL) and supplemented with 10% foetal calf serum (Biotech Line, AS, U.S.A.), 500 $\mu g\,ml^{-1}$ active G-418, 100 $U\,ml^{-1}$ of penicillin, 100 $\mu g\,ml^{-1}$ streptomycin, 2 mM L-glutamine (GibcoBRL) and 150 $\mu g\,ml^{-1}$ hygromycin (Invitrogen AB, Sweden).

Peptides

Zebrafish BK ([Arg^0,Trp^5,Leu^8]-BK), mammalian BK and the derivatives [des-Arg^0,Trp^5,Leu^8]-BK; [Arg^0,Trp^5,Leu^8,des-Arg^9]-BK; Ala-substituted and D-amino acid-substituted analogues were synthesized and purified as described (Jensen & Conlon, 1997). Ornithokinin and HOE140 were kindly provided by Dr Werner Müller-Esterl (Frankfurt, Germany). The model of zebrafish BK shown in Figure 2 is adapted and remodified from the NMR structure of human BK (Verge *et al*., 2002). The picture was created in Swiss-PdbViewer (Guex & Peitsch, 1997).

Determination of inositol phosphate (IP) formation

The experiments were performed as essentially described in Holmqvist *et al*. (2002). Briefly, HEK 293 cells were grown on culture dishes and loaded with 3 $\mu Ci\,ml^{-1}$ *myo*-[2-^{3}H]inositol (1 $mCi\,ml^{-1}$) (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) for 20 h in the culture medium. Cells were harvested using phosphate buffered saline containing 0.2 $g\,l^{-1}$ EDTA, centrifuged at $800 \times g$ for 5 min and suspended in TBM (TES buffered medium = 137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 10 mM glucose and 20 mM 2-([2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino) ethane sulphonic acid (TES) adjusted to pH 7.4 with NaOH) supplemented with 10 mM LiCl. After a 10-min preincubation at 37°C, the stimulations were started by dispensing the cell suspension onto previously prepared 96-well plates containing BK peptides. After 20 min of stimulation at 37°C, cells were centrifuged at $1250 \times g$ for 10 min, supernatants were discarded and the reactions were stopped by adding 100 μl of 0.4 M ice-cold perchloric acid. Plates were frozen down, thawed carefully at 4°C and samples neutralized with 50 μl containing 0.36 M KOH and 0.3 M $KHCO_4$. Precipitates were spun down and the total IP fraction of the supernatant was isolated by anion exchange chromatography (Berridge, 1983). IP fractions were dissolved in an appropriate volume of scintillation cocktail (Optiphase Hisafe 3, Wallac, Turku, Finland) and analyzed in a 1900CA TriCarb® beta-counter (Packard).

Drugs

The following drugs and chemicals were used: [Arg^0,Trp^5,Leu^8]-BK (zebrafish BK) and derivatives, D-arginyl-L-arginyl-L-prolyl-*trans*-4-hydroxy-L-prolyl-L-glycyl-3-(2-thienyl)-L-alanyl-L-seryl-D-1,2,3,4-tetrahydro-3-isoquinolinecarbonyl-L-(2a,3b,7ab)-octahydro-1H-indole-2-carbonyl-L-arginine (HOE140) (gift from Dr Werner Müller-Esterl, Frankfurt, Germany), ^{1}*myo*-[2-^{3}H]inositol (Amersham Pharmacia Biotech, Buckinghamshire, U.K.).

Table 1 Structure of BK from various species

Analogue name	*Sequence*									
	0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
Mammalian BK[a]	—	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg
Zebrafish BK	Arg	Arg	Pro	Pro	Gly	**Trp**	Ser	**Leu**	Phe	Arg
Ornithokinin	—	Arg	Pro	Pro	Gly	Phe	**Thr**	Pro	**Leu**	Arg
des-Arg^9 zebrafish BK	Arg	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	—
des-Arg^0 zebrafish BK	—	Arg	Pro	Pro	Gly	Phe	Ser	Pro	Phe	Arg
HOE140	D-Arg	Arg	Pro	**Hyp**	Gly	**Thi**	Ser	**D-Tic**	**Oic**	Arg

[a]Mouse BK has [Arg^0]. A dash indicates a missing amino acid. For a complete description of the chemical structure of HOE 140, see Methods. Residues shown in bold differ from mammalian sequence.

Statistics

Potencies and maximum responses of the BK peptides were determined by non-linear regression using the sofware package GraphPad Prism version 4.0a. Data are presented as means ±s.e.m. of *n* independent experiments. Statistical comparisons (two groups) were performed by unpaired Student's *t*-test. A probability level of 0.05 or less was considered as statistically significant.

Results

IP response

The functional response with regard to IP accumulation was measured after exposure of HEK 293 cells transfected with the zebrafish BK receptor to zebrafish BK and a diverse panel of analogues (Table 2). Zebrafish BK showed the highest potency ($pEC_{50} = 6.97$) and maximum response, and this value was taken as 100% for comparison with the other analogues. The potency of mammalian BK was not measurable ($pEC_{50} < 5$). The potency of ornithokinin ($pEC_{50} = 6.39$) was significantly ($P < 0.05$) less than for zebrafish BK (Figure 1a). HOE140 was almost inactive at the zebrafish BK receptor (7% of the maximum response of zebrafish BK; $pEC_{50} < 5$) (Table 2). Des-Arg^9-zebrafish BK (analogous to the B1-selective analogue, des-Arg^9-BK in mammals) was also almost without an effect (10%; $pEC_{50} < 5$). Des-Arg^0-zebrafish BK was a full agonist with reduced potency ($pEC_{50} = 6.06$) ($P < 0.01$).

As a negative control, untransfected HEK 293 cells were exposed to mammalian BK, zebrafish BK and ornithokinin.

Table 2 IP response presented as pEC_{50} and maximum response (%)

Peptides/analogues	*pEC_{50} (M)*	*Max (%)*
Zebrafish BK	6.97±0.1	100±0
Mammalian BK	<5.0	**16±1
Ornithokinin	*6.39±0.19	93±19
des-Arg^9	<5.0	**10±3
des-Arg^0	**6.06±0.25	80±21
Ala0	6.90±0.08	102±27
Ala1	***4.76±0.39	**62±3
Ala2	6.81±0.16	93±11
Ala3	**7.53±0.16	77±11
Ala4	<5.0	*27±13
Ala5	6.74±0.16	77±14
Ala6	***5.62±0.19	75±15
Ala7	***5.71±0.14	*74±7
Ala8	***5.68±0.14	*25±14
Ala9	<5.0	*41±8
D-Arg^0	6.74±0.07	66±8
D-Arg^1	6.47±0.20	**38±4
D-Pro^2	6.51±0.23	*49±7
D-Pro^3	*6.31±0.12	*46±3
D-Trp^5	6.89±0.14	**39±4
D-Ser^6	No response	No response
D-Pro^7	No response	No response
D-Leu^8	No response	No response
D-Arg^9	<5.0	No response
HOE140	<5.0	**7±4

Means±s.e.m. of 3–11 experiments in triplicates. *$P<0.05$,**$P<0.01$, ***$P<0.001$ indicates that pEC_{50}(M) is statistically different from zebrafish BK.

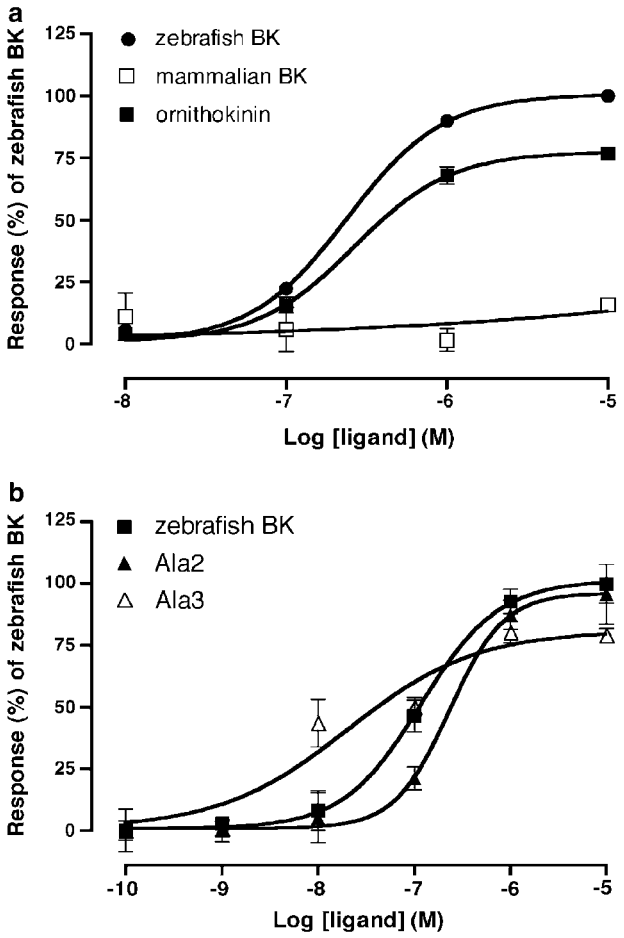

Figure 1 IP accumulation in cells expressing the zebrafish BK receptor. Results are expressed as ligand-stimulated IP production (%) minus basal IP normalized to that of zebrafish BK. Data represent triplicates of one representative experiment. (a) Ornithokinin is a potent agonist at the zebrafish receptor, while mammalian BK acts as a very weak agonist. (b) The Ala^2 analogue is a full agonist with lower potency than zebrafish BK, while the Ala^3 zebrafish BK is more potent than zebrafish BK itself.

The absence of response confirmed lack of expression of endogenous BK receptors in cells (data not shown).

Responses to Ala-substituted zebrafish BK analogues

The substitution Arg^0→Ala in zebrafish BK produced a full agonist with the same potency as wild-type BK ($pEC_{50} = 6.9 \pm 0.08$; 102%). Similarly, Ala substitutions at Arg^2 (Figure 1b) and Trp^5 gave analogues with the same potency as wild-type zebrafish BK (6.81±0.16 and 6.74±0.16, respectively) (Figure 2). However, the maximum response of the Ala^5-substituted peptide was significantly reduced (77%). Ala substitutions at positions Ser^6, Pro^7 and Leu^8 reduced the EC_{50} value by more than one order of magnitude, and the potency of the Arg^1-substituted analogue was reduced 100-fold (4.76±0.39) (Table 2, Figure 2). In contrast, the potency of the analogue substitued at Pro^3 was almost 10-fold higher ($pEC_{50} = 7.53 \pm 0.16$; $P < 0.01$) compared to wild-type BK (Figure 1b). Substitutions at Gly^4 and Arg^9 produced analogues with the lowest responses ($pEC_{50} < 5$), indicating

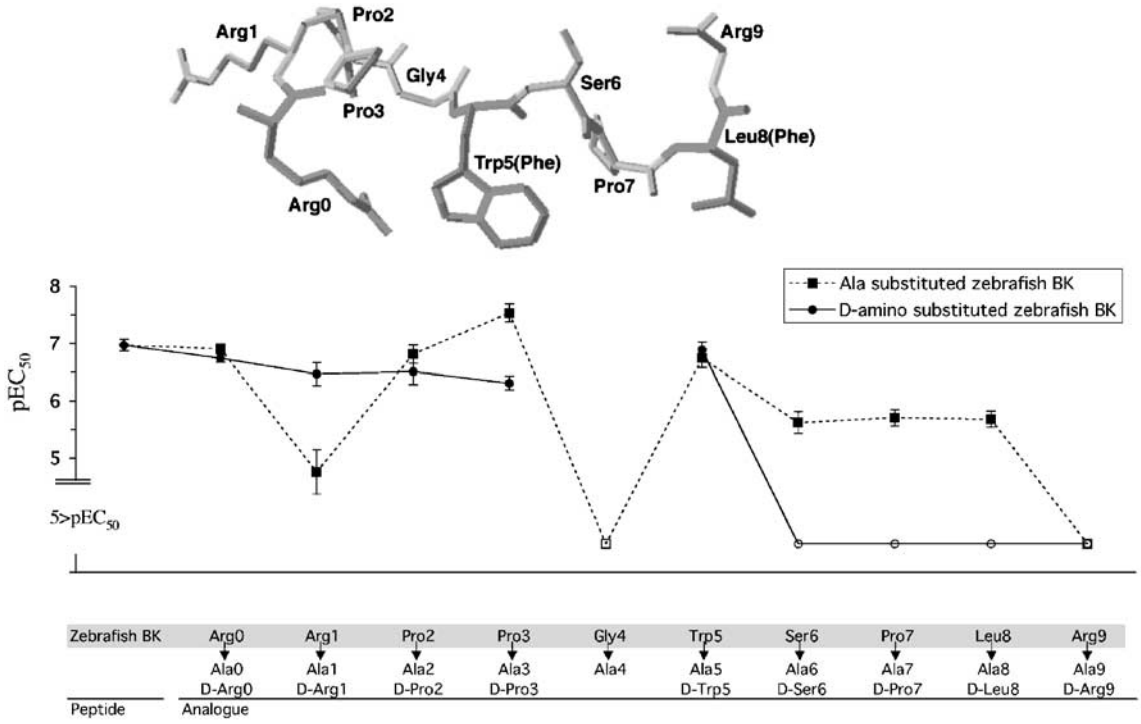

Figure 2 Graphic presentation of pEC_{50} values for Ala-substituted and D-amino-acid-substituted zebrafish BK analogues. Values are from Table 2. The upper part presents the zebrafish BK as a modification of the NMR structure of human BK and the amino acid within parentheses represents the human equivalents. The lower part shows pEC_{50} values for the different analogues compared to zebrafish BK where each mutated position is indicated by an arrowhead. pEC_{50} for wild-type zebrafish BK is indicated by a diamond (far left). Unfilled squares and circles show a pEC_{50} value that was too weak to measure.

that these positions are of particular importance in activation of the receptor (Figure 2).

D-amino-substituted zebrafish BK

Analogues of zebrafish BK substituted at positions Arg^0, Arg^1, Pro^2 and Trp^5 by the corresponding D-amino acid did not display significantly different potencies from the wild-type peptide on receptor-stimulated IP (Figure 2) accumulation, but they all had lower maximum responses, with the exception of D-Arg^0 (Table 2). The D-Pro^3 analogue was significantly less potent with pEC_{50} 6.31 ± 0.12 ($P < 0.05$). The D-Ser^6, D-Pro^7 and D-Leu^8 analogues gave no response and the D-Arg^9 analogue showed a very weak potency ($pEC_{50} < 5.0$) (Figure 2). Dose–response curves for zebrafish BK were constructed in the presence of the inactive analogues at 1 μM concentrations, but the potency of the wild-type peptide was unchanged ($pEC_{50} = 6.97 \pm 0.1$). It is concluded, therefore, that the inactive analogues are devoid of antagonistic properties and have thus lost their affinity for the receptor.

Discussion

The recent cloning of a zebrafish BK receptor, proposed to be orthologous to the mammalian B2 subtype (Duner *et al*., 2002), has allowed us to test for the first time the ability of a wide range of zebrafish BK analogues to activate a fish BK receptor as measured by increased IP production in transfected cells. The analogues were also tested in a cAMP assay with and without forskolin stimulation but gave no response (data not shown), suggesting that the receptor does not couple *via* $G\alpha_i$ or $G\alpha_s$ in the HEK293 cell system. BK with identical amino-acid sequences have been purified from trout (Conlon *et al*., 1996), cod (Platzack & Conlon, 1997) and eel (Takei *et al*., 2001), and the same sequence has been identified in the zebrafish genome database (Bromée *et al*., unpublished). The four species of teleost are not phylogenetically closely related and the complete conservation of the BK structure suggests that the kallikrein–kinin system may be particularly important in teleost fish. A precise regulation of the rate of drinking is essential to the survival of marine teleosts that exist in a hyperosmotic environment and an antidipsogenic action of eel BK in the salt water-adapted eel has been demonstrated (Takei *et al*., 2001).

A complete Ala- and D-amino-acid scan of zebrafish BK has provided insight into those amino acids in the peptide that are important in interaction with the zebrafish BK receptor. Analogues substituted with Ala^1, Ala^4, Ala^7, Ala^8, or Ala^9 acted as partial agonists with reduced potency, and peptides substituted with D-Ser^6, D-Pro^7, D-Leu^8 and D-Arg^9 either gave no response or showed very low potency. These results suggest that all residues at the carboxy-terminal region of the peptide are important for activation of the receptor. Consistent with this, zebrafish des-Arg^9-BK also showed a weak response at the zebrafish BK receptor, adding further support to the assertion that the receptor should be classified, from a pharmacological perspective, as a B2 rather than a B1 receptor, in agreement with the phylogenetic analyses (Duner *et al*., 2002).

Our data demonstrate that the pharmacological properties of the zebrafish BK receptor differ appreciably from mamma-

lian B1 and B2 receptors and also from the ornithokinin receptor in chicken (Schroeder *et al*., 1997) and a putative BK receptor in the smooth muscle of trout stomach (Jensen & Conlon, 1997). An alternative explanation is increased receptor density in the recombinant system relative to the native systems. Mammalian BK was almost inactive at the zebrafish receptor and ornithokinin behaved as a full agonist with only a slightly reduced potency. At the human B2 receptor, HOE140 is a high-affinity antagonist (Wirth *et al*., 1991), but it acts as a full agonist at the chicken ornithokinin receptor (Schroeder *et al*., 1997) and as a potent partial agonist at the trout receptor (Jensen & Conlon, 1997). In contrast, HOE140 acted as a very weak partial agonist at the zebrafish receptor. Deletion of the Arg^0 residue in zebrafish/trout BK or substitution by Ala produces a very weak agonist as measured by the ability to contract isolated smooth muscle from trout stomach (Jensen *et al*., 2000), but this has only a minor effect on the ability of the peptide to stimulate IP accumulation *via* the zebrafish receptor. Similarly, the substitution $Trp^5 \rightarrow Ala$ produced an inactive analogue at the trout receptor, but had no effect at the zebrafish receptor.

The pharmacological properties of the zebrafish BK receptor resemble more closely the putative BK receptor identified in intestinal smooth muscle from the more highly derived teleost, the Atlantic cod (Shahbazi *et al*., 2001). Ala substitutions at positions 4, 8 and 9 produced a marked decrease in the potency of zebrafish/cod BK in contracting cod intestinal smooth muscle, but substitution or deletion at position 1 and substitution at position 5 had only minor effects on potency and maximum response. Similarly, substitution $Pro^3 \rightarrow Ala$ results in an analogue with appreciably increased potency relative to the wild-type peptide in both the cod and zebrafish test systems.

It has been proposed that transmembrane regions TM3 and TM6 of human B1 and B2 receptors contain important sites for interaction with BK agonists and HOE140-related molecules (Nardone & Hogan, 1994; Fathy *et al*., 2000; Leeb-Lundberg *et al*., 2001). Human B2 positions Phe^{259} and Thr^{263} (Leeb *et al*., 1997) in TM6 are conserved in all B2 receptors, including the zebrafish receptor investigated here (see the corresponding zebrafish positions Phe^{265} and Thr^{269} in Figure 1 in Duner *et al*., 2002). Interestingly, the human B1 receptor differs at the corresponding positions with Tyr^{266} and Ala^{270}, suggesting that these positions may account for the higher affinity of des-Arg^9-BK for human B1 than human B2 and the zebrafish receptor described here. The residues Tyr^{295} (TM7) and Gln^{288} (TM7) in human B2 also play a part in the interaction with nonpeptide ligands where Tyr^{295} also has a subtle function in receptor activation (Marie *et al*., 2001). Gln^{288} is conserved in all BK receptor subtypes, while Tyr^{295} is substituted by Phe in human B1, the ornithokinin receptor and the zebrafish BK receptor. Thus, the zebrafish BK receptor has features of both mammalian B1/chicken ornithokinin (Phe^{295}) and mammalian B2 (Phe^{259} and Thr^{263}).

The analogues of human BK, Ala^6-BK and Ala^9-BK showed reduced binding affinity (100- and 27,000-fold, respectively, compared with human BK) measured using membranes of CHO cells expressing the human B2 receptor (Jarnagin *et al*., 1996). The present study shows that the potencies of Ala^6-zebrafish BK and Ala^9-zebrafish BK in stimulating IP accumulation were reduced at least 60-fold, respectively, as compared to zebrafish BK (Table 2). Thus, our results add further support to the importance of Trp^6 and Arg^9 for receptor binding/activation.

It has been suggested that position Phe^8 of human BK is placed in a hydrophobic pocket midway through the transmembrane regions of human B2 (Jarnagin *et al*., 1996). Position 8 in zebrafish BK has a leucine residue, thus maintaining the hydrophobic character. However, the Ala^8 analogue lost only one order of magnitude in potency, suggesting that the hydrophobic side chain is not of major importance for peptide binding.

In summary, our results constitute the first detailed characterization of a piscine BK receptor with regard to ligand interactions. The results suggest important roles in receptor interaction for residues Gly^4, Ser^6, Pro^7 and Leu^8 and Arg^9 in zebrafish BK, whereas residue Pro^3 is proposed to have a limiting effect on receptor activation. Earlier, we designated the zebrafish BK receptor as B2-like based upon phylogenetic analyses (Duner *et al*., 2002). The present data show that the pharmacological properties of the zebrafish receptor are distinct from both the mammalian B1 and B2 receptors; so its subtype assignment remains somewhat ambiguous. Indeed, a second zebrafish BK receptor has been identified in the zebrafish genome (Bromée *et al*., unpublished), which may shed further light on the evolution of the kallikrein–kinin system in fish.

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