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Characterisation and expression of β1-, β2- and β3-adrenergic receptors in the fathead minnow (*Pimephales promelas*)

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ABSTRACT

Complimentary DNAs for three beta-adrenergic receptors (βARs) were isolated and characterised in the fathead minnow. The encoded proteins of 402 (β_1AR), 397 (β_2AR) and 434 (β_3AR) amino acids were homologous to other vertebrate βARs, and displayed the characteristic seven transmembrane helices of G Protein-coupled receptors. Motifs and amino acids shown to be important for ligand binding were conserved in the fathead minnow receptors. Quantitative RT-PCR revealed the expression of all receptors to be highest in the heart and lowest in the ovary. However, the β_1AR was the predominant subtype in the heart (70%), and β_3AR the predominant subtype in the ovary (53%). In the brain, β_1AR expression was about 200-fold higher than that of β_2- and β_3AR, whereas in the liver, β_2AR expression was about 20-fold and 100-fold higher than β_3- and β_1AR expression, respectively. Receptor gene expression was modulated by exposure to propranolol (0.001–1 mg/L) for 21 days, but not in a consistent, concentration-related manner. These results show that the fathead minnow has a beta-adrenergic receptor repertoire similar to that of mammals, with the molecular signatures required for ligand binding. An exogenous ligand, the beta-blocker propranolol, is able to alter the expression profile of these receptors, although the functional relevance of such changes remains to be determined. Characterisation of the molecular targets for beta-blockers in fish will aid informed environmental risk assessments of these drugs, which are known to be present in the aquatic environment.

1. Introduction

Adrenergic receptors (ARs) belong to the G protein-coupled receptor (GPCR) superfamily of proteins, which constitute the largest proportion of membrane signal transducers [38]. There are two main types of adrenoceptors, the αARs and βARs, and for each several subtypes have been identified in mammals: α_{1a}, α_{1b}, α_{1d}, α_{2a}, α_{2b}, α_{2c}, and β_1, β_2, β_3 [13,59]. There is emerging evidence that fish also express the same receptors; for example, α_{1a}-, α_{1b}-, α_{1d} ARs have been characterised in rainbow trout [10]; α_{2b}-, α_{2c}- and α_{2d} ARs in zebrafish [54]; β_1-, β_2- and β_3 ARs in zebrafish [67] and black bullhead [16]; β_2- and β_3AR in rainbow trout [41,42]. Additionally, genomic sequencing has identified homologues in medaka, stickleback, fugu and tetraodon, and a search of GeneBank reveals several partial sequences of teleost adrenoceptors.

The function of the adrenergic receptor system is believed to be the same in fish as it is in mammals, with activation of signal transduction following epinephrine/norepinephrine binding [19]. The recent high-resolution structural studies [11,44,46,68] have provided experimental confirmation of the predicted molecular mechanism of GPCR activation in general, and of adrenoceptors in particular (reviewed by Rosenbaum et al. [52]). Our increased understanding of the structural requirements for receptor interaction and activation is useful when assessing the likelihood of receptors in other species becoming targets for agonists and antagonists designed for human receptors.

Our interest is the potential effects on aquatic organisms, especially fish, of pharmaceuticals present in the aquatic environment. Currently, approximately 150 different drugs have been detected in rivers and waterways [53] and there is concern that some of these, particularly those which have a high usage, are potent at low concentrations, poorly degraded or with a propensity for bioaccumulation, may pose a threat to aquatic organisms [30,61]. For example, ethinylestradiol, a component of the contraceptive pill, has been found to be a highly potent endocrine disrupter in fish at low environmental concentrations [8]. The beta-adrenoceptor blockers (β-blockers) are a group of pharmaceuticals widely prescribed for conditions such as high blood pressure, cardiac arrhythmias, glaucoma, anxiety and migraines, which exert their effects by binding to βARs and thereby preventing the interaction of epinephrine with its receptors. They are present in the aquatic environment at concentrations ranging from <0.8 to 2900 ng/L

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[62,65], and from acute EC_{50} data it appears that atenolol is non-toxic, whilst metoprolol would be classified as toxic and propranolol as very toxic to aquatic organisms [14]. Chronic data with respect to aquatic life and β-blockers are scarce, but recent studies indicate that these human drugs may affect fish at concentrations below toxic levels [43,69]. This suggests the presence of βARs, and we report here the characterisation of three beta-adrenoceptors, β_1, β_2 and β_3, in the fathead minnow. The receptors contain the conserved amino acids and motifs identified as being important for agonist and antagonist binding, and receptor activation. Gene expression data also suggests that similar physiological effects to those seen in mammals may be expected following ligand binding, and modulation of receptor expression was seen following chronic exposure to propranolol. However, the functional importance of such changes requires information at the protein level, and remains to be determined.

2. Materials and methods

2.1. Tissue acquisition

Fathead minnows, *Pimephales promelas*, were bred and maintained at Brunel University as detailed in Giltrow et al. [25]. Liver tissue was used for the characterisation of βARs, whilst receptor expression analysis was performed on liver, brain, heart and ovary tissue from female fish. We also examined receptor expression in these tissues obtained from fathead minnows exposed to different concentrations of propranolol (0.001, 0.01, 0.1 and 1 mg/L) for 21 days [25]. The tissues were immediately snap frozen in liquid nitrogen after removal and stored at −80 °C until use.

2.2. Identification of βARs

Total RNA was extracted from liver using 1 ml of TriReagent (Sigma, Dorset, UK) for every 50–100 mg of tissue. The RNA quality was verified using 1.2% agarose gel electrophoresis and quantified on a Nanodrop ND-1000 spectrophotometer. mRNA was isolated using Genelute mRNA miniprep kit (Sigma, Dorset, UK) and complementary DNA (cDNA) was obtained using Superscript III reagents and protocol (Invitrogen, Paisley, UK).

The oligonucleotide primers and annealing temperatures used in obtaining putative β_1AR, β_2AR and β_3AR fragments are shown in Table 1. AmpliTaq Gold (Applied Biosystems, Warrington, UK) was used in all PCR reactions. Putative βAR fragments were cloned, sequenced and localised to a particular βAR using Blast searches (www.ncbi.nlm.nih.gov). Rapid Amplification of cDNA Ends PCR (RACE PCR; Invitrogen, Paisley, UK and Clontech, California, USA) was used to obtain the remainder of the sequence in each direction. The complete receptor sequences were amplified using primers designed to the 3′ and 5′ untranslated regions (UTRs) and proof reading Taq (Pwo SuperYield DNA polymerase, Roche, Sussex, UK). Following cloning, the receptor sequences were confirmed by 'primer walking' in each direction in triplicate (Dundee University's Sequencing Service, www.dnaseq.co.uk).

2.3. Characterisation of βAR sequences

The fathead minnow βAR sequences were used to search for homologues in other species using Blast (www.ncbi.nlm.nih.gov). Sequence and phylogenetic analyses were performed using the following database entries: For β_1AR: *Homo sapiens* (human) NP_000675, *Danio rerio* (zebrafish) NP_001122161, *Takifugu rubripes* (fugu) ENSTRUP00000031392, *Gasterosteus aculeatus* (stickleback) ENSGACP00000008698, *Oryzias latipes* (medaka) ENSORLP00000006043, *Ovis aries* (sheep) AAB34523, *Mus musculus* (mouse) NP_031445, *Xenopus laevis* (frog) NP_001084152, *Meleagris gallopavo* (turkey) AAA49627; For β_2AR: Human AAA88015, zebrafish adrb2a NP_001096122, zebrafish adrb2b BAH84779, *Oncorhynchus mykiss* (rainbow trout) NP_001117912, *Tetraodon nigroviridis* (tetraodon) ENSTNIP00000020193, fugu AAQ02695, stickleback ENSGACP00000024398, medaka ENSORLP00000009383, sheep NP_001123626, mouse NP_031446, frog NP_001085791, *Ciona intestinalis* (ciona) XP_002121940; For β_3AR: Human NP_000016, zebrafish adrb3a BAH84778, zebrafish adrb3b NP_001128606, rainbow trout adrb3a NP_001118100, rainbow trout adrb3b NP_001117924, *Ameiurus melas* (black bullhead) adrb3b ABH10580, stickleback ENSGACP00000014582, fugu ENSTRUP00000020757, medaka ENSORLP00000014229, sheep AAG31167, mouse NP_038490; For β_{4c}AR: *Salmo salar* (salmon) NP_001133926, turkey AAA62150.

The positions of seven transmembrane helices were predicted using the hydropathy analysis programme TM_PRED (http://www.ch.embnet.org/software/TMPRED_form.html), and refined by manual comparisons to the crystal structures of bovine rhodopsin [44], turkey β_1AR [46] and human β_2AR [11,68] in order to more accurately predict the length of the helices, as structural analysis had revealed these to extend further into the cytoplasm than suggested by hydropathy-based computer modelling. Prediction of palmitoylation sites was carried out using CSS-Palm 2.0 [47]. Potential phosphorylation of serine, threonine and tyrosine residues in the intracellular loop 3 and cytoplasmic tail was identified using NetPhos 2.0, and protein kinase phosphorylation sites were predicted using NetPhosK 1.0 (http://www.cbs.dtu.dk/services/NetPhos) [6].

2.4. Phylogenetic analysis

The βAR amino acid sequences were aligned using ClustalW (2.0.3), and the phylogenetic tree created using the Neigbourhood-Joining algorithm with 1000 bootstrap replicates in ClustalX (2.0.12) [36]. The un-rooted tree was visualised using Dendroscope V2.7.4 (http://www.dendroscope.org), and rooted with *Ciona intestinalis* β_2AR as the outgroup.

2.5. Gene expression

The expression of β_1AR, β_2AR and β_3AR in liver, brain, ovary and heart of female fathead minnows was quantified using real-time PCR (QPCR). Primers were as follows: β_1AR (185 bp) forward 5′CTTCGTATTTTTGAACTGGC3′, reverse 5′CCATTGAGTTCACAAAG CCC3′; β_2AR (224 bp) forward 5′AGGTGATCAAGAGTCGAGTG3′, reverse 5′ATGCTAATTAAGACCACCTC3′; β_3AR (105 bp) forward 5′GGCCCAGCAAAAAACATCC3′, reverse 5′TTCCCATAGTGCTTGCCT CCTC3′. The amplicons were cloned and sequenced to confirm their identities. QPCR standard βARs were prepared by in vitro transcribing cloned amplicons to generate RNA (Riboprobe, Promega), which was serially diluted (10^7–10^1 molecules) prior to use. Assays (20 μl) utilising Quantitect SYBR Green (Qiagen) and 0.5 μM (β_1AR and β_2AR) or 0.1 μM (β_3AR) each of forward and reverse primers were carried out in 96-well plates, with an efficiency greater than 90%. One microliter of each sample mRNA (5 ng/μl), RNA standards and non-template control (sterile water) were assayed in triplicate. The QPCR cycling included a reverse transcription step (30 min at 50 °C, 15 min at 95 °C), followed by 40 cycles of amplification (15 s each at 95 °C, 55 °C and 72 °C for β_1AR; 15 s each at 95 °C, 56 °C and 72 °C for β_2AR; 30 s each at 95 °C, 55 °C and 72 °C for β_3AR), and elongation for 15 min at 72 °C. There was no significant difference between the threshold cycle (Ct) value for each RNA standard concentration between assay plates, and absolute gene expression was calculated from the standard curves and plotted as copies/ng mRNA.