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RESEARCH ARTICLE

In vivo and *in vitro* assessment of cardiac β-adrenergic receptors in larval zebrafish (*Danio rerio*)

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SUMMARY

β-Adrenergic receptors (βARs) are crucial for maintaining the rate and force of cardiac muscle contraction in vertebrates. Zebrafish (*Danio rerio*) have one β1AR gene and two β2AR genes (*β2aAR* and *β2bAR*). We examined the roles of these receptors in larval zebrafish *in vivo* by assessing the impact of translational gene knockdown on cardiac function. Zebrafish larvae lacking β1AR expression by morpholino knockdown displayed lower heart rates than control fish, whereas larvae deficient in both β2aAR and β2bAR expression exhibited significantly higher heart rates than controls. These results suggested a potential inhibitory role for one or both β2AR genes. By using cultured HEK293 cells transfected with zebrafish βARs, we demonstrated that stimulation with adrenaline or procaterol (a β2AR agonist) resulted in an increase in intracellular cAMP levels in cells expressing any of the three zebrafish βARs. In comparison with its human βAR counterpart, zebrafish β2aAR expressed in HEK293 cells appeared to exhibit a unique binding affinity profile for adrenergic ligands. Specifically, zebrafish β2aAR had a high binding affinity for phenylephrine, a classical α-adrenergic receptor agonist. The zebrafish receptors also had distinct ligand binding affinities for adrenergic agonists when compared with human βARs in culture, with zebrafish β2aAR being distinct from human β2AR and zebrafish β2bAR. Overall, this study provides insight into the function and evolution of both fish and mammalian β-adrenergic receptors.

Key words: morpholino, adenylyl cyclase, cardiac output, binding affinity, HEK293 cells.

INTRODUCTION

Adrenergic receptors (adrenoreceptors, ARs) are G-protein-coupled receptors that transduce the cellular effects of adrenaline and noradrenaline and are expressed ubiquitously in vertebrate tissues (e.g. Cavalli et al., 1997; Tanoue et al., 2002). The β1AR subtype is traditionally classified as the 'cardiac' βAR because stimulation of β1AR *in vivo* stimulates heart rate and contractility (Lands et al., 1967b). β2ARs were originally thought to be restricted to the lungs and peripheral vasculature (Lands et al., 1967a); however, later studies have demonstrated significant expression of β2ARs in the mammalian heart. Current models of heart function show that both of these βAR subtypes play critical roles in regulating the rate (chronotropy) and force (inotropy) of heart contraction (e.g. Bernstein, 2002; Brodde, 2008).

When considering heart rate, β1AR appears to be exclusively stimulatory, whereas the role of β2AR is less clear. Resting heart rate in $\beta 1AR^{-/-}$ and $\beta 1AR^{-/-}\beta 2AR^{-/-}$ mice was lower than in wild-types, while β2AR loss of function alone had no effect (Ecker et al., 2006). Also, cardiac myocytes isolated from $\beta 1AR^{-/-}$ mice and exposed to isoproterenol showed an initial increase followed by a sustained decrease in contraction rate compared with baseline levels (Devic et al., 2001), suggesting a dual stimulatory/inhibitory role for the β2AR receptor in these cells. An inhibitory role for cardiac β2AR was recently proposed for larval zebrafish experiencing translational knockdown of M_2 muscarinic receptors because exposure to procaterol (a β2AR agonist) caused a lowering of heart rate (Steele et al., 2009). Because zebrafish have two distinct β2AR receptors (herein termed β2aAR and β2bAR) (Wang et al., 2009), it is not clear whether one or both of the β2ARs are contributing an inhibitory influence on heart function.

Numerous studies have assessed the contribution of adrenergic tone in maintaining resting heart rate in adult fish [for references, see Mendonça and Gamperl (Mendonça and Gamperl, 2009)]; however, considerably less is known about larval fish. Larval zebrafish begin to exhibit a chronotropic response to adrenergic agonists at 4 (Schwerte et al., 2006) or 6 days post-fertilization (d.p.f.) (Bagatto, 2005), and first demonstrate adrenergic tone at 5 d.p.f. (Schwerte et al., 2006). The role of specific βAR subtypes in regulating cardiac function and development beyond the measurement of cardiac frequency in zebrafish has yet to be explored. Both β1AR and β2AR subtypes are linked to stimulatory G-proteins (G_s), which increase adenylyl cyclase activity yielding higher levels of cAMP and thus increasing cardiac chronotropy and inotropy. Whereas β1AR is exclusively linked to G_s proteins, a growing body of evidence suggests that the β2 subtype also associates with inhibitory G_i proteins and can thereby inhibit contraction of heart cells (e.g. Xiao et al., 1999; Bernstein, 2002). This dual coupling of β2AR might help explain why this receptor is not involved to the same extent as β1AR in G_s-mediated cAMP accumulation in heart cells in some species [for references, see Xiao et al. (Xiao et al., 1999; Xiao, 2001)]. Molecular and pharmacological experiments have shown that there is significant

expression of βARs in the fish heart, and that fish hearts are responsive to classic βAR ligands (Nickerson et al., 2001; Kawasaki et al., 2008; Mendonça and Gamperl, 2009; Steele et al., 2009). Some studies on fish βARs have also shown that they can have unexpected affinity for (presumed) subtype-specific agonists. Phenylephrine, a classic α-adrenergic receptor agonist, has similar competitive binding characteristics to noradrenaline for β-adrenergic receptors in catfish liver (Fabbri et al., 1992). Also, β3bARs in red blood cells of rainbow trout have distinct β2AR-like binding characteristics based on their affinity for classic β2AR ligands (Nickerson et al., 2003). To date, the ligand binding affinities of zebrafish βARs, and their ability to initiate intracellular cAMP accumulation *via* agonist stimulation, have yet to be explored.

The first goal of this study was to determine the developmental pattern of cardiac-type β-adrenergic receptor expression in zebrafish and distinguish its role in regulating heart function in early life. Developmental mRNA expression of the classic cardiac-type β-adrenergic receptors (β*1AR*, β*2aAR*, β*2bAR*) was determined using semi-quantitative real-time PCR and qualitative *in situ* hybridization. Zebrafish larvae lacking expression of β1AR, β2aAR and β2bAR either alone or in combination were generated by translational knockdown using antisense oligonucleotide morpholinos. Microscopic imaging techniques were used to determine heart rate, stroke volume and cardiac output in larvae at 4 d.p.f. Larvae were also exposed to a variety of adrenergic ligands to determine any heart rate and cardiac output changes related to agonist and antagonist exposure. The second goal was to characterize the affinity of each of the zebrafish cardiac-type β-adrenergic receptors for classic adrenergic ligands, as well as the ability of each receptor type to associate with G_s proteins within the cell. For this, the zebrafish β1AR and β2ARs were transiently expressed in HEK293 cells in culture to determine their affinity for common adrenergic agonists as well as their ability to initiate cAMP production within these cells.

MATERIALS AND METHODS

Zebrafish husbandry and culture

Adult zebrafish (*Danio rerio*, Hamilton 1822) were obtained from Big Al's Aquarium Services (Ottawa East, Ontario, Canada) and maintained in 10 l acrylic tanks in multi-rack aquatic housing systems (Aquatic Habitats, Apopka, FL, USA). All tanks were supplied with well aerated dechloraminated City of Ottawa tap water at 28°C [for ion composition, refer to Perry and Vermette (Perry and Vermette, 1987)]. Fish were maintained under a 14 h:10 h light:dark cycle and spawning occurred daily at the beginning of the light cycle. To obtain embryos, breeder tanks (1 l; Aquatic Habitats) were placed in each 10 l tank prior to spawning and collected after spawning had been allowed to proceed for at least 15 min. All experiments were performed in accordance with University of Ottawa animal care guidelines and with those of the Canadian Council on Animal Care (CCAC).

At the University of Innsbruck, adult zebrafish (Tübingen line) were housed in small aquaria at 28°C (Schwarz Aquarium Systems, Maschmühlenweg, Germany). Fish were maintained under a 14 h:10 h light:dark cycle and spawning occurred daily at the beginning of the light cycle. To obtain embryos, 8–10 random pairwise crossings were established at the beginning of the spawning period in 2 l breeding tanks. Embryos were collected from each tank every 15–20 min and the clutches were pooled. These experiments were performed in accordance with the animal ethics permission GZ 66.008/4-BrGT/2004 of the Austrian Bundesministerium für Bildung, Wissenschaft und Kultur.

Injection of β-adrenergic receptor morpholinos

Antisense morpholino oligonucleotides (conjugated to the green fluorescent tag carboxyfluorescein) were designed to block translation of β1AR, β2aAR or β2bAR and are described in Table 1. For all experiments, an injection volume of approximately 1 nl per embryo was used. Embryos were injected at the one cell

Table 1. List of primer sets and morpholino sequences used in the present study

	GenBank accession no.	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Product size (bp)	Efficiency (%)	R^2
dTomato plasmid*						
β1AR	XM_680208.2	TGAGCAAGGGCGAGGAGG	TTACTTGTACAGCTCGTCCATG			
β2aAR	XR_029238.1	GTGAGCAAGGGCGAG	Same			
β2bAR	XM_695628.3	TGAGCAAGGGCGAGGAGG	Same			
Cell culture amplicon†						
β1AR		ATGAACGCGCTTCTTTTCTC	GCGTAAAGTAAAACCCGAAGTG	1468		
β2aAR		CTGTCAGGTCATGGGAAACA	TTGAGTGTGCTAGCCTTTTTGA	1454		
β2bAR		AAGCTCATGGAGGGAGACAA	CGGTTGTAAGTTGGGACATTT	1551		
In situ probe synthesis						
β1AR		CAGAGGCTCCAGACGCTCAC	GACATCCTGCCGTTTCTCTC	983		
β2aAR		CTAATGCCTCCACCAAAAGC	GAAGGCAGAGTTTGCGTACC	896		
β2bAR		GGAGGGAGACAATACGCTGA	TTCCCATTTTTGTTTTTGGTG	1200		
Real-time PCR						
β1AR		GGGTTACTGGTGGTGCCATT	GCGTGACGCAAAGTACATC	110	96.2	0.989
β2aAR		GCTTCCAGCGTCTTCAGAAC	CCGAAGGGAATCACTACCAA	91	89.4	0.989
β2bAR		CTCGTTCCTACCCATCCACA	ATGACCAGCGGGATGTAGAA	150	103.0	0.991
18S‡	N/A	GGCGGCGTTATTCCCATGACC	GGTGGTGCCCTTCCGTCAATTC	117	98.3	0.997
Morpholino sequence (5′–3′)						
β1AR		ACGGTAGCCCGTCTCCCATGATTTG				
β2aAR		GTATTGAGGACCTTATGTTTCCCAT				
β2bAR		GATCAGCGTATTGTCTCCCTCCATG				
Control		CCTCTTACCTCAGTTACAATTTATA				

Data are given for zebrafish β-adrenergic receptor (AR) sequences 1, 2a and 2b.
*Corresponding morpholino sequence added to 5′ end of the forward primer.
†*Bam*HI restriction sequence added to 5′ end of forward primers, *Not*I restriction sequence added to 5′ end of reverse primer.
‡18S primer sequences as per Esbaugh et al., 2009.

developmental stage (approximately 15–30 min post-fertilization) for all morpholino experiments. All working stocks of morpholino were diluted prior to injection in 1× Danieau buffer [58 mmol l^{-1} NaCl, 0.7 mmol l^{-1} KCl, 0.4 mmol l^{-1} $MgSO_4$, 0.6 mmol l^{-1} $Ca(NO_3)_2$, 5.0 mmol l^{-1} Hepes (pH 7.6)] and 0.05% Phenol Red (for visualization of the injection volume). Working concentrations of the morpholino solutions were 4 ng nl^{-1} for single knockdown of β1AR and β2aAR, and 3 ng nl^{-1} for β2bAR. For dual knockdowns, a working concentration of either 8 or 7 ng nl^{-1} was created by combining these concentrations. Matching concentrations of a standard control morpholino (for sequence, see Table 1; Gene Tools, LLC, Philomath, OR, USA) were used in both the single and dual knockdown experiments. Injections were performed using either a Narishige IM 300 Microinjector system in Ottawa (Narishige International USA Inc., Long Island, NY, USA) or a pneumatic picopump (World Precision Instruments, Berlin, Germany) in Innsbruck. After injection, embryos were placed in 30 ml Petri dishes containing E3 medium with 0.03‰ Ethylene Blue and incubated at 28°C.

To test for the sequence binding specificity of the β1AR, β2aAR and β2bAR morpholinos, *in vitro*-synthesized fusion constructs were made in which the β1AR, β2aAR and β2bAR morpholino target sequences were separately introduced upstream of and in frame with the red fluorescent protein dTomato (Shaner et al., 2004) coding sequence. Each of these constructs was cloned in the forward direction into a pCS2+ expression vector. Constructs were then amplified from these plasmids by PCR using SP6 and T3 primers (IDT, Coralville, IA, USA), run on a 0.8% native agarose gel, and purified by gel extraction (Sigma-Aldrich Inc., St Louis, MO, USA). Capped mRNAs were synthesized from each purified PCR product using a mMESSAGE mMACHINE® RNA transcription kit (AM1340; Ambion Inc., Austin, TX, USA) as per the manufacturer's protocol. Embryos were injected at the one cell stage with each dTomato mRNA construct individually (100 pg nl^{-1}) or together with the corresponding morpholino (4 ng nl^{-1}). To test for cross-reactivity of the β2 morpholinos, co-injections were also performed with the β2aAR morpholino/β2bAR dTomato mRNA, and *vice versa*.

Heart rate measurements – University of Ottawa

For baseline heart rate measurements, 4 d.p.f. larvae were placed individually in a small volume of 100 mg l^{-1} Tris-buffered MS-222 (ethyl-3-aminobenzoate methanesulfonate salt, Sigma-Aldrich Inc.) at 28°C. After 3 min, the heart rate was measured by observing the embryo under a dissecting microscope and counting heart beats for 30 s. Each larva was then placed in a fresh solution also containing 100 mg l^{-1} MS-222, with 10^{-4} mol l^{-1} adrenaline (general adrenergic receptor agonist), 10^{-4} mol l^{-1} isoproterenol (βAR agonist), 10^{-4} mol l^{-1} procaterol (β2AR agonist) or 10^{-4} mol l^{-1} propranolol (βAR antagonist). These concentrations were chosen after trials with other concentrations to determine the dose required to produce the heart rate effects, and are in keeping with concentrations used in other studies on zebrafish larvae (e.g. Schwerte et al., 2006; Steele et al., 2009). After 10 min of exposure to these chemicals, heart rate was measured again. Heart rates in β1/β2aAR and β1/β2bAR morphants (Fig. 6) were acquired this way; all other heart rates were measured as described below.

Heart rate, stroke volume and cardiac output measurements – University of Innsbruck

Larvae (4 d.p.f.) were individually anaesthetized in 100 mg l^{-1} Tris-buffered MS-222 at 28°C. Once immobilized, larvae were embedded in a small volume of 2% low melting point agarose prepared with 100 mg l^{-1} MS-222. The animal was then covered in 1 ml of 100 mg l^{-1} MS-222 and placed on the temperature-controlled stage (28°C) of an inverted microscope (Zeiss Axiovert 25, Zeiss, Vienna, Austria). A digital high speed video camera (Basler A504k, Basler, Ahrensburg, Germany) attached to the microscope and connected to a personal computer captured images of the larval ventricle (dimensions of 240×240 pixels, 30 frames s^{-1}) under 40-fold magnification. Images from larvae were acquired for approximately 1 min prior to the addition of drugs to the surrounding media to obtain baseline (i.e. anaesthetized) values for heart rate, stroke volume and cardiac output. A 1 µl sample of a 10^{-1} mol l^{-1} solution of adrenaline, isoproterenol, procaterol or propranolol was added to the 1 ml of solution bathing the larva and gently mixed to create a final concentration of 10^{-4} mol l^{-1} for each treatment. After 10 min, images from each larval ventricle were acquired for 1 min to obtain the treatment values. Heart rate, stroke volume and cardiac output were measured/calculated from the captured images as per Kopp et al. (Kopp et al., 2007).

Tissue collection – adults, embryos and larvae

All adult tissues (muscle, brain, liver, gut, heart, kidney, eye and gill) and pooled samples of larvae [from 1 h post-fertilization (h.p.f.) to 10 d.p.f.] were collected and stored prior to analysis as per Steele et al. (Steele et al., 2009).

RNA extraction, cDNA synthesis and real-time PCR

All RNA extraction and cDNA synthesis were performed as described by Steele et al. (Steele et al., 2009). Briefly, total RNA was extracted from tissue and larvae samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol. cDNA was synthesized from 2 µg of total RNA using RevertAid Maloney's murine leukaemia virus reverse transcriptase (Fermentas International Inc., Burlington, ON, Canada). For the current study, Brilliant II™ SYBR Green Master Mix (Stratagene, Santa Clara, CA, USA) was used for all real-time PCR reactions. Otherwise, all real-time PCR (including standard curve validation and data analysis) was performed as per Steele et al. (Steele et al., 2009).

Whole-mount *in situ* hybridization

PCR products for β*1AR*, β*2aAR* and β*2bAR* were amplified from adult heart cDNA using the primers listed in Table 1. From these PCR products, *in situ* RNA probes were developed as per Steele et al. (Steele et al., 2009). All larvae were reared and fixed, and *in situ* hybridization performed as per Steele et al. (Steele et al., 2009).

Synthesis of expression constructs

Full-length coding regions for β*1AR*, β*2aAR* and β*2bAR* were amplified from adult zebrafish heart cDNA using the primers listed in Table 1. All PCR products were run on a 0.8% native agarose gel and gel purified using a GenElute gel extraction kit (Sigma). Gel-purified PCR products were ligated into pDrive cloning vector according to the manufacturer's instructions (cat. no. 231122; Qiagen Inc., Valencia, CA, USA). Several positive clones from each group were selected and sequenced to confirm orientation and correct reproduction of each sequence. One clone was then selected from each group and digested with *Bam*HI and *Not*I restriction enzymes according to the manufacturer's protocols (Invitrogen). Gel-purified restriction products were subsequently ligated into pcDNA3 expression vector (Invitrogen) also digested with *Bam*HI and *Not*I to ensure proper ligation of the insert in the forward direction.

Ligation was performed with T4 DNA ligase according to the manufacturer's protocol (Fermentas International Inc.). Each ligation was transformed into subcloning efficiency DH5α cells (Invitrogen) and incubated on agar plates containing 50 μg ml^{-1} ampicillin. Positive colonies were grown overnight at 37°C in 150 ml of LB media containing 50 μg ml^{-1} ampicillin. Plasmid DNA was purified from the resulting culture using a HiSpeed Plasmid Midi kit (cat. no. 12643; Qiagen Inc.) according to the manufacturer's protocol. Human β1AR (*Eco*RI) and β2AR (*Eco*RI and *Sal*I) in the CMV-based expression vector pRK5 (Lattion et al., 1999) were generously provided by Dr Susanna Cotecchia (University of Lausanne, Switzerland).

Cell culture and transfection

Human embryonic kidney 293 (HEK293) cells (CRL-1573; American Type Culture Collection, Manassas, VA, USA) seeded in 100 mm dishes were grown in minimal essential medium (MEM; Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS; PAA Laboratories Inc., Etobicoke, ON, Canada) and gentamicin (10 μg ml^{-1}; Invitrogen) at 37°C in a humidified 5% CO_2 environment. Cells (2.5×10^6 cells dish^{-1}) were transiently transfected with human (hβ1AR, hβ2AR) and zebrafish (zfβ1AR, zfβ2aAR, zfβ2bAR) receptors with a total of 5 μg DNA per 100 mm dish using a modified calcium phosphate procedure (Tumova et al., 2004). For radioligand binding studies, 5 μg of receptor plasmid DNA were employed per transfection dish. For whole-cell cAMP studies, empty pCMV5 vector was added to normalize the total amount of DNA to 5 μg per 100 mm dish as the quantity of receptor DNA required to obtain submaximal receptor expression was less than 5 μg. HEK293 cells used in experiments were from 40 to 50 passages.

Crude membrane preparation

Transfected HEK293 cells were washed with phosphate-buffered saline (PBS), trypsinized, pooled into 150 mm dishes and incubated at 37°C in a 5% CO_2 environment for ~48 h prior to radioligand saturation studies. Crude membrane preparations from cells grown in 150 mm dishes were prepared by centrifugation washes as previously described (D'Aoust and Tiberi, 2010). Final pellets were homogenized using a Brinkman Polytron for 15 s in 3 ml of cold resuspension buffer (62.5 mmol l^{-1} Tris-HCl pH 7.4, 1.25 mmol l^{-1} EDTA pH 8.0). A fraction of membrane preparations (0.6 ml) was used immediately for saturation studies and the remaining homogenates were frozen in liquid nitrogen, and stored at –80°C until used for competition studies.

Radioligand binding assays

Binding reactions were carried out with 100 μl of membrane preparations and 50 μl of [^{3}H]dihydroalprenolol (DHA, 97–102 Ci mmol^{-1}; where 1 Ci≈3.7×10^{10} Bq; Perkin-Elmer, Boston, MA, USA) in the absence or presence of 'cold' competing drugs in a total volume of 500 μl of assay buffer (final in assays: 50 mmol l^{-1} Tris-HCl pH 7.4, 120 mmol l^{-1} NaCl, 5 mmol l^{-1} KCl, 4 mmol l^{-1} $MgCl_2$, 1.5 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} EDTA pH 8.0) at 20°C for 1 h. For saturation studies, fresh membrane preparations were incubated with increasing concentrations of [^{3}H]DHA (0.005–5 nmol l^{-1} for hβ2AR and zfβ2bAR; 0.05–25 nmol l^{-1} for hβ1AR, zfβ1AR and zfβ2aAR) in the absence or presence of 1 μmol l^{-1} alprenolol hydrochloride (cat. no. A8676) to delineate total and non-specific binding, respectively. For competition studies, frozen membranes were thawed on ice and incubated with 50 μl [^{3}H]DHA (~0.5 nmol l^{-1} for hβ2AR and zfβ2bAR; ~2.5 nmol l^{-1} for hβ1AR, zfβ1AR and zfβ2aAR) and increasing concentrations of competing ligands dissolved in double distilled water [dobutamine hydrochloride (cat. no. D0676), final concentration in assays 0.1–1000 μmol l^{-1}; (–)-adrenaline (+)-bitartrate salt (cat. no. E4395), final concentration in assays 0.1–1000 μmol l^{-1}; (*R*)-(–)-isoproterenol (cat. no. 286303), final concentration in assays 0.1–100 μmol l^{-1}; (±)-noradrenaline (+)-bitartrate salt (cat. no. A0937), final concentration in assays 0.1–1000 μmol l^{-1}; (*R*)-(–)-phenylephrine (cat. no. P6126), final concentration in assays 0.1–1000 μmol l^{-1}; procaterol hydrochloride (cat. no. P9180), final concentration in assays 0.1–1000 μmol l^{-1}]. Drugs were from Sigma-Aldrich. Binding reactions were stopped by rapid filtration through glass fibre filters (GF/C, Whatman, Piscataway, NJ, USA) and bound radioactivity was quantified by liquid scintillation counting (Beckman Counter, LS6500). Protein concentrations were measured using the Bio-Rad assay kit (Bio-Rad Laboratories Inc., Mississauga, ON, Canada) with bovine serum albumin (BSA) as standard. Binding curves were analysed using the non-linear curve-fitting program GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA) to calculate the equilibrium dissociation constant (K_d, nmol l^{-1}) and maximal binding capacity (B_{max}, pmol mg^{-1} of membrane proteins) of [^{3}H]DHA (saturation studies), and the equilibrium dissociation constant of unlabelled adrenergic drugs (K_i, nmol l^{-1}) at [^{3}H]DHA-labelled receptors (competition studies). Affinity ratios were calculated by dividing the ligand affinity of zfβ1AR and zfβ2AR by that measured with the same ligand at hβ1AR and hβ2AR, respectively. Affinities measured with different ligands at hβ2AR and zfβ2AR were divided by the corresponding ligand affinity of hβ1AR and zfβ1AR, respectively.

Whole-cell cAMP assays

Transfected HEK293 cells were seeded in 12-well plates and cultured in MEM with 10% FBS (v/v) and gentamicin (10 μg ml^{-1}) for 24 h. The medium was then removed and cells were cultured in labelling MEM containing 5% FBS (v/v), gentamicin (10 μg ml^{-1}) and [^{3}H]adenine (1 μCi ml^{-1}) overnight. The next day, labelling medium was aspirated and cells were incubated with 1 ml of 20 mmol l^{-1} Hepes-buffered MEM containing 1 mmol l^{-1} isobutylmethylxanthine (phosphodiesterase inhibitor; Sigma-Aldrich) in the absence [0.1% (v/v) ethanol] or presence of adrenergic drugs dissolved in double-distilled water (final in assays: 100 μmol l^{-1} adrenaline, 1 μmol l^{-1} procaterol) or ethanol [final in assays: 10 μmol l^{-1} (*S*)-(–)-propranolol hydrochloride; Sigma-Aldrich] at 37°C for 30 min. Following the incubation period, plates were put on ice, medium was aspirated and 1 ml of lysis solution [2.5% (v/v) perchloric acid, 0.1 mmol l^{-1} cAMP and [^{14}C]cAMP (~3.3 nCi, 9000–11,000 d.p.m.)] was added to each well. Cells were lysed for 30 min at 4°C and lysates were transferred to tubes containing 0.1 ml of a neutralizing solution (4.2 mol l^{-1} KOH), vortexed and clarified using low-speed centrifugation (500 ***g***, 15 min) at 4°C. [^{3}H]cAMP in supernatants was purified by sequential chromatography columns using Dowex AG 50W-4X resin (Bio-Rad Laboratories Inc.) and alumina N Super I (MP Biomedicals Canada, Montréal, Québec, Canada) as previously described (Johnson et al., 1994). [^{3}H]cAMP levels (CA) divided by the total amount of intracellular [^{3}H]adenine uptake (TU) was calculated and used as a relative index of adenylyl cyclase activity (expressed as CA/TU×1000). Receptor expression (B_{max}) was determined using a saturating concentration of [^{3}H]DHA on fresh membranes prepared from one 100 mm dish of cells as described above.

Table 2. Stroke volume and cardiac output of 4 d.p.f. zebrafish β-adrenergic receptor or control morphants before (MS-222) and after exposure to the adrenergic agonists adrenaline, isoproterenol and procaterol or the antagonist propranolol

	Control	β1	Control	β2a	Control	β2b	Control	β2a/β2b
MS-222–adrenaline								
SV	0.23±0.02	0.21±0.03	0.28±0.03	0.23±0.02†	0.21±0.01	0.22±0.01	0.18±0.01	0.19±0.02
CO	51.25±3.31	42.84±5.52	61.91±6.28	49.30±5.31†	46.31±2.88	48.11±3.31	36.36±2.35	44.04±4.34†
Adrenaline								
SV	0.23±0.02	0.21±0.02	0.28±0.03	0.23±0.02	0.18±0.02	0.23±0.02	0.18±0.01	0.23±0.03
CO	51.77±4.57	43.70±4.65	65.65±6.18	51.57±4.27	41.69±5.32	52.91±5.58	37.26±3.29	51.28±7.71
MS-222–isoproterenol								
SV	0.23±0.01	0.19±0.02	0.21±0.02	0.20±0.02	0.19±0.02	0.21±0.02	0.24±0.02	0.26±0.01
CO	49.11±2.48	36.05±2.59	44.61±4.50	43.57±4.00	40.77±4.23	45.06±3.79	50.64±4.71	58.19±3.86
Isoproterenol								
SV	0.22±0.02	0.19±0.02	0.22±0.02	0.21±0.02	0.20±0.01	0.24±0.02	0.24±0.02	0.28±0.01
CO	47.27±3.98	39.02±4.23	47.81±4.06	46.98±4.47	44.32±2.90	53.53±4.05	52.38±4.54	65.19±3.24*
MS-222–procaterol								
SV	0.18±0.01	0.19±0.01	0.23±0.01	0.25±0.02	0.23±0.01	0.26±0.02		
CO	36.40±1.71	34.75±2.90	46.87±2.94	50.81±4.69	46.87±2.94	55.20±3.21		
Procaterol								
SV	0.19±0.02	0.22±0.02	0.21±0.02	0.30±0.02*	0.21±0.02	0.26±0.01		
CO	36.53±3.23	41.83±4.74	41.52±3.36	61.60±3.91*	41.52±3.36	54.64±1.87		
MS-222–propranolol								
SV	0.25±0.02	0.21±0.02	0.13±0.02	0.24±0.02†	0.22±0.02	0.21±0.01	0.20±0.02	0.20±0.02
CO	50.91±4.62	41.07±4.24†	26.28±3.51	49.68±3.88†	46.42±3.68	47.96±3.45	40.36±4.07	43.07±4.77
Propranolol								
SV	0.27±0.03	0.25±0.02	0.19±0.02*	0.23±0.02	0.27±0.02*	0.23±0.02	0.22±0.02	0.20±0.02
CO	43.12±4.42*	33.72±3.00*	29.80±3.43	36.29±3.25*	42.26±3.05	42.50±4.29	35.57±3.28	36.12±3.65

Column headings indicate morphant type, row headings indicate treatment. CO, cardiac output (nl min^{-1}); SV, stroke volume (nl $beat^{-1}$). All agonists used were 1×10^{-4} mol l^{-1} and MS-222 concentration was 100 mg l^{-1}.

*Significant difference between same measurement within morphant group due to chemical exposure. †Significant difference between control and corresponding β-adrenergic receptor morphant (P<0.05, N=8–10).

Statistical analyses

All statistical analyses presented in Table 2 and Figs 1, 3, 4, 5 and 6 were performed using SigmaStat statistical analysis software (v. 3.5; Systat Software Inc., San Jose, CA, USA). In Table 2 and Figs 5 and 6, all comparisons between control (MS-222 treated only) and drug-treated (adrenergic ligand in MS-222 solution) fish within morphant groups were made using Student's paired *t*-test. All comparisons between morphant groups within the MS-222 treatment (i.e. control) were made using Student's unpaired *t*-test. Real-time PCR data in Figs 1, 3 and 4 were compared using a one-way ANOVA on ranks (because of failure of normality and equal variance test) with a Tukey *post hoc* test.

Statistics for geometric (K_d and K_i) and arithmetic (B_{max}) means with the 95% lower and upper confidence intervals were used to report binding values (Tables 3 and 4). Arithmetic means (±standard error) were calculated to describe all other data in Figs 8 and 9. Student's one-sample and unpaired *t*-test and one-way ANOVA (followed by Newman–Keuls *post hoc* test) were used to perform the statistical analysis presented in Tables 3 and 4 and Figs 8 and 9. Statistical tests were performed using GraphPad Prism version 5.03 for Windows. All statistical analyses were two-sided and performed with a level of significance established at $P<0.05$.

RESULTS

Developmental and adult tissue mRNA expression of β*1AR*, β*2aAR* and β*2bAR*

The mRNA expression of β*1AR* was detectable as early as 1 h.p.f., had increased approximately 200-fold by 12 h.p.f. and at 6 and 8 d.p.f. was significantly higher than expression at 1 h.p.f. (Fig. 1A). The expression of β*2aAR* was detectable beginning at 6 h.p.f. and expression was significantly higher than at this early stage at 4, 8 and 10 d.p.f. (Fig. 1B). β*2bAR* expression was below detection levels at 6 h.p.f., but at 8 d.p.f. expression levels were significantly higher than those at 1 h.p.f. (Fig. 1B). Using *in situ* hybridization, the expression of all three transcripts in 3 d.p.f. larvae was compared. β*1AR*, β*2aAR* and β*2bAR* mRNA all appeared to be expressed in the heart region (arrows, Fig. 2), and also in different regions of the brain (Fig. 2). When comparing adult tissue mRNA, all three transcripts were expressed in the heart. β*1AR* expression was highest in the brain and heart (Fig. 3A), β*2aAR* was highest in the gill (Fig. 3B), while β*2bAR* expression was not significantly different from that in the liver in any tissue (Fig. 3C). The expression of β*1AR* was higher than that of β*2bAR* in the eye, gill, brain, heart and kidney, while it was significantly lower in the muscle (Fig. 4). β*2aAR* expression was lower than β*2bAR* expression in the liver, whereas both β*1AR* and β*2aAR* expression were lower than β*2bAR* expression in the gut (Fig. 4).

βAR morphants – general observations

β1AR morphants showed no observable physical abnormalities when compared with control morphant fish. In general, β1AR knockdown larvae had significantly lower heart rates than control larvae. β2aAR morphants were also physically similar to control morphants in all experiments. In one experiment (adrenaline), cardiac output was significantly lower in β2aAR morphants *versus* controls, whereas in another experiment (propranolol), cardiac output was significantly higher (Table 2).

Some percentage of every β2bAR morpholino-injected clutch (10–50%) had a phenotype different from control morphants. This phenotype generally presented itself as a curled body often accompanied by an enlarged pericardial cavity. Because of the differences in body shape, which could lead to changes in blood flow, and in particular the enlarged pericardial cavity, which can affect the proper function of the heart, only β2bAR morphants that

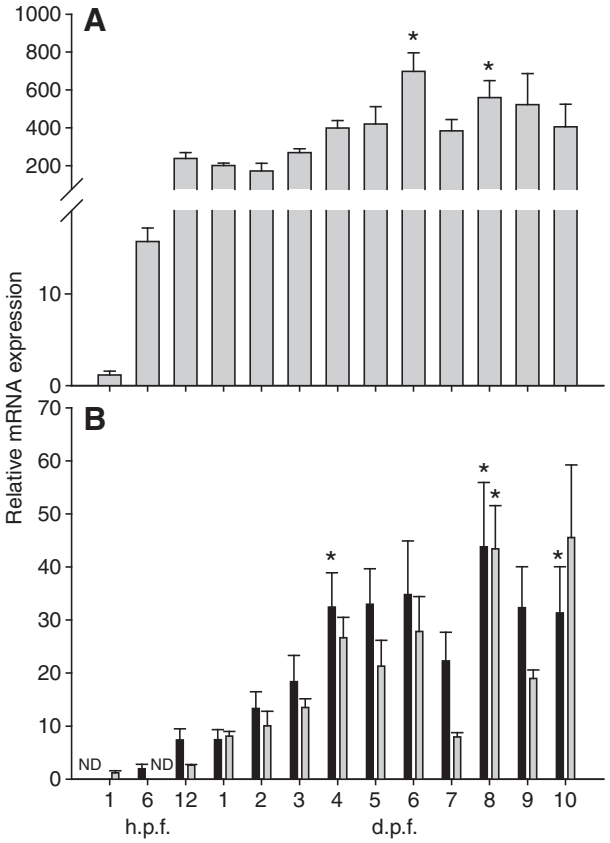

Fig. 1. mRNA expression of the β-adrenergic receptor genes β*1AR* (A), β*2aAR* (black bars; B) and β*2bAR* (grey bars; B) in zebrafish larvae at 1, 6 and 12 h post-fertilization (h.p.f.) and 1–10 days post-fertilization (d.p.f.). Expression of each gene of interest is standardized to 18S ribosomal RNA expression and is shown relative to its own level of expression at 1 h.p.f. (for β*1AR* and β*2bAR*) or 6 h.p.f. (for β*2aAR*). ND, not detectable. *Significant difference from own expression level at 1 h.p.f. (for β*1AR* and β*2bAR*) or 6 h.p.f. (for β*2aAR*). Values are means + s.e., *N*=4.

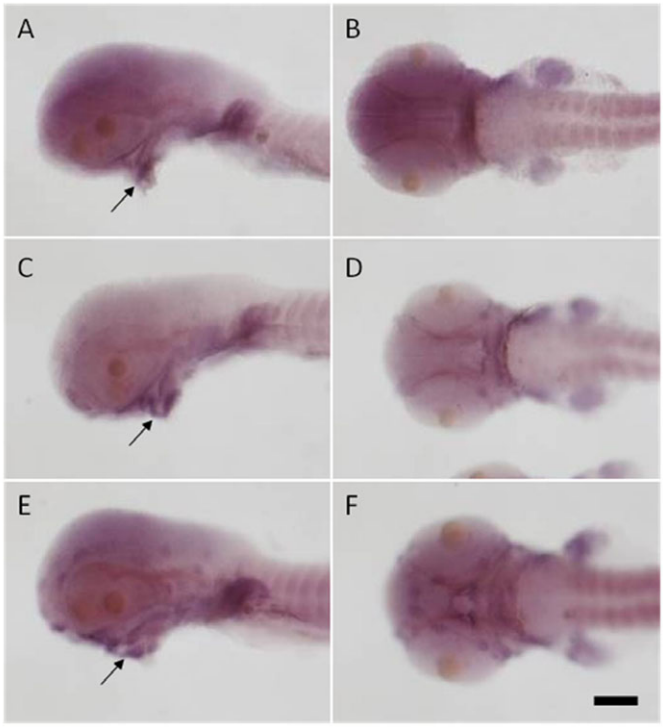

Fig. 2. *In situ* hybridization of β*1AR* (A,B), β*2aAR* (C,D) and β*2bAR* (E,F) in 3 d.p.f. larval zebrafish. Pictures on the right (A,C,E) are lateral views and those on the left (B,D,F) are ventral. Yolk sacs have been removed from larvae. Heart region is indicated by arrows. Bar, 250 μm.

appeared physically similar to control morphants were used in these experiments. In two out of four experiments, β2bAR morphants had significantly higher heart rates than control morphants (Fig. 5).

Dual β2AR morphant larvae had consistently higher heart rates than control morphants in every experiment conducted (Fig. 6). These morphants also occasionally had the β2bAR morphant phenotype described above, although it was not as common in the dual β2AR morphants (and these fish were not used in subsequent experiments).

βAR morphants – drug treatments

Treatment of 4 d.p.f. control morphants with 10^{-4} mol l^{-1} adrenaline caused a significant increase in heart rate in all experiments (Figs 5 and 6). Adrenaline (βAR agonist) also caused a significant increase in heart rate in β2bAR and β1/β2aAR morphants, but not in any of the other βAR morphant groups (Figs 5 and 6). Stroke volume and cardiac output were not affected by adrenaline exposure in any of the morphants tested (Table 2). Isoproterenol (βAR agonist) exposure caused a significant increase in heart rate in all control groups (Figs 5 and 6). It also caused a significant increase in cardiac output in the dual β2a/β2bAR morphants (Table 2). Procaterol (β2AR agonist) exposure caused a significant decrease in heart rate in two out of five groups of control morphants, and a significant decrease in heart rate in β1/β2aAR and β1/β2bAR morphants (Fig. 6). Procaterol had the greatest overall effect on β2aAR morphants, in which heart rate, stroke volume and cardiac output were all significantly higher upon exposure to the drug (Fig. 5; Table 2). Propranolol (βAR antagonist) caused highly significant decreases in heart rate in all control morphants and every βAR morphant group examined (Figs 5 and 6). Two out of four groups of control morphants showed a significant increase in stroke volume as a result of propranolol exposure, and one control group showed a significant increase in cardiac output (Table 2). β1AR and β2aAR morphants also had significantly lower cardiac output during propranolol exposure (Table 2).

Morpholino controls – dTomato red fluorescent protein

Similar results were found in all experiments testing the efficacy of the β1AR, β2aAR and β2bAR morpholinos in blocking dTomato protein synthesis; therefore, only the results of the β2bAR experiment are presented here, as an example (Fig. 7). Injection of the morpholino sequence-tagged dTomato capped mRNAs alone caused the 4 d.p.f. larvae to express the red fluorescent protein in all three cases (e.g. Fig. 7). Co-injection of each of the capped mRNAs with its corresponding morpholino consistently blocked the production of the dTomato protein in all three cases, as demonstrated by the lack of red fluorescence in these fish (e.g. Fig. 7), suggesting that the morpholinos were binding specifically to their antisense sequence tagged to the 5′ end of the dTomato mRNA and thereby blocking the production of this protein (and therefore presumably the production of their native adrenergic receptor proteins). To test for cross-reactivity of the two β2AR morpholinos, each morpholino was injected with the dTomato mRNA of the opposite β2AR. As

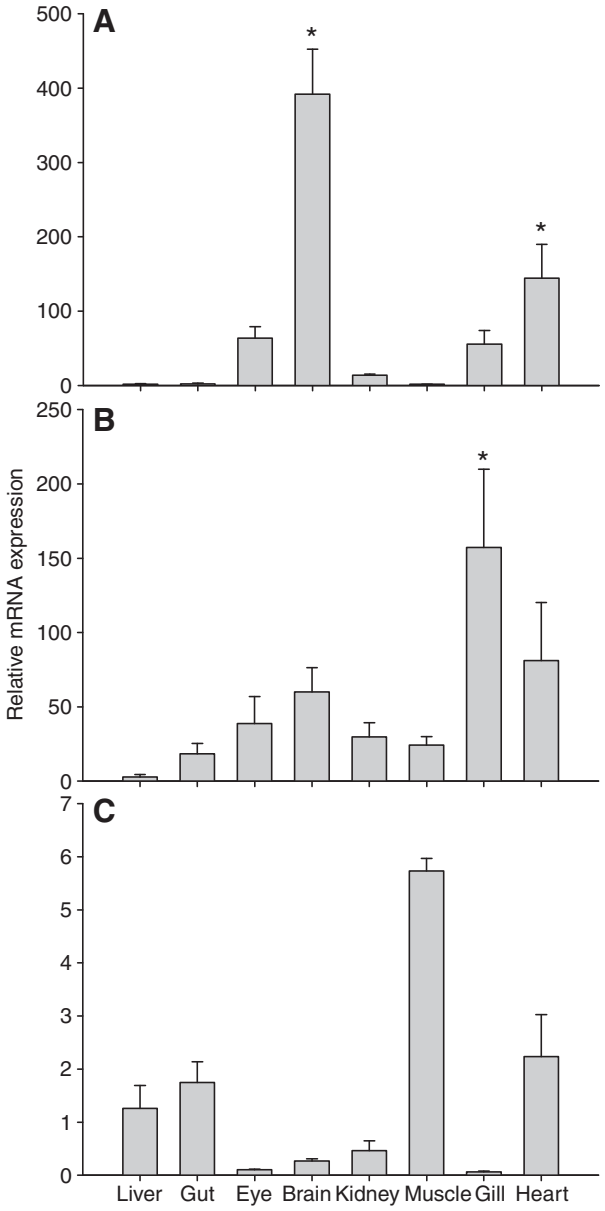

Fig. 3. Relative mRNA expression of β*1AR* (A), β*2aAR* (B) and β*2bAR* (C) in various adult zebrafish tissues. All values are standardized to 18S ribosomal RNA expression and the level in each tissue is expressed relative to mRNA expression in the liver within each gene of interest. *Significant difference from the level of expression in the liver within each gene of interest (P<0.05). Values are means + s.e., N=4.

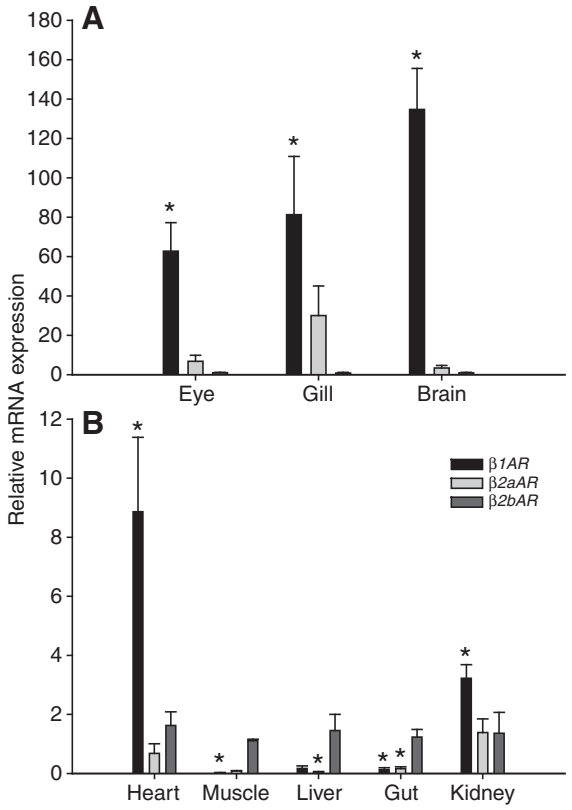

Fig. 4. Relative mRNA expression of β*1AR*, β*2aAR* and β*2bAR* in adult zebrafish eye, gill and brain (A), and heart, muscle, liver, gut and kidney (B). All values are standardized to 18S ribosomal RNA expression and are expressed relative to β*2bAR* mRNA expression in the specified tissue. *Significant difference from the level of expression of β*2bAR* within each tissue (P<0.05). Values are means + s.e., N=4.

seen in Fig. 7D, injecting the β2aAR morpholino with the β2bAR dTomato mRNA did not block the synthesis of the red fluorescent protein. The same result was seen for the β2aAR dTomato protein/β2bAR morpholino combination, suggesting both β2AR morpholinos are efficiently binding to their own gene targets.

Zebrafish βAR ligand binding properties

To gain insight into the pharmacological properties of zebrafish adrenergic receptors, transfected HEK293 cells, a common cellular model for G-protein-coupled receptors, were used (Thomas and Smart, 2005). Notably, HEK293 cells express very low levels of endogenous human β-adrenergic receptors (Fig. 10). Equilibrium dissociation constants (K_d) and maximal binding capacity (B_{max}) of the non-selective β-adrenergic radioligand [^{3}H]DHA in HEK293 cell membranes expressing human and zebrafish β-adrenergic receptors are reported in Table 3. Representative saturation and competitive binding curves (for zfβ2bAR) are provided in Fig. 8. The hβ1AR and hβ2AR were expressed at B_{max} values in HEK293 cells using transfection conditions leading to maximal expression in this cellular system (5 μg dish^{-1}). Interestingly, zfβ2aAR exhibited lower B_{max} values than hβ2AR and zfβ2bAR. Likewise, zfβ1AR was expressed at significantly lower levels than hβ1AR. Importantly, the lower B_{max} of zfβ1AR and zfβ2aAR is not explained by their lower K_d for [^{3}H]DHA as these values are indistinguishable from K_d of hβ1AR, which had a higher B_{max} than either zfβ1AR or zfβ2aAR. Moreover, it is unlikely that B_{max} values measured here are linked to differences in receptor transfection efficiency in HEK293 cells. Indeed, it has previously been shown that transfection efficiency in HEK293 cells is similar regardless of the receptor expression construct used (Tumova et al., 2003). Alternatively, these data potentially suggest that zfβ1AR and zfβ2aAR have distinct determinates regulating their optimal folding and trafficking conformations in HEK293 cells relative to human adrenergic receptors and zfβ2bAR. Additionally, these data demonstrated that zebrafish β-adrenergic receptors bound to [^{3}H]DHA with high affinity. However, zfβ2aAR displayed a ~3-fold lower affinity for [^{3}H]DHA in comparison with hβ2AR. The selectivity ratio of hβ2AR over hβ1AR was ~3-fold, a value that was recapitulated when comparing zfβ2bAR and zfβ1AR. Interestingly, no [^{3}H]DHA

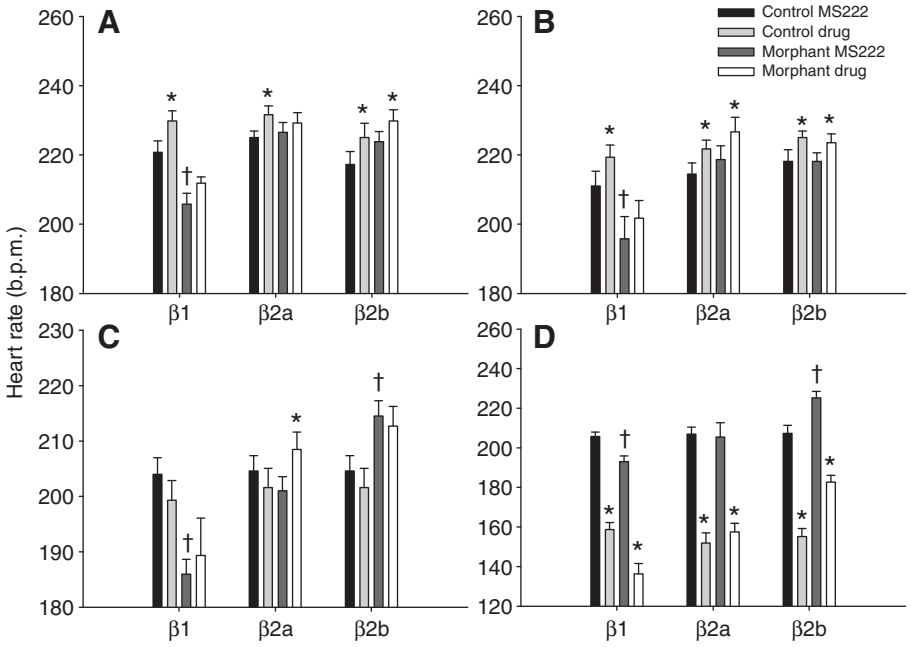

Fig. 5. Heart rates of β1AR, β2aAR and β2bAR knockdown larvae (morphants) exposed to 10^{-4} mol l^{-1} adrenaline (A), isoproterenol (B), procaterol (C) and propranolol (D). Control fish were injected with a standard control morpholino. MS-222 indicates heart rate of larvae when anaesthetized only, prior to drug exposure. *Significant difference between MS-222- and drug-treated fish within morphant group. †Significant difference between control and morphant fish in MS-222 (P<0.05, N=8–10). Note: scale of y-axis in D differs from the scale used in A–C.

selectivity was observed between zfβ1AR and zfβ2bAR. Overall, differences in affinity and selectivity of K_d values for [^{3}H]DHA potentially suggest differences between ligand binding properties of human and zebrafish adrenergic receptors. This idea was further tested using competition studies with a wider range of adrenergic compounds.

Inhibitory constants (K_i) for different ligands (adrenaline, noradrenaline, isoproterenol, procaterol, phenylephrine and dobutamine) are shown in Table 4 along with affinity and selectivity ratios for these compounds (Fig. 9). While K_i values of adrenaline, noradrenaline and procaterol were essentially not different between zfβ1AR and hβ1AR, affinities of other synthetic adrenergic drugs (isoproterenol, phenylephrine and dobutamine) were differed significantly between these two receptors (Table 4, Fig. 9). Additionally, ligand affinities were all significantly different between zfβ2aAR and hβ2AR. With the exception of adrenaline and procaterol, which had similar K_i values for zfβ2bAR and hβ2AR, other tested drugs displayed significant differences in their affinity for human and zebrafish β2-adrenergic receptors. Notably, procaterol, a selective β2-adrenergic receptor agonist, had a ~10-fold lower affinity for zfβ2aAR relative to hβ2AR (Fig. 9). Altogether, the affinity and selectivity of adrenergic drugs suggest that zfβ1AR and zfβ2bAR are zebrafish orthologues of hβ1AR and hβ2AR, respectively. Meanwhile, zfβ2aAR may represent another zebrafish β2-adrenergic receptor isoform with distinct pharmacological properties (Table 4, Fig. 9E). Indeed, zfβ2aAR

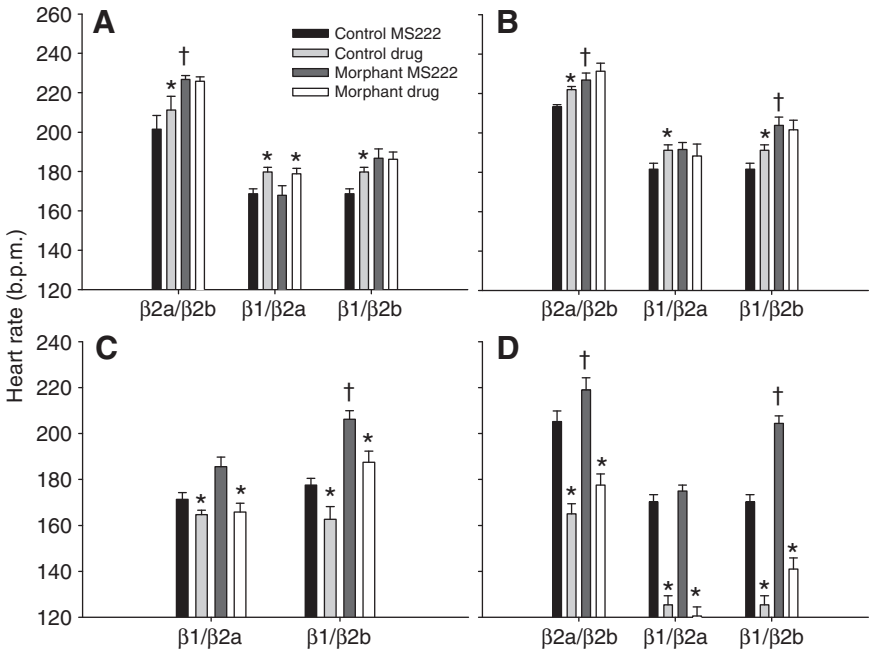

Fig. 6. Heart rates of dual β2a/β2bAR, β1/β2aAR and β1/β2bAR knockdown larvae (morphants) exposed to 10^{-4} mol l^{-1} adrenaline (A), isoproterenol (B), procaterol (C) and propranolol (D). Control fish were injected with a standard control morpholino. MS-222 indicates heart rate of larvae when anaesthetized only, prior to drug exposure. *Significant difference between MS-222- and drug-treated fish within morphant group. †Significant difference between control and morphant fish in MS-222 (P<0.05, N=8–10).

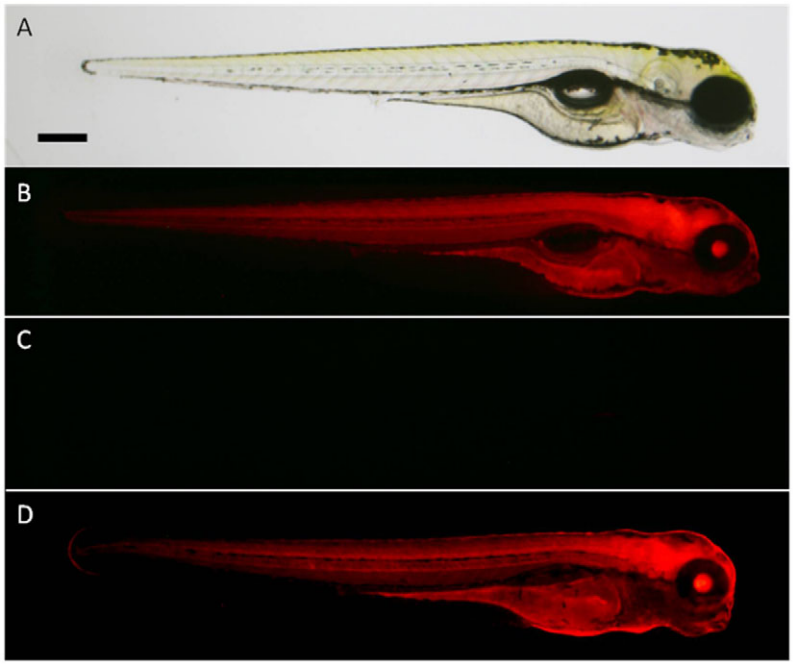

Fig. 7. Lateral images of zebrafish larvae at 4 d.p.f. after being injected with a red fluorescent dTomato capped mRNA (100 pg) tagged with the β2bAR morpholino sequence, alone or in conjunction with β2bAR or β2aAR morpholinos (4 ng, see Materials and methods). (A) Representative bright field image of a 4 d.p.f. injected larva. (B) Red fluorescent image of a β2bAR dTomato-injected larva. (C) Red fluorescent image of β2bAR dTomato plus β2bAR morpholino-injected larva. (D) Red fluorescent image of a β2bAR dTomato plus β2aAR morpholino-injected larva. Bar, 250 μm.

strikingly displayed higher affinity for the α1-adrenergic receptor agonist phenylephrine when compared with hβ2AR and zfβ2bAR. Overall, while ligand K_d and K_i values suggest that zfβ1AR, zfβ2aAR and zfβ2bAR behave pharmacologically as β-adrenergic receptors, the distinct drug selectivity points to important functional differences in the binding mechanisms and ligand discrimination of human and zebrafish β1- and β2-adrenergic receptors.

Zebrafish βAR G_s coupling properties

The ability of different zebrafish β-adrenergic receptors expressed at similar levels to stimulate adenylyl cyclase activity was tested using adrenaline and procaterol. Adrenaline (100 μmol l^{-1}) robustly stimulated adenylyl cyclase activity in HEK293 cells overexpressing human and zebrafish β1ARs and β2ARs (~10-fold over basal) in comparison to mock-transfected cells (~2-fold over basal). In contrast to cells transfected with hβ2AR, procaterol (1 μmol l^{-1}) partially stimulated adenylyl cyclase activity in HEK293 cells expressing hβ1AR relative to adrenaline exposure. Interestingly, procaterol behaved as a full agonist in cells expressing zfβ1AR (Fig. 10C). In agreement with the idea that zfβ2bAR is the zebrafish orthologue of hβ2AR, procaterol evoked a strong and weak stimulation of adenylyl cyclase activity in HEK293 cells expressing zfβ2bAR and zfβ2aAR, respectively. The lower intrinsic activity of procaterol relative to adrenaline in cells transfected with zfβ2aAR may be explained by the lower procaterol affinity for zfβ2aAR in comparison to zfβ2bAR and hβ2AR. Propranolol did not produce detectable adenylyl cyclase activation in cells expressing human or zebrafish adrenergic receptors (Fig. 10). Collectively, these whole-cell cAMP studies suggest that zebrafish β-adrenergic receptors exhibit differences in procaterol-mediated adenylyl cyclase activation.

DISCUSSION

The results of the present study show that β1AR has a stimulatory role in the zebrafish heart, and that the two β2AR subtypes have unique cardioinhibitory roles *in vivo*. Wang and colleagues also noted a similar trend in zebrafish β1AR morphants, reporting a significant reduction in heart rate at 3 and 4 d.p.f. but not at 2 and 5 d.p.f. (Wang et al., 2009). Comparatively, in $\beta 1AR^{-/-}$ mice, heart rate was as much as 25% lower than in wild-types (Ecker et al., 2006). These data conform to the widely accepted canon that β1ARs are stimulatory in *in vivo* systems. It is interesting to note that knocking down β1AR together with either β2aAR or β2bAR did not cause a significant decrease in heart rate in zebrafish (Fig. 6). This is in contrast to mice, where $\beta 1AR^{-/-}\beta 2AR^{-/-}$ knockouts have significantly lower heart rates than wild-types in anaesthetized (Rohrer et al., 1999) or waking (Ecker et al., 2006) animals. These differences could be attributed to species-specific differences in β1AR signalling, or to the activity of other adrenergic receptor subtypes in the heart (see below).

Morpholino knockdown of β2aAR had no effect on heart rate; however, stroke volume and cardiac output both increased significantly in one experiment but decreased significantly in another, a phenomenon which is not explainable using the current data set (Table 2). β2bAR appears to play a more significant role in the regulation of heart rate *in vivo* in these larvae. Loss of function of

Table 3. K_d and B_{max} values of [^{3}H]DHA in membranes from HEK293 cells expressing human (h) and zebrafish (zf) β-adrenergic receptors

	hβ1AR	zfβ1AR	hβ2AR	zfβ2aAR	zfβ2bAR
K_d (nmol l^{-1})	1.24 (0.84–1.82)	1.05 (0.78–1.42)	0.49 (0.28–0.84)	1.25* (0.84–1.86)	0.47† (0.26–0.85)
B_{max} (pmol mg^{-1})	27.0 (23.9–30.1)	4.36‡ (3.73–4.99)	20.0 (18.6–21.6)	1.64* (1.18–2.10)	16.1† (14.4–17.8)

Saturation curves (N=6) were individually analysed using GraphPad Prism version 5.03. Equilibrium dissociation constant (K_d, nmol l^{-1}) and maximal binding capacity (B_{max}, pmol mg^{-1} membrane protein) values for [^{3}H]DHA are expressed as geometric and arithmetic means, respectively. The 95% lower and upper confidence intervals are shown in parentheses.

Statistical analysis was performed using one-way ANOVA followed by Newman–Keuls *post hoc* test. ‡P<0.05 when compared with human (h)β1AR; *P<0.05 when compared with hβ2AR; †P<0.05 when compared with zebrafish (zf)β2aAR.

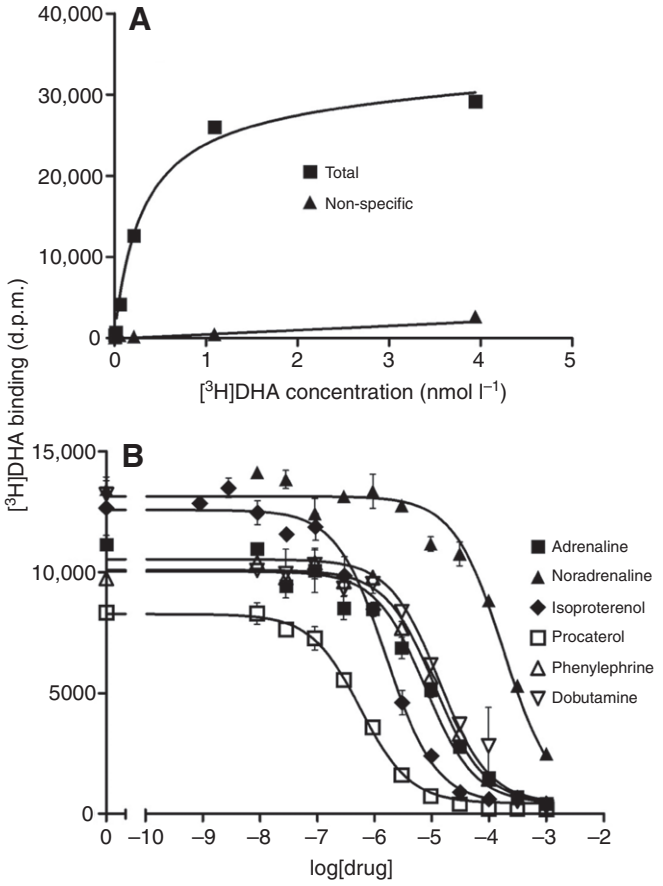

Fig. 8. Saturation (A) and competitive (B) curves for HEK293 cells transfected with zebrafish β2bAR (chosen as representatives for curves generating data for Tables 3 and 4). Saturation curves of [^{3}H]DHA were determined in the presence of 1 μmol l^{-1} alprenolol. Competitive curves for zebrafish β2bAR-transfected cells were performed with 0.5 nmol l^{-1} [^{3}H]DHA (for others, see Results). Ligand concentration (mol l^{-1}) is given as log values.

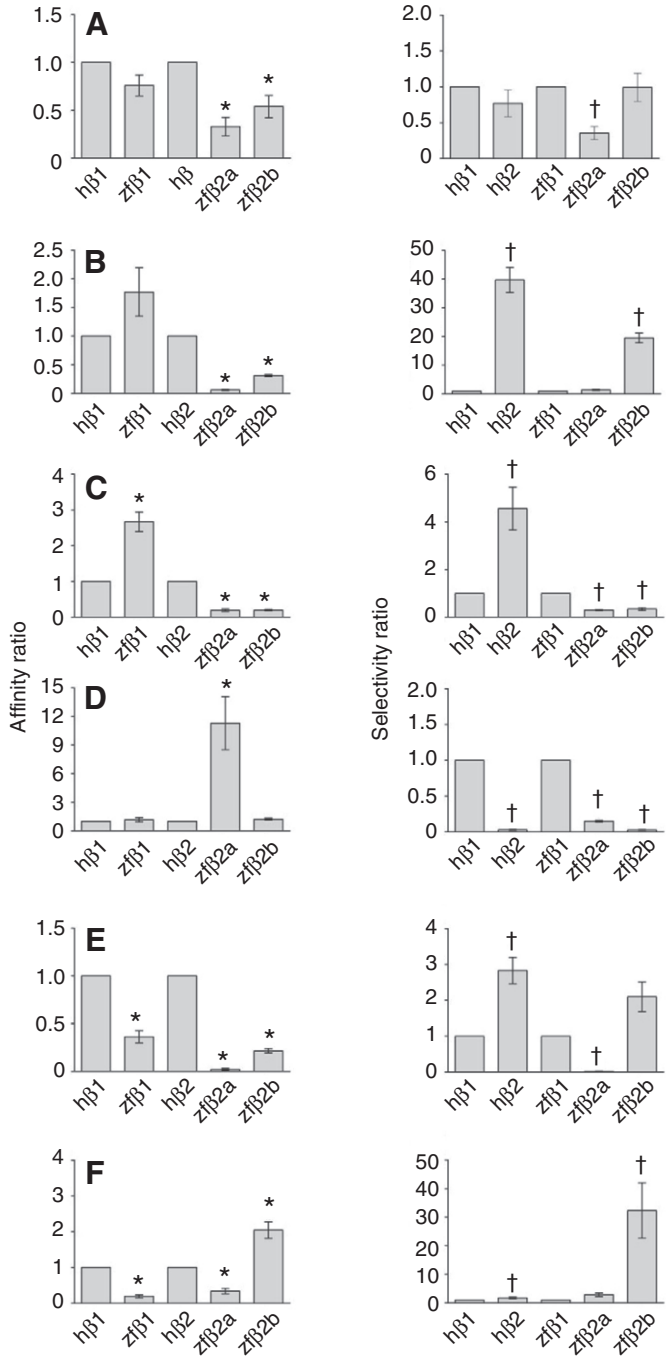

Fig. 9. Ligand affinity (left) and selectivity (right) ratios for human and zebrafish β-adrenergic receptors expressed in HEK293 cells. (A) Adrenaline, (B) noradrenaline, (C) isoproterenol, (D) procaterol, (E) phenylephrine and (F) dobutamine. Affinity ratios for zebrafish (zf)β1AR and zfβ2AR (a and b isoforms) were calculated relative to human (h)β1AR and hβ2AR, respectively. Selectivity ratios for hβ2AR and zfβ2AR (a and b isoforms) were computed relative to hβ1AR and zfβ1AR, respectively. *P<0.05 when compared with a value of 1 (affinity ratio). †P<0.05 when compared with a value of 1 (selectivity ratio).

β2bAR alone caused a significant increase in heart rate in 2 out of 3 experiments presented (Fig. 5). When knocked down in conjunction with either β2aAR or β1AR, β2bAR loss of function caused an even more robust and reproducible increase in heart rate (Fig. 6). This was despite the fact that β2aAR is more highly expressed than β2bAR in the zebrafish heart (Wang et al., 2009) (present study, Fig. 3). One possible explanation for this phenomenon is that while β1 and β2 adrenergic receptors are generally the most plentiful β-adrenergic receptor found in the heart, they are not the only G-protein-coupled receptor that can affect heart rate. The β3AR subtype plays a minimal but not insignificant role in cardiovascular function in most species studied. Both isoproterenol and the β3AR-specific agonist CL-316243 cause a brief decrease in the rate of contraction in myocytes cultured from β*1AR*/β*2AR*$^{-/-}$ mice (Devic et al., 2001). Likewise, β3ARs in the heart of the freshwater eel exert negative inotropic effects by linking with pertussis toxin-sensitive (presumably $G_{i/o}$) proteins (Imbrogno et al., 2006). β3ARs have also been found in the heart of rainbow trout (Nickerson et al., 2003) and possibly winter flounder (Mendonça and Gamperl, 2009). While rainbow trout appear to express β3aAR mRNA in the heart (Nickerson et al., 2003), Wang and colleagues reported minimal to non-existent expression of β3aAR or β3bAR mRNA anywhere but in the blood of adult zebrafish (Wang et al., 2009). It is therefore unlikely that these β3ARs play a significant role in regulating heart rate in the β2AR morphants of the current study; however, the present data cannot entirely rule out the possibility.

The present *in vivo* findings led us to hypothesize that one or both of the zebrafish β2ARs has a negative chronotropic role in

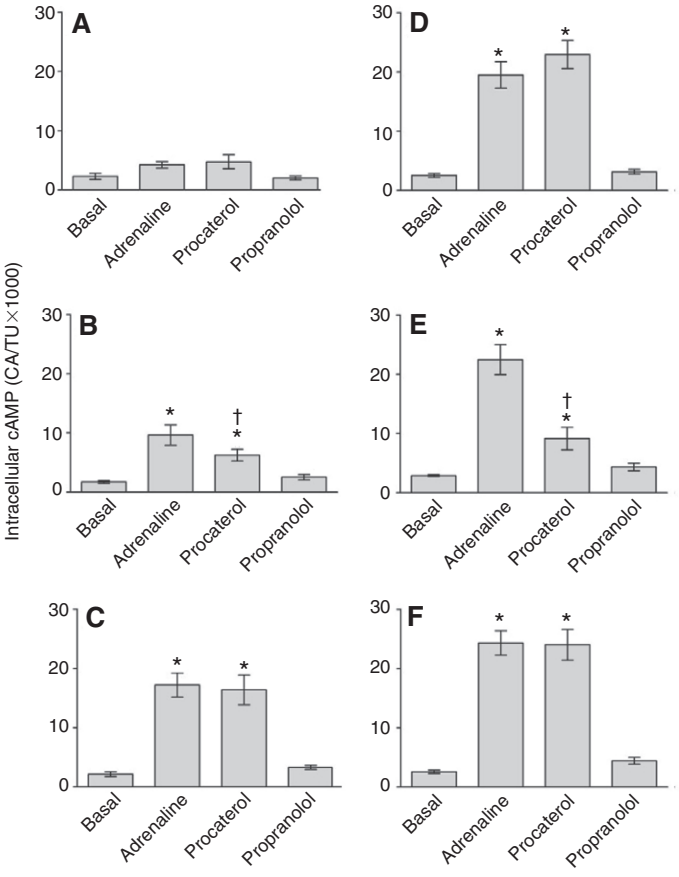

Fig. 10. Drug-mediated adenylyl cyclase activity [expressed as [^{3}H]cAMP levels (CA) over the total amount of intracellular [^{3}H]adenine uptake (TU)] in HEK293 cells expressing human (h) and zebrafish (zf) β-adrenergic receptors. Arithmetic means ± s.e. of raw data (N=5–8) are reported. *P<0.05 when compared with basal condition and †P<0.05 when compared with adrenaline condition. (A) Mock-transfected cells, (B) hβ1AR, (C) zfβ1AR, (D) hβ2AR, (E) zfβ2aAR and (F) zfβ2bAR. Maximal binding capacity (B_{max}) values (in pmol mg^{-1} protein) were as follows: mock transfected, 0.09±0.03; hβ1AR, 3.00±1.01; zβ1AR, 4.63±2.12; hβ2AR, 3.49±0.66; zfβ2aAR, 2.94±0.80; and zfβ2bAR, 2.95±0.44. Drug concentrations were as follows: adrenaline, 100 μmol l^{-1}; procaterol, 1 μmol l^{-1}; and propranolol, 10 μmol l^{-1}. All significant differences were maintained when cAMP accumulation in βAR-transfected cells was corrected for accumulation in mock-transfected cells as shown in A (data not shown).

the zebrafish heart, because of associations with G_i proteins and/or differences in their association with G_s proteins as compared with other βARs. Therefore, cell culture experiments in which each of the zebrafish (and human) βARs were expressed in HEK293 cells were used to determine the effect of various agonists on intracellular cAMP accumulation. Data obtained in HEK293 cells are important as they are the first to demonstrate that the zebrafish β1 and β2 adrenergic receptor proteins behave like those previously described in other species, in that they associate with G_s proteins. However, these results by themselves do not help rationalize the increase in heart rate seen in dual β2AR zebrafish morphants. Many factors including subcellular localization (e.g. caveolae) (Rybin et al., 2000), changes in conformation, dual coupling to G_s and G_i proteins, and agonist-mediated internalization of the receptor can all play a role in how β2AR affects different cell signalling pathways (for reviews, see Xiao et al., 2003; Zheng et al., 2004).

Stimulation of mammalian β1 and β2 adrenergic receptors increases intracellular cAMP in cardiomyocytes (Freyss-Beguin et al., 1983; Kuschel et al., 1999). Despite this, β2AR activation does not appear to increase cAMP-dependent protein kinase A (PKA) activity in normal canine (Kuschel et al., 1999) or murine (Devic et al., 2001) cardiomyocytes, nor does it increase phosphorylation of proteins involved in the excitation–contraction pathway of these cells (Kuschel et al., 1999). Regardless of this disassociation within the classic G_s–cAMP/PKA pathway, it is obvious that β2AR signalling is involved in regulating chronotropic and inotropic activity of the heart, possibly mediated by its additional association with G_i proteins. In support of this, the present study shows that loss of β2AR function *in vivo* causes increased heart rate in zebrafish larvae (Figs 5 and 6). Also, the stimulation of β2ARs by isoproterenol causes an initial increase in contraction rate followed by a sustained decrease in murine cardiomyocytes (Devic et al., 2001; Wang et al., 2008), suggesting some cardioinhibitory role for the receptor. This is further supported by the observation that disruption of G_i activity by pertussis toxin (PTX) enhances the β2AR-mediated contractile response of murine (for reviews, see Xiao, 2001; Xiao et al., 2003) and canine (Kuschel et al., 1999) cardiomyocytes. Indeed, while the β2AR–G_i complex does not seem to directly inhibit global cAMP production, it does seem to affect downstream PKA activity and also the association of β2ARs with G_s proteins. For example, the β2AR-G_i complex activates phosphoinositide 3-kinases (PI3Ks) which provide a cell survival effect for cardiomyocytes. When PI3K activity is blocked in isolated rat myocytes, β2AR stimulation causes a more robust positive contractile response without a concurrent overall increase in intracellular cAMP compared with when β2ARs are stimulated without blocking PI3K (Jo et al., 2002). Overall, therefore, the fact that cAMP levels are increased in HEK293 cells expressing β1AR and β2AR in the present study does not negate the possibility that β2AR–G_i associations limit the contractile response of the heart, as is suggested by the present *in vivo* data.

Table 4. K_i values of unlabelled drugs in membranes from HEK293 cells expressing human (h) and zebrafish (zf) β-adrenergic receptors

	Adrenaline	Noradrenaline	Isoproterenol	Procaterol	Dobutamine	Phenylephrine
hβ1AR	1924 (1303–2842)	1258 (744–2043)	74.9 (49.6–113)	7025 (4869–10137)	1296 (923–1821)	7580 (5060–11356)
zfβ1AR	1394 (1189–1634)	1977 (1492–2620)	207 (169–254)‡	9066 (6309–13026)	193 (110–338)‡	2562 (1813–3619)‡
hβ2AR	1376 (590–3210)	55911 (25691–121679)	478 (160–1424)	156 (93.4–257)	2172 (1183–3985)	20837 (8883–48882)
zfβ2aAR	481 (234–991)*	2717 (1837–4020)*	66.3 (49.1–89.4)*	1214 (718–2053)*	715 (470–1088)*	115 (23.7–561)*
zfβ2bAR	1076 (484–2396)†	18277 (9445–35369)*,†	94.4 (30.9–289)*	162 (101–260)†	4299 (2461–7510)*,†	4081 (2267–7346)*,†

Equilibrium dissociation constant (K_i, nmol l^{-1}) for different ligands expressed as geometric means with the 95% lower and upper confidence intervals shown in parentheses (N=4–6).

Statistical analysis was performed using Student's unpaired t-test to compare ligand affinity between hβ1AR and zfβ1AR. One-way ANOVA followed by Newman–Keuls *post hoc* test was used to compare hβ2AR, zfβ2aAR and zfβ2bAR. ‡P<0.05 when compared with hβ1AR; *P<0.05 when compared with hβ2AR; †P<0.05 when compared with zfβ2aAR.

This is the first study to examine the ligand binding affinities and cellular activity of the zebrafish β1 and β2 adrenergic receptors. The first result of note is that the zebrafish β1AR had a similar binding affinity profile for the endogenous ligands adrenaline and noradrenaline when compared with the human β1AR. The zebrafish protein, however, had a lower binding affinity for isoproterenol and a higher affinity for dobutamine and phenylephrine than hβ1AR (Table 4). This finding is in keeping with previous observations that fish receptors can have different receptor binding properties from those expected based on mammalian data (Janssens and Grigg, 1988; Fabbri et al., 1992). Perhaps the most interesting outcome of the competitive binding experiments is the obvious difference in binding affinities between the two zebrafish β2ARs, with β2aAR having the greatest divergence in binding profile from that of human β2AR (Table 4). Each of the zebrafish β2ARs had unique mRNA expression profiles in adult tissues (Fig. 3B,C). Also, the zebrafish β2ARs had differential effects on heart rate when knocked down individually in zebrafish larvae, with the β2bAR subtype potentially playing the more critical role at this stage (Figs 5 and 6). β2aAR has been shown to be involved in pigment formation in the larval zebrafish (Wang et al., 2009). It would be interesting to further investigate the potential sub-function of each of these β2ARs in zebrafish, both in the heart and in other tissues where β2AR function is critical, such as the liver (e.g. Dugan et al., 2008).

Comparing the binding affinities of the different βAR agonists used in this study revealed some unexpected specificities of the β1 and β2 adrenergic receptors. Each of the β2ARs (including the human receptor) had a high affinity for phenylephrine, a classic α1AR agonist (Table 3). Fabbri and colleagues showed that phenylephrine is as potent as noradrenaline and adrenaline at displacing [^{3}H]DHA binding in catfish liver membranes (Fabbri et al., 1992). Significant displacement of the βAR ligand [^{125}I]ICP by phenylephrine has also been demonstrated in liver membranes of *Xenopus laevis*, the Australian lungfish (*Neoceratodus fosteri*) and the axolotl (*Ambystoma mexicanum*) (Janssens and Grigg, 1988). Thus, there is a growing body of evidence that mammalian and non-mammalian adrenoreceptors do not always conform to the same functional paradigms. The present data for cAMP activation in human and zebrafish βAR-transfected cells also highlight some of these differences. Adrenaline caused a robust increase in intracellular cAMP in whole HEK293 cells transfected with all five of the βARs (Fig. 10), supporting similar findings in other studies, which show that activation of both β1 and β2 adrenergic receptors with adrenaline causes cellular cAMP accumulation (e.g. Green et al., 1992). Procaterol, a classic β2AR agonist, also induced cAMP accumulation in both β1 and β2 adrenergic receptor-transfected cells; however, cAMP levels were significantly lower in the zfβ2aAR-transfected cells exposed to procaterol *versus* adrenaline (Fig. 10). These data suggest that procaterol (1) behaves as a strong partial or full agonist to human and zebrafish β1ARs, respectively, and (2) is not as effective as the endogenous catecholamine adrenaline at increasing cAMP accumulation in zfβ2aAR-transfected cells. The current *in vivo* data show that in two control morphant groups, procaterol exposure caused a significant decrease in heart rate (Fig. 6), and a significant increase in heart rate in zebrafish experiencing β2aAR knockdown (Fig. 5). Zebrafish lacking M_2 muscarinic receptor function also show a negative chronotropic response to procaterol (Steele et al., 2009). Considering the activity of the receptors in the present HEK293 experiments, it is possible that these effects are mediated by β1AR, β2ARs, or both receptor types. *In vitro* assessment of β2AR agonist effects on rat cardiomyocyte chronotropy suggests that some of these chemicals increase the rate of cell contraction *via* a β1AR-mediated pathway (Freyss-Beguin et al., 1983; Juberg et al., 1985). It would seem, therefore, that even mammalian βARs do not always interact with synthetic ligands in a predictable fashion.

In conclusion, it appears that the β-adrenergic receptors are necessary for regulating heart function during early life in zebrafish, with β1AR and β2bAR being most strongly implicated in controlling heart rate. It is also apparent that while the zebrafish β-adrenergic receptors are equally capable of instigating cAMP production as their human counterparts, they have distinct binding affinities for different ligands. While morpholino knockdown of β2aAR and β2bAR suggests that one or both of these receptors may be cardioinhibitory, experiments expressing each of these receptors individually in HEK293 cells imply that stimulation of both β2AR subtypes increases intracellular cAMP levels. The β2ARs may cause inhibition by another indirect pathway, such as by interaction with other signalling cascades (e.g. PI3K) *via* an association with G_i proteins. Further research into how these receptors behave at the cellular level in native tissues would be key in clarifying how these unique paralogues function.

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