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Conserved structural, pharmacological and functional properties among the three human and five zebrafish α_2-adrenoceptors

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1 Zebrafish has five distinct α_2-adrenoceptors. Two of these, α_{2Da} and α_{2Db}, represent a duplicated, fourth α_2-adrenoceptor subtype, while the others are orthologue of the human α_{2A}-, α_{2B}- and α_{2C}-adrenoceptors. Here, we have compared the pharmacological properties of these receptors to infer structural determinants of ligand interactions.

2 The zebrafish α_2-adrenoceptors were expressed in Chinese hamster ovary cells and tested in competitive ligand binding assays and in a functional assay (agonist-stimulated [^{35}S]GTPγS binding). The affinity results were used to cluster the receptors and, separately, the ligands using both principal component analysis and binary trees.

3 The overall ligand binding characteristics, the order of potency and efficacy of the tested agonists and the G-protein coupling of the zebrafish and human α_2-adrenoceptors, separated by $\sim$350 million years of evolution, were found to be highly conserved. The binding affinities of the 20 tested ligands towards the zebrafish α_2-adrenoceptors are generally comparable to those of their human counterparts, with a few compounds showing up to 40-fold affinity differences.

4 The α_{2A} orthologues and the zebrafish α_{2D} duplicates clustered as close pairs, but the relationships between the orthologues of α_{2B} and α_{2C} were not clearly defined. Applied to the ligands, our clustering methods segregated the ligands based on their chemical structures and functional properties. As the ligand binding pockets formed by the transmembrane helices show only minor differences among the α_2-adrenoceptors, we suggest that the second extracellular loop – where significant sequence variability is located – might contribute significantly to the observed affinity differences.
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Abbreviations: B_{max}, receptor density; CHO, Chinese hamster ovary; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; PCA, principal component analysis; PTX, pertussis toxin; RT, room temperature; TM, transmembrane (domain); XL, extracellular loop

Introduction

G-protein-coupled receptors (GPCRs) form a large family of cell surface receptors that control intracellular second messenger systems by activating guanine nucleotide binding regulatory proteins (G proteins). The GPCRs share in their structure a bundle of seven α-helical transmembrane segments (TM1–TM7) that form a ligand binding pocket. The only GPCR with a known high-resolution crystal structure is bovine rhodopsin (Palczewski *et al*., 2000). In rhodopsin, the binding pocket is covered by the second extracellular loop (XL2) that connects TM4 and TM5; XL2 is probably found at a similar location in most rhodopsin-like GPCRs (Shi & Javitch, 2004).

Adrenoceptors are GPCRs that mediate the physiological effects of the hormones/neurotransmitters adrenaline and noradrenaline, and are target molecules of several clinically important drugs. Adrenoceptors can be divided into three main classes, α_1-, α_2- and β-adrenoceptors, each of which is represented by three subtypes in humans. Three different genes encode the three distinct mammalian α_2-adrenoceptor subtypes, α_{2A}, α_{2B} and α_{2C}; the rodent (and bovine) orthologue of the human α_{2A} is occasionally misleadingly called α_{2D} on the basis of its pharmacological properties. We have recently isolated and mapped the chromosomal location of a fourth, duplicated α_2-adrenoceptor subtype from zebrafish (*Danio rerio*), a teleost fish used as an experimental model in developmental biology and genetics and, more recently, also in behavioural studies (Peitsaro *et al*., 2003; Anichtchik *et al*., 2004; Ruuskanen *et al*., 2004). Despite the potential confusion caused initially by the inappropriate naming of the rodent

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orthologues of α_{2A}-adrenoceptors as α_{2D}, we have named the true fourth paralogous α_2-adrenoceptor subtype as α_{2D}, and the two duplicates of this receptor as α_{2Da} and α_{2Db}, which is in accordance with the IUPHAR guidelines and the nomenclature guidelines for naming zebrafish genes (Ruuskanen *et al*., 2004). The number and chromosomal locations of the α_2-adrenoceptor genes in different species support the notion that the different receptor subtypes have been generated *via* two rounds of block/chromosome (or even whole genome) duplication, with a third round of duplication in the teleost lineage to bring the total number of α_2-adrenoceptor genes up to eight, as in the pufferfish (*Fugu rubribes*) (Ruuskanen *et al*., 2004).

Here, we have tested the ligand binding affinities of the five zebrafish α_2-adrenoceptors with respect to 20 ligands known to bind to the human α_2-adrenoceptors. A pharmacological and functional characterisation of the zebrafish α_2-adrenoceptors is the first step in validating the zebrafish model for *in vivo* pharmacological and behavioural research of the α_2-adrenergic system. In addition, the comparative biology approach of our previous work (Ruuskanen *et al*., 2004) may be further expanded to enhance our understanding of α_2-adrenoceptor–ligand interactions: a correspondence between differences in amino acids facing the ligand binding cavity and in binding affinities across α_2-adrenoceptor subtypes and species may reveal amino-acid residues important for specific ligand binding characteristics. Conversely, a correlation between structural features of a group of ligands and similar variations in binding affinity across subtypes and species may provide useful information for drug design.

Methods

Isolation of the zebrafish α_2-adrenoceptor clones, sequence analysis and classification of the subtypes, based on sequence alignments, phylogenetic analyses and chromosomal mapping, have been described in detail elsewhere (Ruuskanen *et al*., 2004).

Expression vectors

Fragments containing the coding sequences for the zebrafish α_{2A}-, α_{2B}-, α_{2C}-, α_{2Da}- and α_{2Db}-adrenoceptors were generated either from genomic DNA or phage or cosmid clones containing these intronless genes (see Ruuskanen *et al*., 2004) using PCR with primers flanking the coding regions and containing artificial restriction enzyme recognition sites. Primers were designed to contain as little noncoding sequence as possible and to avoid any extra ATG codons at the 5′-end of the product in order to maximise transcription efficiency. See the Supplementary Material for the primer sequences and locations. PCR products were purified and digested with the appropriate restriction enzymes and ligated into the pREP4 (InVitrogen, NV Leek, The Netherlands) expression vector. The expression vector constructs were sequenced with vector- and gene-specific primers and found to be identical to the genomic sequences.

Cell culture and transfections

Adherent Chinese hamster ovary (CHO-K1) cells (American Type Culture Collection, Manassas, VA, U.S.A.) were transfected and cultured as reported previously (Marjamäki *et al*., 1998). Briefly, the pREP4-based expression constructs were transfected into the cells using the Lipofectin reagent kit (Life Technologies Inc., Rockville, MD, U.S.A.) with slight modifications to the manufacturer's instructions. Hygromycin B ($600\,\mu g\,ml^{-1}$)-resistant cell cultures were examined for their ability to bind the α_2-adrenoceptor antagonists [^{3}H]rauwolscine, [ethyl-^{3}H]RS-79948-197 and [^{3}H]RX821002 (see below). The transfected cell clones with the highest expression levels were chosen for further experiments and were subsequently maintained in $250\,\mu g\,ml^{-1}$ hygromycin B. For some experiments, cells were grown for 18 h in serum-free medium supplemented with $200\,ng\,ml^{-1}$ pertussis toxin (PTX) before harvesting.

Membrane preparation

All procedures were performed at $+4^{\circ}C$. The harvested cells were suspended in hypotonic lysis buffer (10 mM Tris-HCl, 0.1 mM EDTA, 0.32 mM sucrose, pH 7.4) and homogenised using an Ultra-Turrax homogeniser (3×10 s at 8000 r.p.m.). The homogenate was centrifuged at $500 \times g$ for 10 min to remove cell nuclei, unbroken cells and aggregates. The supernatant was collected, centrifuged at $23{,}000 \times g$ for 30 min, and the pellet was rehomogenised and again centrifuged at $23{,}000 \times g$ for 30 min. The membrane pellet was suspended in hypotonic lysis buffer and stored at $-70^{\circ}C$ until used.

Saturation binding assays

Transfected cell lines were screened for their binding of three α_2-adrenoceptor radioligands. [^{3}H]Rauwolscine and [^{3}H]RX821002 binding experiments were conducted as reported previously (Halme *et al*., 1995). [Ethyl-^{3}H]RS-79948-197 binding was determined using similar methods. Briefly, cell homogenates or membrane preparations were incubated with serial dilutions of the radioligands (in 50 mM potassium-phosphate buffer (pH 7.4) at $25^{\circ}C$ for 30 min). Incubations were terminated using rapid filtration through glass fibre filters (Whatman GF/B), the filters were washed (50 mM Tris-HCl, 10 mM EDTA, pH 7.4), placed into scintillation vials with OptiPhase 'HiSafe' III (Wallac, Turku, Finland) and bound radioactivity was measured in a scintillation counter (Wallac 1410). Nonspecific binding was determined in parallel tubes in the presence of an excess of either adrenaline or phentolamine ($10\,\mu M$), and specific binding was defined as the difference between total and nonspecific binding. Protein concentrations were determined with the method of Bradford (1976), with bovine serum albumin as the reference standard. All incubations were performed in duplicate or triplicate and repeated at least three times. Equilibrium dissociation constants (K_d) and receptor expression levels (B_{max}) were calculated from the results of saturation binding experiments using the GraphPad Prism package (GraphPad Prism Software, San Diego, CA, U.S.A.).

In order to assess the expression level of α_2-adrenoceptors in zebrafish brain, 21 wild-type adult zebrafish were killed in a crushed ice–water bath and the brains were removed and pooled for membrane preparation. The yield of membranes was sufficient to perform a single saturation binding assay with [ethyl-^{3}H]RS-79948-197. The fish were obtained as a gift from the Department of Biology, Åbo Akademi University, Turku,

Finland and were used under the guidelines and with the permission of the Animal Use and Care Committee of Åbo Akademi University.

Competition binding assays

Competitive ligand binding assays were carried out essentially as reported previously (Halme *et al*., 1995). The competing ligands used in this study were chosen (1) on the basis of known affinity differences among the human α_2-adrenoceptors (oxymetazoline, chlorpromazine), (2) to maximise chemical diversity of the ligands by choosing ligands and their derivatives from several structural classes (cf. Table 1 and Figure 4: imidazol(in)es 1–6, yohimbane-derivatives 7–9, tricyclic antipsychotics 10–11, 'bulky antagonists' 12–16, catecholamines 17–20) and (3) on functional basis (clonidine, dexmedetomidine, oxymetazoline, UK14,304, 2-amino-1-phenylethanol, dopamine, (−)-adrenaline and (−)-noradrenaline act as agonists or partial agonists on human α_2-adrenoceptors, while atipamezole, idazoxan, L-657.743, rauwolscine, yohimbine, chlorpromazine, clozapine, ARC239, prazosin, spiperone, spiroxatrine and WB-4101 are antagonists). The radioligands were employed at concentrations close to their affinity constants (K_d) at each particular receptor, and serial dilutions of the competing ligands were incubated with cell membrane preparations (in 50 mM potassium-phosphate buffer, pH 7.4). The apparent affinities (apparent K_i) of these ligands at each receptor were determined *via* nonlinear regression analysis (GraphPad Prism), assuming a homogeneous class of binding sites. For the conversion of IC_{50} into K_i values, the Cheng–Prusoff equation was applied (Cheng & Prusoff, 1973). The statistical significance of differences between orthologues or with respect to the two α_{2D}-adrenoceptor paralogues was evaluated with *t*-tests. The affinities of WB-4101, clonidine, dexmedetomidine, oxymetazoline, dopamine and (−)-noradrenaline towards the human α_{2A}-adrenoceptor were obtained in similar experiments with membranes from stably transfected S115 cells. A set of 11 ligands were tested on both CHO and S115 cells expressing the human α_{2A}-adrenoceptor, with good agreement between the two types of host cell lines (see Results). Linear regression analysis of pK_i-values between different receptors/host cells was made using the GraphPad Prism software.

Cluster analysis

Two independent methods were used to cluster (1) the eight receptors and (2) the 20 ligands on the basis of the $\log_{10}$-transformed binding affinity data from Table 1. In the case of binary trees, pairwise Euclidean distances were calculated (using the $\log_{10}$-transformed binding affinities) between columns, to compare the receptors, or between rows, to compare the ligands. Binary trees were constructed using the program Fitch of the Phylip package (Felsenstein, 1993). A standard multivariate analysis procedure, principal component analysis (PCA) (see Chatfield & Collins, 1989), was applied directly to the $\log_{10}$-transformed binding affinity values.

[^{35}S]GTPγS binding assay

Noradrenaline has been detected in zebrafish brain in concentrations similar to those of rat brain (Anichtchik *et al*., 2004). Adrenaline is another endogenous adrenoceptor agonist. In addition to these natural ligands, the synthetic agonists UK14,304, oxymetazoline and dexmedetomidine, which display different efficacies and potencies at the three human α_2-adrenoceptors, were used. Agonist-induced stimulation of [^{35}S]GTPγS binding was measured as follows: membrane suspensions were thawed and 5–10 μg sample^{-1} of membrane protein were added to the reaction buffer (final concentrations: 30 μM ascorbic acid, 50 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 150 mM NaCl, 1 μM GDP and 0.1 nM [^{35}S]GTPγS (pH 7.4) at room temperature (RT)) in a total volume of 0.3 ml. In addition, the three human α_2-adrenoceptor subtypes were tested using transfected CHO cells under the same conditions and with approximately equal expression levels as for the zebrafish receptors. The cell lines expressing the human α_2-adrenoceptors have been previously studied under slightly different conditions (Pohjanoksa *et al*., 1997; Peltonen *et al*., 1998). The samples were preincubated for 30 min at RT with serial dilutions of the agonists before [^{35}S]GTPγS was added for the 60 min incubation period. The incubations were terminated by rapid filtration through Whatman GF/B filters, the filters were rinsed (in cold 20 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$, pH 7.4) and the bound radioactivity was counted in a liquid scintillation counter.

Molecular modelling

Structural models of the three human and the five zebrafish α_2-adrenoceptor subtypes were constructed based on the X-ray structure of bovine rhodopsin (1HZX; Palczewski *et al*., 2000), using Modeller 6.0 (Sali & Blundell, 1993). The construction of the structural models of the human α_2-adrenoceptor subtypes is reported in more detail elsewhere (Xhaard *et al*., submitted). The pairwise sequence alignments used to construct these structural models were all derived from Malign (Johnson & Overington, 1993). In the bovine rhodopsin structure two cysteines, C3.25 in TM3 and Cxl2.50 in XL2, form a disulphide bridge, which constrains XL2 above the binding cavity; XL2 directly interacts with bound 11-*cis*-retinal (Palczewski *et al*., 2000) (C3.25 is numbered according to the Ballesteros & Weinstein convention (1995), where the most conserved residue in each of the TM segments is numbered using the TM number followed by an index of 50. In addition, we expand the indexing system to XL2, where the conserved cysteine involved in a disulphide bridge is analogously labelled as Cxl2.50). In all α_2-adrenoceptors, cysteines are present at 3.25 and xl2.50, which suggests a similar position of XL2 as well as direct interactions with bound ligands in the model structures of the α_2-adrenoceptors (Xhaard *et al*., submitted).

Materials

[^{3}H]Rauwolscine, rauwolscine and [^{35}S]GTPγS were from NEN Life Science Products Inc. (Boston, MA, U.S.A.), [ethyl-^{3}H]RS-79948-197 and [^{3}H]RX821002 were from Amersham Pharmacia Biotech (Buckinghamshire, U.K.). Spiperone and spiroxatrine were gifts from Juvantia Pharma Ltd, Turku, Finland, L-657.743 (MK-912) was a gift from Merck & Co. (Whitehouse Station, NJ, U.S.A.) and dexmedetomidine and atipamezole were gifts from Orion Pharma, Turku, Finland.

Table 1 Competition binding results of different ligands at three human (h) and five zebrafish (z) α_2-adrenoceptors using [^{3}H]RX821002, [^{3}H]rauwolscine or [ethyl-^{3}H]RS-79948-197 as radioligands, expressed as apparent K_i values (nM) and their 95% confidence intervals (CI) from three to five separate experiments

	Receptor Apparent K_i *(CI)*							
Ligand	*h* α_{2A}	*z* α_{2A}	*h* α_{2B}	*z* α_{2B}	*h* α_{2C}	*z* α_{2C}	*z* α_{2Da}	*z* α_{2Db}
Atipamezole (1)	1.6 (1.3–2.1)	13 (7.4–24)**	1.5 (0.8–2.8)[a]	5.0 (3.2–7.9)[a,**]	4.3 (2.5–7.7)[a]	2.1 (1.9–2.3)[a]	5.1 (3.0–8.8)	6.90 (4.1–12)
Clonidine (2)	10 (5–23)[a]	89 (62–130)***	44 (21–94)[a]	250 (190–320)[a,**]	110 (51–240)[a]	55 (46–67)[a]	120 (65–210)	150 (98–240)
Dexmedetomidine (3)	1.3 (0.5–3.4)[a]	2.2 (1.5–3.0)	4.7 (2.2–10)[a]	7.6 (5.0–12)[a]	6.5 (2.8–16)[a]	12 (9.9–15)[a]	4.1 (3.3–5.0)	3.7 (2.6–5.3)
Idazoxan (4)	17 (13–21)	85 (48–150)**	24 (18–34)[a]	40 (23–68)	17 (6.7–26)[a]	17 (13–22)	52 (31–85)	94 (42–210)
Oxymetazoline (5)	2.1 (1.5–3.0)[a]	5.1 (3.2–8.0)*	1100 (530–2300)[a]	1200 (1000–1400)[a]	130 (70–250)[a]	1300 (860–2000)[a,**]	1100 (540–2400)	440 (300–660)
UK14,304 (6)	32 (28–37)	40 (23–69)	320 (200–510)[a]	1200 (940–1600)[a,**]	190 (140–270)[a]	700 (510–970)[a,***]	260 (200–320)	280 (210–370)
L657.743 (7)	0.8 (0.5–1.2)	6.9 (3.7–13)*	0.7 (0.5–1.2)[a]	1.2 (1.0–1.6)[a]	0.09 (0.06–0.14)[a]	1.0 (0.9–1.1)[a,***]	1.6 (1.1–2.2)	1.3 (0.7–2.1)
Rauwolscine (8)	1.9 (1.3–3.0)	1.0 (0.6–1.6)	1.1 (0.7–1.8)[a]	1.4 (0.6–3.2)	0.2 (0.1–0.5)[a]	0.5 (0.3–0.7)	2.3 (1.2–4.4)	2.3 (1.7–3.1)
Yohimbine (9)	5.9 (4.9–7.1)[b]	5.2 (3.4–7.8)	7.5 (6.4–8.9)[b]	9.3 (7.0–12)[a]	4.6 (3.9–5.5)[b]	3.4 (3.0–3.7)[a,*]	6.4 (4.5–9.2)	4.0 (3.1–5.1)
Chlorpromazine (10)	990 (500–1900)	110 (36–330)**	43 (20–100)[a]	1.1 (0.8–1.6)[a,*]	330 (220–1900)[a]	83 (72–95)[a,*]	18 (13–26)	19 (10–36)
Clozapine (11)	32 (15–66)	3.3 (2.6–4.3)***	12 (5.0–28)[a]	9.3 (5.8–14)	2.1 (1.1–3.9)[a]	3.2 (1.8–5.7)	12 (9.5–16)	24 (15–38)
ARC239 (12)	2100 (860–5100)	1800 (870–3900)	9.6 (3.9–26)[a]	36 (31–42)[a,*]	66 (36–630)[a]	280 (210–370)[a]	55 (46.8–65)	44 (19–110)
Prazosin (13)	1030 (540–2050)	330 (200–540)	66 (31–150)[a]	300 (260–360)[a,***]	31 (18–56)[a]	100 (78–130)[a,*]	68 (50–92)	64 (48–86)
Spiperone (14)	540 (440–660)	45 (28–71)***	12 (3.7–38)[a]	51 (38–67)[a,**]	11 (5.4–23)[a]	63 (44–91)[a]	15 (11–22)	18 (9.6–33)
Spiroxatrine (15)	320 (240–430)[b]	150 (92–240)*	2.4 (1.2–5.0)[a]	93 (62–140)[a,***]	3.1 (1.7–5.6)[a]	35 (22–56)**	11 (3.7–33)	11 (4.7–86)
WB-4101 (16)	5.4 (2.0–15)[a]	11 (3.7–30)	60 (30–120)[a]	51 (45–59)[a]	1.9 (1.0–3.9)[a]	19 (16–24)[a,***]	31 (22–43)	16 (11–24)
2-Amino-1-phenylethanol (17)	1300 (940–1700)[b]	5400 (3100–9400)**	4200 (2500–7100)[b]	9400 (7000–12,600)[a]	8100 (6200–10,600)[b]	5100 (3700–7000)[a]	3700 (2700–5100)	4000 (2200–7200)
Dopamine (18)	2000 (1300–3000)[a]	790 (520–1200)***	6300 (2800–14,400)[a]	4400 (3800–5200)[a]	1200 (710–2300)[a]	3900(2100–7200)[a]	1300 (820–1900)	1700 (790–3700)
(−)-Adrenaline (19)	150 (83–250)	140 (94–220)	710 (330–1600)[a]	910 (720–1200)[a]	130 (65–270)[a]	1080 (700–1700)[a]	500 (240–1100)	470 (300–740)
(−)-Noradrenaline (20)	110 (32–400)[a]	260 (140–490)	680 (360–1300)[a]	647 (500–830)[a]	250 (120–550)[a]	580 (330–1000)[a]	380 (270–540)	510 (310–840)

*$P<0.05$, statistical significance of differences between the receptor orthologues A, B or C.
**$P<0.01$.
***$P<0.001$.
[a]Using [^{3}H]rauwolscine.
[b]Using [ethyl-^{3}H]RS-79948-197.
The ligands are grouped into five crosscorrelating clusters. Numbers after the ligand names refer to Figure 4.

Other ligands were from Sigma-Aldrich (St Louis, MO, U.S.A.). Cell culture reagents were supplied by Life Technologies Inc. (Rockville, MD, U.S.A.). Other reagents were of analytical grade and were purchased from commercial suppliers.

Results

Saturation binding assays

The binding affinities of all three tested radioligands towards the five zebrafish α_2-adrenoceptors were in the low nanomolar range and thus similar to their human orthologues. For example, for [ethyl-^{3}H]RS-79948-197, the K_d and B_{max} were: α_{2A}, 0.61 ± 0.08 nM and 3050 ± 130 fmol mg^{-1}; α_{2B}, 0.25 ± 0.03 nM and 459 ± 8 fmol mg^{-1}; α_{2C}, 0.12 ± 0.02 nM and 553 ± 5 fmol mg^{-1}; α_{2Da}, 0.31 ± 0.03 nM and 2260 ± 230 fmol mg^{-1}; and α_{2Db}, 0.27 ± 0.12 nM and 2270 ± 280 fmol mg^{-1} protein (the results for α_{2B}- and α_{2C}-adrenoceptors are from cell homogenates instead of membranes, which results in three- to four-fold lower B_{max} values). These values are comparable to the observed [ethyl-^{3}H]RS-79948-197 binding in membranes prepared from the zebrafish brain homogenate: the single experiment with 21 pooled zebrafish brains resulted in a K_d of 0.1 nM and a B_{max} of 475 fmol mg^{-1} protein using similar methods as above. For the three cell lines expressing each of the three human α_2-adrenoceptors used in competition binding and [^{35}S]GTPγS binding assays, the B_{max} values were ~ 1000 fmol mg^{-1} protein (results from cell homogenates).

Competition binding assays

The binding affinities of the tested ligands towards the three human α_2-adrenoceptors and the five zebrafish α_2-adrenoceptors are summarised in Table 1 as apparent K_i values (assuming single-site binding). All ligands were tested on receptors expressed in CHO-K1 cells, with the exception of clonidine, dexmedetomidine, oxymetazoline, WB-4101, dopamine and (−)-noradrenaline binding to human α_{2A}, which were conducted with receptor expressed in the S115 host cell line. However, this should not have an effect on the outcome of the results as the 11 ligands that were tested on this receptor subtype in both CHO-K1 and S115 cell lines showed an excellent correlation ($r = 0.96$) between the host cell types without a significant systematic effect on the ligand binding affinities. Differences in the cellular background have usually played minor roles compared to differences in buffer conditions (Deupree *et al*., 1996). As the buffer conditions in the current investigation were kept constant, the apparent K_i values in Table 1 should be comparable among all ligands and receptors tested here. In general, the binding affinities are

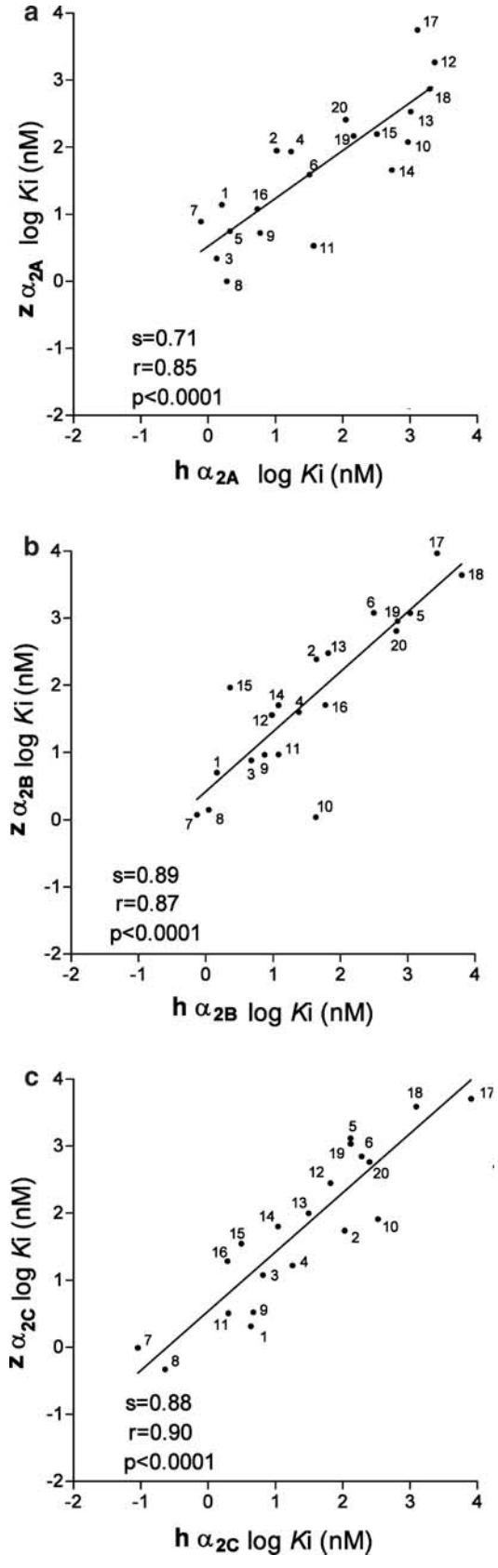


Figure 1 Linear regression analysis of logarithmic K_i values (nM) for 20 competing ligands between the orthologous human (h) and zebrafish (z) α_2-adrenoceptor subtypes (a–c). Regression lines, slopes (*s*), Spearman's correlation coefficients (*r*) and *P*-values for difference of the linear regression line from a line of zero slope are shown. Ligands are numbered as follows: (1) atipamezole, (2) clonidine, (3) dexmedetomidine, (4) idazoxan, (5) oxymetazoline, (6) UK14,304, (7) L-657.743, (8) rauwolscine, (9) yohimbine, (10) chlorpromazine, (11) clozapine, (12) ARC239, (13) prazosin, (14) spiperone, (15) spiroxatrine, (16) WB-4101, (17) 2-amino-1-phenylethanol, (18) dopamine, (19) (−)-adrenaline and (20) (−)-noradrenaline.

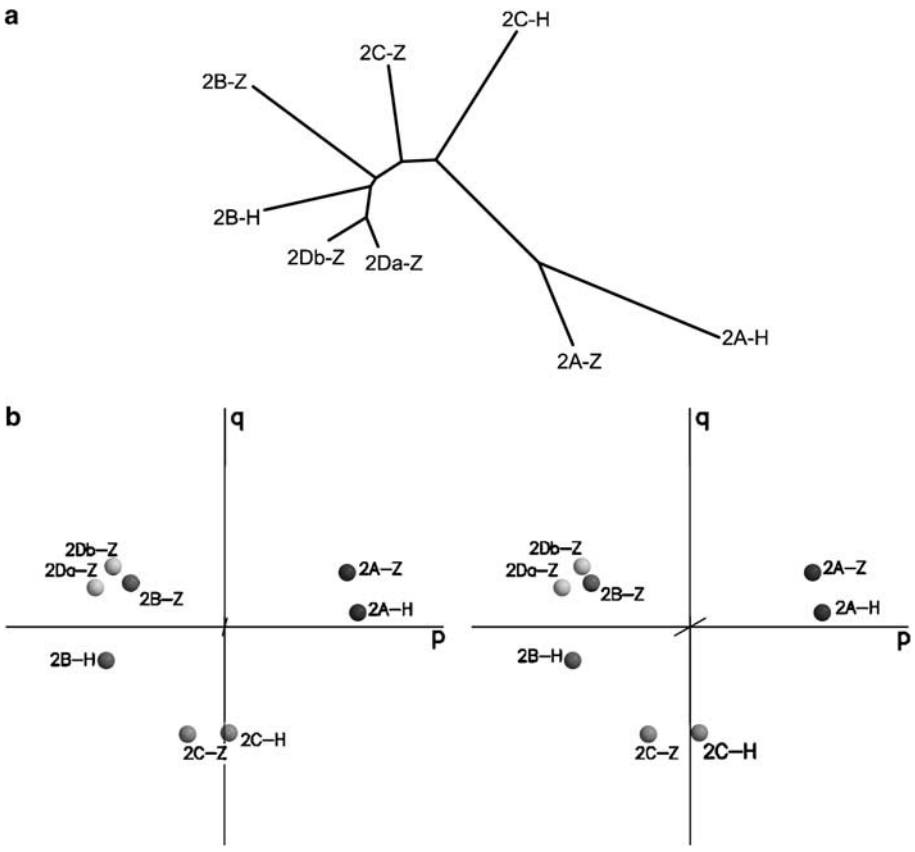


Figure 2 Clustering of the receptors according to the binding affinity data from Table 1, using the panel of 20 ligands. (a) Binary clustering. The average percent standard deviation of the tree from the distance data that it was constructed from is 5.3. (b) PCA (stereo view). Spheres depict the relative relationships among the receptors. The percentage of the total variance in the data depicted along the three most significant axes is 50.8%, *p*-axis; 20.2%, *q*-axis; 14.1%, *r*-axis. H, human; Z, zebrafish. (Note: The width of the stereo figures should be 120–130 mm to be viewed properly.)

very similar between the orthologues, with only very few compounds showing ~10-fold differences in affinity. The largest differences, ~40-fold, are seen in the binding affinities of chlorpromazine and spiroxatrine towards the human and zebrafish α_{2B}-adrenoceptors. Some compounds (oxymetazoline, chlorpromazine, ARC239, spiroxatrine) show over 100-fold differences between paralogues (subtype selectivity). Between zebrafish α_{2Da} and α_{2Db} there are no statistically significant differences in the binding affinities for any of the tested ligands. Figure 1 illustrates the linear regression analyses of the pK_i values between the A, B and C orthologues: all slopes are slightly less than unity, reflecting a general tendency of ligands with high affinities towards the human receptor subtypes to display somewhat lower affinities at the zebrafish orthologues (see, for example, L657.743 with subnanomolar K_i-values towards the human α_2-adrenoceptors).

Cluster analysis of the receptors

We have applied two independent clustering methods to the binding affinity data of Table 1. The pharmacology of the receptors towards the panel of 20 ligands is depicted in the binary tree (Figure 2a) and the results from PCA (Figure 2b), which show close clustering of α_{2Da} and α_{2Db}, and of the human and zebrafish α_{2A}-adrenoceptors, but the pairings of the human and zebrafish orthologues of α_{2B} and α_{2C} are not resolved. The zebrafish α_{2Da} and α_{2Db}, 75% identical over their entire sequences, are most similar in terms of their evolutionary relationship and almost identical in terms of their pharmacological binding affinities for the 20 ligands ($r = 0.98$; Figure 3). The results (PCA) viewed in three dimensions are coincident with the branching order shown in the binary tree and are in agreement with comparisons of the pharmacology between zebrafish α_{2Da} and the human subtypes A–C (Figure 3).

Cluster analysis of the ligands

The clustering of the ligands as a binary tree and using PCA generally results in the segregation of the ligands according to their chemical structures and gross functional characteristics. In the binary tree (Figure 4a), all agonist ligands (clonidine, oxymetazoline, UK14,304, 2-amino-1-phenylethanol, dopamine, (−)-adrenaline and (−)-noradrenaline) but one (dexmedetomidine) cluster together and away from the remaining inverse agonist/antagonist ligands. According to PCA, all of these agonists (purple and cyan spheres, Figure 4b), including dexmedetomidine, cluster apart from the inverse agonists/antagonist ligands.

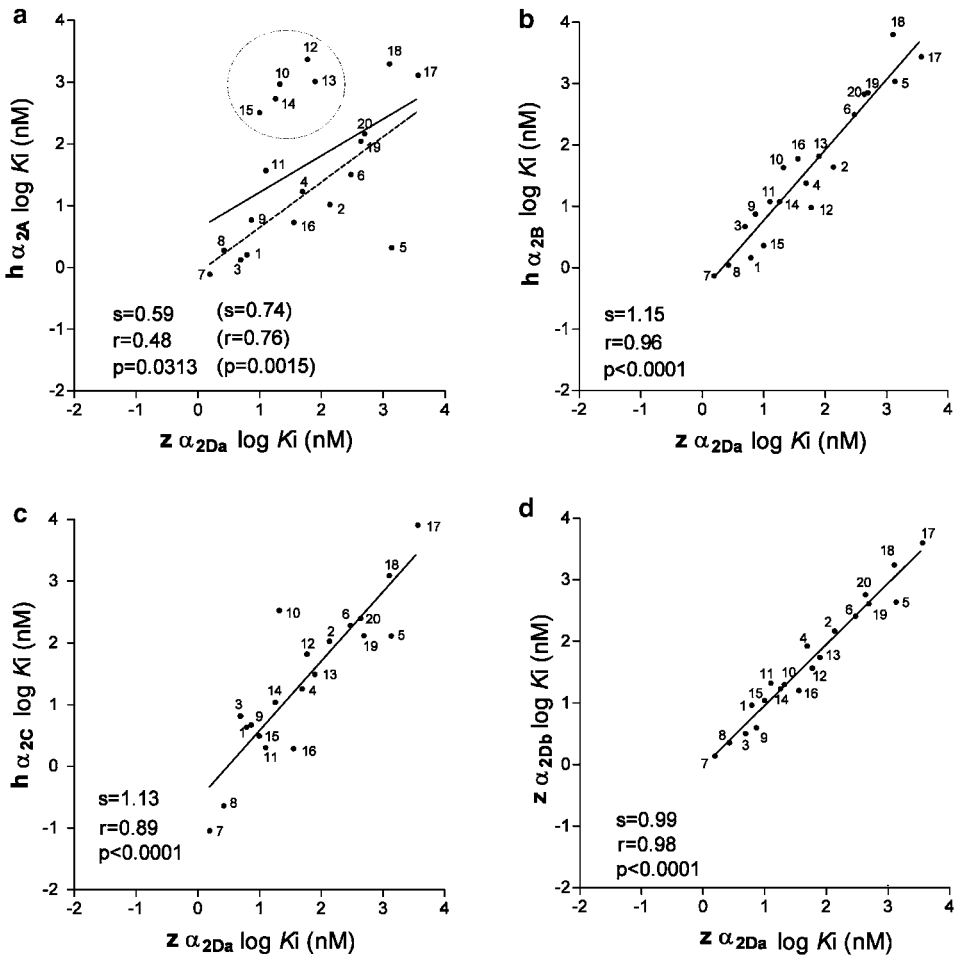


Figure 3 Linear regression analysis of logarithmic K_i values (nM) for 20 competing ligands between the zebrafish (z) α_{2Da} and the paralogous human (h) α_2-adrenoceptors (a–c), and the paralogous zebrafish α_{2Db} (d). Regression lines, slopes (*s*), Spearman's correlation coefficients (*r*) and *P*-values for difference of the linear regression line from a line of zero slope are shown. Ligand order and labelling as in Table 1 and Figure 1. In (a), the outlying group of five antagonists (ARC239, chlorpromazine, prazosin, spiperone and spiroxatrine) is circled and the regression line resulting from analysis without these ligands is shown as a dotted line and the corresponding slope, Spearman's correlation coefficient and *P*-value are within parentheses.

[^{35}S]GTPγS binding assay

Table 2 summarises the agonist efficacies and potencies of (−)-adrenaline, (−)-noradrenaline, UK14,304, oxymetazoline and dexmedetomidine on the three human α_2-adrenoceptors and the five zebrafish α_2-adrenoceptors expressed in CHO cells. For the human and zebrafish α_{2A}-adrenoceptors, adrenaline and noradrenaline show high efficacy and rather low potency, while UK14,304, oxymetazoline and dexmedetomidine show partial agonism and higher potency. For the human α_{2B}, the maximal effect of the natural ligands in absolute terms is lower than that for the human α_{2A} and α_{2C}. The zebrafish α_{2B} shows an even smaller response to adrenaline and noradrenaline. Moreover, no clear stimulation is seen for UK14,304, oxymetazoline or dexmedetomidine on the zebrafish α_{2B} (see Discussion). The zebrafish α_{2C} shows a relatively low response to the natural ligands and no response to UK14,304 or oxymetazoline. The order of the potencies is similar between the zebrafish α_{2Da} and α_{2Db}, while the efficacies of all ligands tested are lower on the α_{2Db} than on the α_{2Da}. When α_2-adrenoceptor expressing CHO cells were treated with PTX before being harvested, the responses to (−)-adrenaline were completely abolished for all receptors, indicating coupling to G_i proteins, which are inactivated by PTX (Freissmuth *et al*., 1999).

Model structures of the α_2-adrenoceptors

The residues predicted to line the binding cavities of α_{2Da} and α_{2Db} are nearly identical – based on the modelled structures of the human and zebrafish α_2-adrenoceptors (Figure 5) and schematic representations of the binding cavities (Figure 6) – reflecting their close evolutionary relationship. In general, the human and zebrafish α_2-adrenoceptors appear extensively conserved in their ligand binding pockets. In all, 27 amino-acid side chains from the TMs are predicted to line the ligand binding pocket and all but three of them are conserved. The three differences within the ligand-accessible TM regions concern mostly aliphatic changes, that is, [C-C/S-T/C-S/C-C] (sequential presentation of the amino acids at a given position in the eight receptors studied here: human α_{2A} – zebrafish α_{2A}/human α_{2B} – zebrafish α_{2B}/human α_{2C} – zebrafish α_{2C}/zebrafish α_{2Da} – zebrafish α_{2Db})5.43, [V-I/I-I/I-I/I-I]5.39 and [V-V/I-I/V-I/V-V]2.57. The 'typical' α_2-adrenoceptor cavity appears

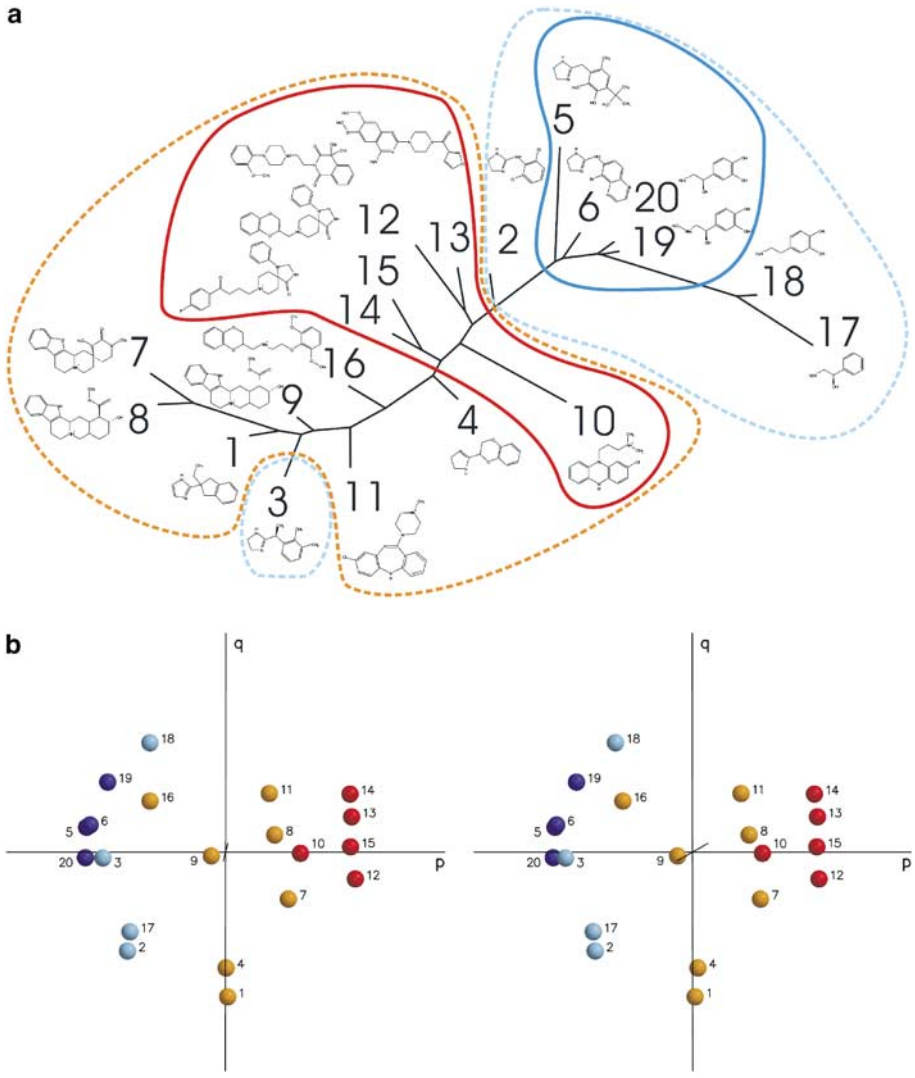


Figure 4 Clustering of the 20 ligands according to the binding affinity data from Table 1, on the three human and five zebrafish α_2-adrenoceptors. (a) Binary clustering. The chemical structures are shown for the 20 ligands and they are numbered as in Table 1. The average percent standard deviation of the tree from the original distance data that it was constructed from is 11.1. (b) PCA (stereo view). Spheres depict the relative positions of the ligands, numbered according to Table 1. The percentage of the total variance in the data depicted along the three most significant axes is 45.6%, *p*-axis; 19.4%, *q*-axis; 16.3%, *r*-axis. In both (a and b), agonists are indicated in purple and cyan; and antagonists in red and gold. (Note: The width of the stereo figures should be 120–130 mm to be viewed properly.)

extensively hydrophobic with one-third of the side chains being aromatic (Xhaard *et al*., submitted). Most of the side chains located at the bottom of the binding cavity are identical with bovine rhodopsin, or represent conservative changes (Figure 5a). TM3 and TM5 have two polar regions where the small catecholic ligands form important polar interactions: the carboxylate of D3.32, which is involved in a charge–charge interaction with the cationic amine of catecholamines, and the hydroxyls of S5.42 and S5.46, which form hydrogen bonds with the catechol hydroxyl groups (Marjamäki *et al*., 1999; Nyrönen *et al*., 2001; Peltonen *et al*., 2003). These amino acids, especially together with W6.48, F6.51, F6.52, Y6.55, V3.33 and C3.36, which can form aromatic or hydrophobic contacts, are common to all α_2-adrenoceptors (Figures 5b and 6).

In bovine rhodopsin (Palczewski *et al*., 2000), and probably also in the dopamine D2 receptors (Shi & Javitch 2004), XL2 forms a β-hairpin composed of strands β3 and β4, with several possible points of contact with bound ligands. In α_2-adrenoceptors, we have modelled XL2 as a β-hairpin using the putative disulphide bridge between Cxl2.50 and C3.25 as a spatial restraint. From XL2, there are three variable positions that can be reasonably predicted to be exposed to the binding cavity and may be in contact with bound ligands: [I-I/L-L/L-L/I-L]xl2.52 involves conservative substitutions, while positions [R-I/Q-Q/Q-M/E-E]xl2.49 and [E-D/K-Q/G-Q/L-L]xl2.51 are more variable, and contain mainly long, often charged side chains. Other side chains from XL2 may be able to reach the binding cavity surface as well, but there is little information provided by the rhodopsin structure that can be used to model that region of the α_2-adrenoceptors.

Discussion

A total of 20 ligands representing several structural classes were used in competition binding assays to characterise ligand

Table 2 Characterization of [^{35}S]GTPγS binding to membranes from CHO cells transfected to express human (h) and zebrafish (z) α_2-adrenoceptors

	Receptor																	
	h α_{2A}		*z* α_{2A}		*h* α_{2B}		*z* α_{2B}		*h* α_{2C}		*z* α_{2C}		*z* α_{2Da}		*z* α_{2Db}	
Ligand	E_{max}	$-log\ EC_{50}$	E_{max} (%)	$-log\ EC_{50}$	E_{max} (%)	$-log\ EC_{50}$	E_{max} (%)	$-log\ EC_{50}$	E_{max} (%)	$-log\ EC_{50}$	E_{max} (%)	$-log\ EC_{50}$	E_{max} (%)	$-log\ EC_{50}$	E_{max} (%)	$-log\ EC_{50}$
(−)-Adrenaline	248±36	6.3±0.3	317±100	6.8±0.1	171±13	5.9±0.1	25±1[a]	5.6±0.1	236±50	6.5±0.2	172±6	5.1±0.2	297±37	6.1±0.2	115±14	6.2±0.5
(−)-Noradrenaline	256±60	5.7±0.1	328±66	6.2±0.1	171±24	6.1±0.2	24±9	6.3±0.7	224±38	6.3±0.1	142±27	5.2±0.2	273±64	6.2±0.1	117±10	6.2±0.1
UK14,304	171±43	7.4±0.0	256±58	7.3±0.2	60±7	6.3±0.1	—	—	168±28	6.7±0.2	—	—	63±23	6.0±0.1	38±7	6.0±0.1
Oxymetazoline	32±9	7.8±0.8	10±6	6.8±0.8	102±17	5.7±0.2	—	—	23±5	7.0±0.2	—	—	114±28	5.9±0.1	34±6	5.7±0.4
Dexmedetomidine	71±14	8.0±0.8	246±74	8.1±0.2	146±13	8.1±0.1	—	—	175±32	8.1±0.1	33±18	7.4±0.4	207±56	8.0±0.1	86±4	7.8±0.1

Values are means±s.d. of three or more independent experiments performed in duplicate.
E_{max} (% over basal) indicates the maximal stimulation. EC_{50} is the ligand concentration (M) causing 50% of the maximal effect.
[a]Data from two independent experiments performed in duplicate. Results from experiments where the E_{max} was too small to be determined reliably are marked with (−).

binding properties of the three human and the five zebrafish α_2-adrenoceptor subtypes. The generally surprisingly similar pharmacological profiles of the zebrafish and human α_2-adrenoceptors (see Figures 1 and 3) probably reflect equal requirements to bind endogenous catecholamines: in structural models of the receptor proteins, amino acids that form the putative catecholamine binding pocket, located on the TMs and XL2, are highly conserved (Figures 5 and 6). It is likely that antagonists bind at least partially within the same pocket where agonists bind (Xhaard *et al*., submitted); the radiolabelled antagonists are displaced in competition binding assays. Antagonists are generally larger than agonists and their interaction with more distant side chains could give rise to the observed pharmacological differences among the α_2-adrenoceptors.

Only three positions differ within the TM segments in the structural models. Conservative substitutions of aliphatic amino acids are found at positions 2.57 and 5.39. The role of serine/cysteine at position 5.43 in the α_2-adrenoceptors has been extensively studied with regard to catecholamine binding (Rudling *et al*., 1999; Nyrönen *et al*., 2001; Peltonen *et al*., 2003). For the human α_{2A}, in the case of agonist low-affinity binding (competitive assays against the antagonist radioligand [^{3}H]RX821002), phenethylamines and UK14,304 prefer cysteine over serine at position 5.43, while the binding of clonidine and *para*-amino-clonidine is not affected. However, high-affinity agonist binding (competitive assays against the agonist [^{3}H]UK14,304) and receptor activation are only marginally affected by the mutation of cysteine to serine in the human α_{2A} at position 5.43 (Peltonen *et al*., 2003). In addition, C5.43 has been shown to be more favourable than S5.43 for the antagonist yohimbine; this at least partly accounts for the observed affinity difference for yohimbine between the mouse and human α_{2A}-adrenoceptors (Link *et al*., 1992).

In contrast to the TM helices, most of the amino-acid variation within the binding cavity of the human and zebrafish α_2-adrenoceptors is concentrated along XL2 (Figure 6), and may contribute to the observed differences in binding affinities. XL2 has been implicated in ligand binding in other GPCRs closely related to α_2-adrenoceptors, for example, in the dopamine D2 receptor, where point mutations of xl2.49 and xl2.51–xl2.52 most significantly reduced the affinity of *N*-methylspiperone (Shi & Javitch, 2004), and in the α_1-adrenoceptor subtypes A and B, where mutations of xl2.51–xl2.53 affected the binding of the antagonist WB-4101 (Zhao *et al*., 1996). The disulphide bridge linking Cxl2.50 to C3.25 constrains the position of XL2 in the rhodopsin structure. The cysteines are conserved throughout most Family A GPCRs and likely function similarly to constrain the XL2 region, especially in the vicinity of xl2.50. When amino-acid sequences from diverse α_2-adrenoceptors are compared, the region xl2.48-TM5 of XL2 has the same length, eight residues shorter in comparison to the equivalent region in bovine rhodopsin, whereas wider variation occurs over the TM4-xl2.47 region (Figure 6). In comparison to the amino-terminal region of XL2 (TM4-xl2.47), Xl2.48-TM5 is more conserved, supporting a location for the carboxyl-terminal region closer to the protein core as is seen in the bovine rhodopsin X-ray structure. Among the α_2-adrenoceptors, Cxl2.50 and Nxl2.53 are conserved while Pxl2.48 is conserved in all α_2-adrenoceptors except for α_{2A}, α_{2Da} and α_{2Db} of zebrafish.

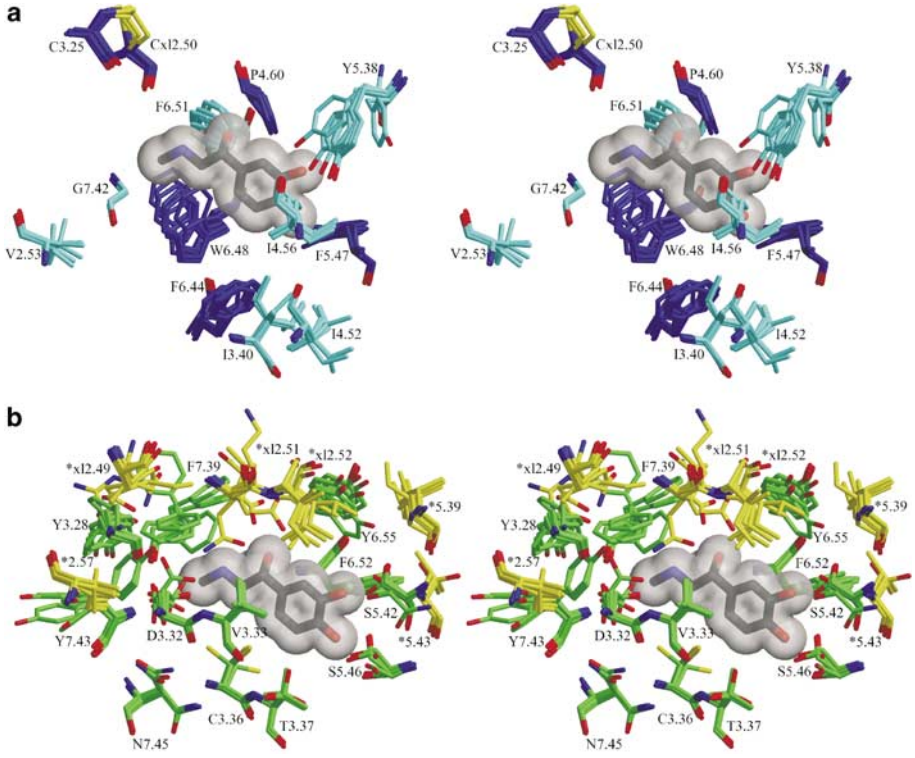
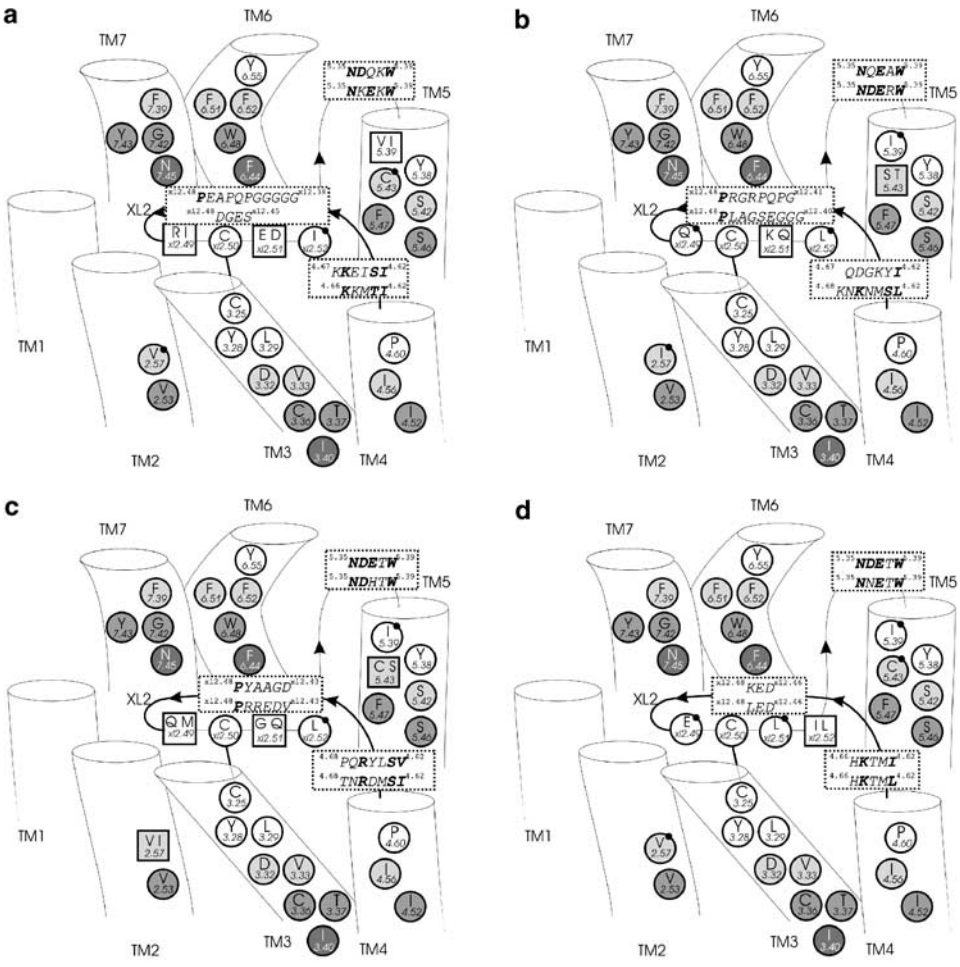


Figure 5 Superimposed structural models of the binding cavities of the three human and the five zebrafish α_2-adrenoceptor subtypes; adrenaline has been manually docked (a stereo view). (a) Residues common with bovine rhodopsin (blue), or representing conservative changes (cyan; in bovine rhodopsin: M2.53, L3.40, M4.52, C4.56, F5.38, Y6.51, A7.42). (b) Residues differing from rhodopsin but conserved among the α_2-adrenoceptors (green); the six variable residues within the α_2-adrenoceptor binding cavity, labelled with (*) (yellow; cf. Figure 6). Structural models for the human and zebrafish α_2-adrenoceptors were constructed based on homology with bovine rhodopsin whose structure is known (see Methods, and Xhaard *et al*., submitted). Adrenaline was positioned based on key amino-acid contacts identified in Peltonen *et al*. (2003). (Note: The width of the stereo figures should be 120–130 mm to be viewed properly.)

Pxl2.48 is not exposed to the binding cavity surface in the structural models but is located at the β-turn of XL2. Other changes along the carboxyl-terminal region of XL2 involve conservative substitutions: [I-I/L-L/L-L/I-L]xl2.52, [R-I/Q-Q/Q-M/E-E]xl2.49 and [E-D/K-Q/G-Q/L-L]xl2.51 are variable but correspond to mainly long side chains, often charged. Aside from the region xl2.49–xl2.52, it is not possible to reliably model the rest of XL2 due to ambiguities in alignments of rhodopsin with the α_2-adrenoceptors that result from insertions and deletions, and a lack of amino-acid similarity.

The ligand binding affinities of the human and zebrafish orthologues show close agreement (Figure 1). In general, the ligand binding affinities are more similar between orthologues (up to 40-fold differences) in comparison to the paralogues (up to 100-fold differences for a few compounds). Based on the pharmacology, both the binary tree and PCA clearly cluster α_{2Da} and α_{2Db} together, as well as the α_{2A}-adrenoceptors of human and zebrafish (Figure 2). The α_{2D} duplicates are most similar in sequence, having a single conservative exchange (α_{2Da}, isoleucine; α_{2Db}, leucine) within the proposed binding cavity (Figure 6). Of the six variable sites seen in the α_2-adrenoceptors in the binding cavity (Figure 6e), three differences occur between the α_{2A}-adrenoceptors of human and zebrafish: arginine/isoleucine at xl2.49; glutamate/aspartate at xl2.51 and valine/isoleucine at 5.39. For α_{2B}-adrenoceptors, only two positions differ (lysine/glutamine at xl2.51; serine/threonine at 5.43) between human and zebrafish, yet the pharmacology of the human receptor is more similar to the zebrafish α_{2D} duplicates (with 5 (α_{2Da}) and 4 (α_{2Db}) differences with the human α_{2B}) than to its zebrafish α_{2B}-orthologue (Figures 1–3). There are four differences in the binding sites of the human and zebrafish α_{2C}-adrenoceptors (valine/isoleucine at 2.57; glutamine/methionine at xl2.49; glycine/glutamine at xl2.51 and cysteine/serine at 5.43), whereas from two to six positions differ from the α_{2A}- and α_{2B}-adrenoceptors (Figure 6e). In terms of the pharmacology, as clustered (Figure 2), the α_{2C} orthologues appear no more similar to each other than to the other human and zebrafish α_2-adrenoceptors.

Pharmacological binding affinities must be considered with caution when used to assign receptor subtype classifications to newly identified receptors. This is especially true in this case where a small number of amino-acid replacements is associated with the observed differences in receptor pharmacology, compounded by the fact that these sites within human/zebrafish orthologues have not been strictly conserved over the $\sim$350 million years since bony fishes and tetrapods diverged. Indeed, a residue difference present in one orthologue of a pair is often identical with the residue at the equivalent location in a paralogous receptor (Figure 6e). Complicating the issue, amino-acid differences distant from the binding cavity may also affect receptor pharmacology (Venkataraman *et al*., 1997).



e

Subtype - species	Position 2.57	xl2.49	xl2.51	xl2.52	5.39	5.43
2A-H	V	R	E	I	V	C
2A-Z	V	I	D	I	I	C
2B-H	I	Q	K	L	I	S
2B-Z	I	Q	Q	L	I	T
2C-H	V	Q	G	L	I	C
2C-Z	I	M	Q	L	I	S
2Da-Z	V	E	L	I	I	C
2Db-Z	V	E	L	L	I	C

Figure 6 Sequence variation within the putative ligand binding cavities of the human and zebrafish α_2-adrenoceptors. (a–d) Schematic representation of the binding cavity based on the three-dimensional model structures depicted in Figure 5. Residues predicted to be exposed to the binding cavity are highlighted. Human *versus* zebrafish (a) α_{2A}, (b) α_{2B} and (c) α_{2C}, and (d) the zebrafish α_{2Da} and α_{2Db} duplicates. The predicted binding cavity is located within the TM bundle in close contact with the XL2 that connects TM4 to TM5 and links TM3 *via* a disulphide bridge (as occurs in the bovine rhodopsin X-ray structure). Amino-acid residues are numbered according to the Ballesteros and Weinstein nomenclature, expanded to XL2. The conserved cysteine of XL2 involved in the disulphide bridge is referred to as xl2.50. Positional conservation and variation are indicated as follows: conserved positions in the compared receptors are circled and the amino acid indicated in the single letter amino-acid code; a dot on the circle indicates that the position is *not* conserved across one or more of the paralogous receptors; variable positions in the compared receptors are boxed and the variation indicated (in (a–c) the amino acid in human is followed by that in zebrafish; in (d) the amino acid in α_{2Da} is followed by that in α_{2Db}). Those residues whose location with respect to the proposed binding site is uncertain, belonging to XL2, are shown in a dashed box. Shades of gray are used to indicate the location of the residue with respect to the extracellular surface: light towards the surface and darker away from the surface. (e) Sequence variation within the proposed binding cavity at the six sites where amino-acid differences occur between the human and zebrafish α_2-adrenoceptors.

When ligands are clustered based on their binding affinities at the eight receptors, the use of PCA allows distinction of the agonist ligands from the antagonists (Figure 4b); this is also generally supported by the binary tree (Figure 4a). Why this apparent segregation occurs between agonist and antagonist ligands is not clear to us, since binding affinity does not necessarily reflect a ligand's functional properties. However, some similar patterns can be seen in Table 1: a subset of four of these agonist ligands (oxymetazoline, UK14,034, (−)-adrenaline and (−)-noradrenaline) generally have higher affinities for α_{2A}-adrenoceptors relative to other α_2-adrenoceptors; they are located on adjacent branches of the binary tree and cluster as a group according to PCA (purple spheres, Figure 4b). A group of antagonists that generally have lower affinity for α_{2A}-adrenoceptors (chlorpromazine, ARC239, prazosin, spiperone, spiroxatrine), relative to the other subtypes, can be segregated at a central position on the tree and cluster together according to PCA (red spheres, Figure 4b); these antagonists are located at the opposite end along the principal axis from the agonist ligands. A negative charge at xl2.51, only present in α_{2A}-adrenoceptors, might be involved in reducing the affinity for these large compounds.

For ligand binding and functional assays, we have expressed zebrafish receptors in a mammalian cell line. A fish α_2-adrenoceptor expressed in a mammalian cell line has previously been shown to be functional (Svensson *et al*., 1993). The use of a heterologous expression system is furthermore supported by the high conservation of the G proteins across human and zebrafish species: for example, the human $G_i\alpha_1$-subunit shares ~97% sequence identity with its putative zebrafish homolog (Accession number NP_957265; first ranking hit on a BlastP search of the zebrafish genome at http://www.ncbi.nlm.nih.gov/genome/seq/DrBlast.html). Moreover, the functional response here is similar to native systems, and CHO cells are by far the predominant host system for α_2-adrenoceptors. Unfortunately, for receptor cycling, the picture may or may not be different, but as far as ligand binding and G-protein coupling are considered, CHO cells can be considered to provide an appropriate environment for this particulate case.

The rank orders of the potencies of the agonists tested are conserved between the human and zebrafish α_{2A}-adrenoceptors. The natural ligands noradrenaline and adrenaline show very low efficacy on the zebrafish α_{2B} (see Table 2). Likewise, the human α_{2B} shows lower efficacy with these agonists than its human paralogues, although the differences are not as large as among the zebrafish paralogues. Furthermore, UK14,304, oxymetazoline and dexmedetomidine show no activation of the zebrafish α_{2B}, perhaps because the GRK site (EESSSS), which might be important for G-protein coupling, is not encoded by the zebrafish α_{2B} gene (Ruuskanen *et al*., 2004). Alternatively, agonists designed for human α_2-adrenoceptors may be inactive on zebrafish α_{2B} because of other structural differences. This may also be true for UK14,304 and oxymetazoline for the zebrafish α_{2C}. Despite the poor responses of the zebrafish α_{2B} and α_{2C} for all or some of the agonists used, differentiation between partial and full agonists is possible as with the human α_2-adrenoceptors.

In conclusion, this work provides ligand binding and functional characterisation of the zebrafish counterparts of the three human α_2-adrenoceptors, as well as the first pharmacological and functional profiling of a previously uncharacterised, fourth α_2-adrenoceptor subtype with two duplicates in zebrafish, α_{2Da} and α_{2Db}. Structural models of the ligand binding core of α_2-adrenoceptors indicate a highly conserved TM pocket and a somewhat more variable XL2 domain, which may contribute to a significant extent to the observed differences and similarities in ligand binding between the receptor orthologues and paralogues.

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