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Binding Properties of Three Neuropeptide Y Receptor Subtypes from Zebrafish: Comparison with Mammalian Y1 Receptors

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ABSTRACT. Neuropeptide Y (NPY) and peptide YY (PYY) are two related 36-amino-acid peptides found in all vertebrates and are involved in many physiological processes. Five receptor subtypes have been cloned in mammals (Y1, Y2, Y4, Y5, and y6). We have recently cloned three NPY/PYY receptor subtypes in zebrafish, called Ya, Yb, and Yc. Here we report on a direct comparison of the pharmacological properties of these three receptors *in vitro* using porcine NPY with alanine substitutions in positions 33–36 as ligands and three analogues with internal deletions: [Ahx$^{8-20}$]NPY, [Ahx$^{8-20}$, Pro^{34}]NPY, and [Ahx$^{5-24}$]NPY. In all cases, the zYc receptor was the most sensitive to the modifications of the NPY molecule and zYa was the least sensitive (except for the Arg → Ala replacement at position 33). Our data identified zYa as a receptor that can bind ligands specific for Y1, Y2, and Y4 receptors, while zYb and zYc were more Y1-like. All peptides with internal deletions bound to the zYa receptor with affinities similar to that of intact pNPY. Neither the Y1-selective antagonists BIBP3226 and SR120819A nor the Y2-selective BIIE0246 bound to any of the zebrafish receptors, although the amino acids identified as important for BIBP3226 binding were almost completely conserved. These results may prove helpful in molecular modeling of the three-dimensional receptor structure. BIOCHEM PHARMACOL **60**;12: 1815–1822, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. alanine scan; G-protein-coupled receptor; neuropeptide Y (NPY); peptide YY (PYY); binding; zebrafish

The NPY** family of peptides consists of NPY, PYY, and PP in mammals. Fish lack PP, but some species have a third peptide called PY [1]. Over the 36 amino acids the peptides share a common hairpin-like three-dimensional structure, called the "PP-fold", as shown by x-ray diffraction [2] and by NMR [3]. NPY effects in the mammalian brain include increased feeding, inhibition of reproductive behavior, and shifts in circadian rhythms, while in the periphery NPY causes vasoconstriction [4, 5]. PYY is primarily an endocrine peptide in the gastrointestinal tract where it is released after food intake, influencing gut motility and acid secretion. PYY has, like NPY, been cloned and localized in the brain in zebrafish (*Danio rerio*) [6], sea bass (*Dicentrarchus labrax*) [7], and in river lamprey (*Lampetra fluvialitis*) [8].

At least six receptors for NPY family peptides are known in mammals and named Y1–y6, and pharmacological indications for up to three more receptor subtypes have been reported [9–11]. The Y1, Y4, and y6 receptors form a subfamily of more closely related receptors with about 50% sequence identity. As additions to this subfamily, our group has cloned three receptors from zebrafish named zYa [12], zYb [13], and zYc [14], as well as a receptor from the Atlantic cod (*Gadus morhua*), Yb, with high identity to the zYb and zYc receptors [15, 16]. The zYb and zYc receptors are 75% identical to each other and both are 50% identical to zYa [14]. The Y2 and Y5 receptors seem to form two additional subfamilies with 25–30% identity to each other and to the receptors of the Y1 subfamily, i.e. with an identity almost as low as these receptors display to other neuropeptide receptors such as the neurokinin receptors. Despite strong pharmacological evidence for the existence of an NPY-selective Y3 receptor [17], this receptor still remains to be cloned. The functions and distribution of the fish receptors have not yet been explored.

Alanine scanning where sets of analogues are produced with single amino acids changed, one in each peptide, for

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** *Abbreviations:* Ahx, 6-aminohexanoic acid; NPY, neuropeptide Y; p, porcine; PP, pancreatic polypeptide; PYY, peptide YY; TM, transmembrane; and z, zebrafish.

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alanine is a rational and powerful tool to map which parts of a ligand are important for receptor recognition. Previous studies of the Y1 and Y2 receptors [18, 19] pointed to the carboxy-terminus of NPY as the most important part of the ligand for binding to Y1 and Y2 receptors. Here, we used four pNPY analogues where each of the last four amino acids of the NPY molecule was replaced by alanine. For the binding to Y1 receptor the amino-terminus is also involved, since there is a gradual loss of affinity for Y1 receptors with progressive amino-terminal truncation of the NPY molecule [9–11]. In contrast, amino-terminally truncated analogues, such as NPY2–36 and NPY13–36, bind to Y2 receptors with the same affinity as the parent molecule. Another approach to reduce the size of NPY receptor ligands is to replace parts of the central loop with a linker between the polyproline helix (positions 1–8) and the α-helix (positions 15–32). The compound [Ahx$^{8-20}$]NPY, [20], where amino acids 8–20 have been replaced by Ahx, has been identified as a full agonist at the Y1 receptor. Other ligands used here were [Ahx$^{8-20}$, Pro^{34}]NPY, which does not bind to the Y2 receptor and [Ahx$^{5-24}$]NPY, which is Y2-selective [20, 21].

We have embarked on a project aimed at characterizing the NPY/PYY system in zebrafish. The zebrafish is being increasingly used in studies of vertebrate development. The embryos are transparent, making them ideal for whole-mount *in situ* hybridization studies. In addition, the gene map for the zebrafish is becoming very detailed [22]. These types of information will provide further insight into the functions of the NPY receptors and shed light on the evolution of the NPY receptor family.

Here, we present a detailed pharmacological characterization of the three zebrafish receptors Ya, Yb, and Yc and compare these data with previous data from the mammalian Y1 receptor in order to further classify the fish receptors as well as facilitate structural modeling of the Y1 subfamily of receptors.

MATERIALS AND METHODS

Cloning and Generation of Stable Cell Lines

The three receptors were cloned by polymerase chain reaction (PCR) with degenerate primers followed by screening of a zebrafish genomic library. First, the zebrafish Ya and Yb receptors were cloned [12, 13] using primers based on conserved parts of the Y1 receptor in mammals and *Xenopus laevis*. Based on the new sequence information from the zYa and zYb receptor genes together with the Y1 receptor sequences, a panel of new degenerate primers was designed. These primers were subsequently used to clone the zYc receptor [14]. The coding regions were inserted into the eukaryotic vector pTEJ-8 [23]. For the zYa, zYb, and zYc receptors, Chinese hamster ovary (CHO) cells were stably transfected with 2 μg of DNA using lipofectin, [12–14]. Clones resistant to G418 were picked three weeks later. The previously published data on NPY and PYY analogue binding to the zYa and zYb receptors, however, come from receptors transiently expressed in green monkey kidney (COS-7) cells. The cloning of the three receptors as well as the generation of stable cell lines have been described in detail elsewhere [12–14]. The guinea pig Y1, Y2, and Y4 receptors [24–26] used for the BIIE0246 binding were stably expressed in CHO cells as described above, while the guinea pig Y5 receptor was transiently expressed in human embryonic kidney cells (HEK-293 EBNA).

Sequence Analysis

All sequence comparisons and alignments were done using the DNAStar software MEGALIGN.

Peptides and Ligands

The peptides were synthesized by solid-phase peptide synthesis using fluorenylmethoxycarbonyl (Fmoc) strategy and tertiary butyl/trityl side-chain protection. Rink amide resin was used for solid phase. Cleavage from the resin and side-chain deprotection was performed in one step using trifluoracetic acid. Peptides were precipitated from diethyl ether, containing 0.05% HCl in order to receive peptide hydrochlorides, and lyophilized from water. Purification was performed by preparative HPLC and peptides were characterized by HPLC (purity) and mass spectrometry (identity) as described previously [18, 20, 21]. Four alanine-substituted pNPY analogues were used where each of the last four amino acids in the NPY sequence (Arg^{33}, Gln^{34}, Arg^{35}, and Tyr^{36}) had been changed to L-alanine: p[Ala^{33}]NPY, p[Ala^{34}]NPY, p[Ala^{35}]NPY, and p[Ala^{36}]NPY [19]. One analogue proposed to have weak Y4 receptor selectivity [27] was modified at two positions (p[Glu^{4}, Pro^{34}]NPY). Three loop-deleted peptides where parts of the loop were substituted with a 6-aminohexanoic acid bridge were also used. In [Ahx$^{8-20}$]NPY, amino acids 8–20 were substituted [20]. [Ahx$^{8-20,}$,Pro^{34}]NPY is the same molecule as [Ahx$^{8-20}$]NPY but with Gln^{34} changed to a proline. This peptide does not bind to the Y2 receptor. The peptide [Ahx$^{5-24}$]NPY, where amino acids 5–24 are substituted, is Y2 receptor-selective [20, 21]. Previously published results [12–14] using analogues of porcine NPY (i.e. p[Leu^{31}, Pro^{34}]NPY, pNPY2–36, pNPY3–36, pNPY13–36, and p[D-Trp^{32}]NPY) are also presented in Table 1 in order to compare the binding profiles. The zebrafish NPY used to determine non-specific binding was synthesized at Eli Lilly, while p[Leu^{31}, Pro^{34}]NPY, pNPY2–36, pNPY3–36, pNPY13–36, and p[D-Trp^{32}]NPY were purchased from BACHEM. Along with the peptides, two Y1-selective non-peptidic antagonists were tested: BIBP3226 ((*R*)-*N*^{2}-(diphenylacetyl)-*N*-[(4-hydroxy-phenyl)methyl]argininamide) [28] (synthesized at Boehringer Ingelheim KG) and SR120819A (1-[2-(2-naphthylsulfamoyl)-3-phenylpropionamido]-3-[4-[*N*-(dimethylaminomethyl)-*cis*-cyclohexylmethyl]-amidino]phenyl]propionyl]pyrrolidine, (*R*, *R*) stereoisomer [29] (synthesized at Sanofi), as well as one Y2-selective antagonist: BIIE0246 ((*S*)-*N*^{2}-[[1-[2-[4-[(*R*, *S*)-5,11-dihydro-6(6*h*)-oxodibenz[*b*, *e*]azepin-11-yl]-1-pipera-zinyl]2-oxoethyl] cyclopentyl] acetyl]-*N*-[2-[1, 2,-dihydro-3, 5

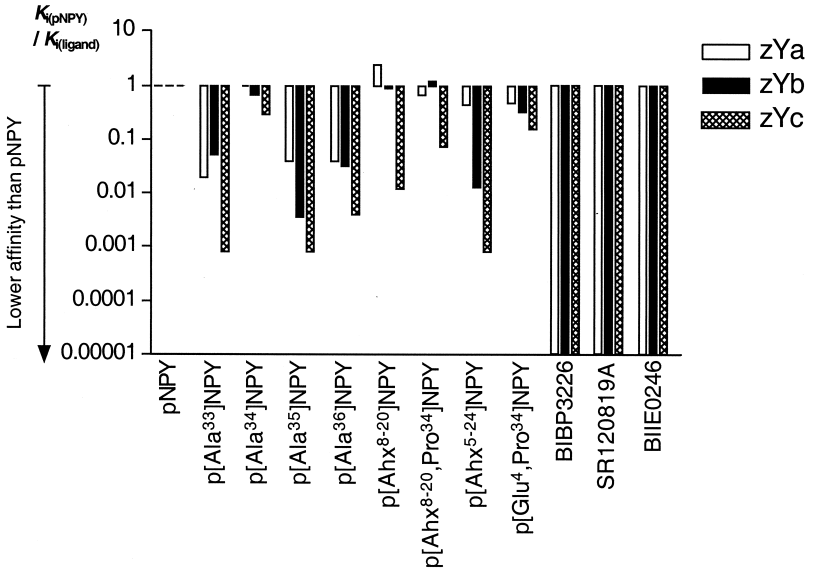

FIG. 1. K_i values for each peptide analogue compared with the K_i value for pNPY. A value < 1 indicates that the modification decreases the affinity for the receptor.

(4*H*)-dioxo-1,2-diphenyl-3*H*-1, 2, 4-triazol-4-yl] ethyl]-argininamide) [30] (synthesized at Boehringer Ingelheim KG).

Binding Assays

The thawed aliquots of membranes from each of the cell lines stably expressing the zYa, zYb, and zYc receptors were resuspended in 25 mM HEPES buffer (pH 7.4) containing 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, and 2 g/L of bacitracin and homogenized. Competition experiments were performed in a final volume of 100 μL. Various concentrations of the peptides pNPY, p[Ala^{33}]NPY, p[Ala^{34}]NPY, p[Ala^{35}]NPY, p[Ala^{36}]NPY, [Ahx$^{8-20}$]NPY, [Ahx$^{8-20}$, Pro^{34}]NPY, [Ahx$^{5-24}$]NPY, and p[Glu^{4}, Pro^{34}]NPY as well as the three non-peptidic antagonists SR120819A, BIBP3226, and BIIE0246 were included in the incubation mixture along with 30–40 pM ^{125}I-pPYY (Amersham). Due to the extreme affinities of NPY and PYY to the zebrafish receptors, we used double-iodinated PYY (iodinated at tyrosines 21 and 27) that has a specific activity of 4000 Ci/mmol. Incubations were terminated after 2 hr (RT) by filtration through GF/C filters (Filtermat A), which had been presoaked in 0.3% polyethyleneimine, using a TOMTEC cell harvester. The filters were washed with 5 mL of 50 mM Tris (pH 7.4) at 4° and dried at 60°. The dried filters were treated with MeltiLex A (Wallac) melt-on scintillator sheets and the radioactivity retained on the filters determined using the Wallac 1450 Microbeta counter. Inhibition constants (K_i) were calculated by using the Cheng and Prusoff equation. The dissociation constant (K_d) for ^{125}I-pPYY to the zebrafish Yc receptors was taken from [14]. For the zYa and zYb receptors, saturation experiments were performed in a final volume of 200 μL with about 10 μg (Ya) and 0.5 μg (Yb) of protein for 2 hr at room temperature. Non-specific binding was defined as radioactivity remaining on the filter in the presence on 100 nM zNPY. In order to explore the importance of the radioligand, pNPY, pNPY3–36, pNPY13–36, p[Ala^{34}]NPY, [Ahx$^{5-24}$]NPY, bPP, and BIBP3226 were used to compete with 50 pM monoiodinated ^{125}I-hPP (Eurodiagnostica) at the zYa receptor. As a control for the Y2-selective antagonist, we used our panel of guinea pig receptors [24–36]. BIIE0246 was used to displace ^{125}I-pPYY at the guinea pig Y1, Y2, and Y5 receptors as described above for the zebrafish receptors. ^{125}I-hPP was used as radioligand at the guinea pig Y4 receptor. The results were analyzed using the Prism 2.0 software package (GraphPad). Protein concentrations were measured using the Bio-Rad Protein Assay (Bio-Rad) with BSA as standard.

RESULTS

Binding

The radioligand ^{125}I-pPYY identified a single class of high-affinity-binding sites at all three stably expressed zebrafish receptors zYa, zYb, and zYc. The K_d values for ^{125}I-pPYY were 12 ± 1 pM, 3.2 ± 0.4 pM, and 16 ± 2 pM, respectively, and the B_{max} values were 40 ± 7, 150 ± 20, and 700 ± 30 fmol/mg prot, respectively (the K_d and B_{max} values for the zYc receptor were previously reported by Ringvall *et al.* [14]). The K_i values for pNPY were 60, 16, and 26 pM, respectively. All four of the alanine substitutions in the NPY molecule resulted in decreased affinity for the zYb and zYc receptors (see Fig. 1 and Table 1). As previously reported for mammalian Y1 and Y2 receptors, the effects were greatest with substitution of Arg^{33}, Arg^{35} or Tyr^{36}. The Arg^{33} → Ala^{33} substitution caused a 50-, 20-, and 1200-fold loss of affinity at the zYa, zYb, and zYc receptors, respectively. For [Ala^{35}]NPY, the loss in affinity was 25-, 250-, and 6000-fold at the three different recep-

TABLE 1. Potencies of NPY and NPY-related ligands to inhibit ^{125}I-pPYY binding to membranes prepared from Chinese hamster ovary cells stably expressing the zebrafish Ya, Yb, and Yc receptors

Peptide	Receptor subtype		
	zYa	zYb	zYc
pNPY	0.06 ± 0.01	0.016 ± 0.005	0.026 ± 0.005
p[Ala^{33}]NPY	3.1 ± 0.5	0.28 ± 0.02	30 ± 10
p[Ala^{34}]NPY	0.06 ± 0.01	0.022 ± 0.003	0.08 ± 0.03
p[Ala^{35}]NPY	1.5 ± 0.5	4 ± 1	150 ± 70
p[Ala^{36}]NPY	1.5 ± 0.1	0.47 ± 0.07	6.2 ± 0.3
[Ahx$^{8–20}$]NPY	0.025 ± 0.002	0.017 ± 0.02	2.1 ± 0.3
[Ahx$^{8–20}$, Pro^{34}]NPY	0.087 ± 0.006	0.013 ± 0.001	0.34 ± 0.07
[Ahx$^{5–24}$]NPY	0.13 ± 0.01	1.1 ± 0.4	240 ± 90
p[Glu^{4}, Pro^{34}]NPY	0.12 ± 0.02	0.05 ± 0.01	0.16 ± 0.005
BIBP3226	> 3000	> 3000	> 3000
SR120819A	> 3000	> 3000	> 3000
BIIE0246	> 10000	> 10000	> 10000
pNPY2–36	0.038 ± 0.009*	3.4 ± 0.8*	2.1 ± 0.7*
pNPY3–36	0.06 ± 0.01*	40 ± 10*	40 ± 20*
pNPY13–36	0.036*	90 ± 4*	60 ± 40*
p[Leu^{31}, Pro^{34}]NPY	0.039 ± 0.007*	0.012*	0.022 ± 0.005*
p[D-Trp^{32}]NPY	110 ± 20*	6.4 ± 1.3*	210 ± 90*
bPP	0.36 ± 0.05*	2.8 ± 0.6*	30 ± 10*
K_d ^{125}I-pPYY	0.012 ± 0.002	0.0032 ± 0.0004	0.016 ± 0.002

Data represent inhibition constants K_i ± SEM (nM), for three to six experiments performed in duplicate (except for pNPY13–36 at zYa and p[Leu^{31}, Pro^{34}]NPY at the zYb, data from a single experiment performed in duplicate). Non-specific binding was defined in the presence of 100 nM zNPY. Non-linear regression analyses were carried out using the GraphPad Prism 2.0 Software. K_i values were taken from [14].

tors, and for [Ala^{36}]NPY it was 25-, 30-, and 250-fold, respectively. The Gln^{34} to Ala^{34} substitution had little or no impact on binding to zYa and zYb receptors and affected the binding at the zYc receptor only by 3-fold.

The loop-deleted peptides [Ahx$^{8–20}$]NPY and [Ahx$^{8–20}$, Pro^{34}]NPY both displayed a similar affinity as pNPY for the zYa and zYb receptors. In contrast, the two peptides bound to zYc with 80- and 10-fold lower affinity, respectively, than pNPY. The smallest loop-deleted molecule, the Y2-selective agonist [Ahx$^{5–24}$]NPY, bound to zYa with only 2-fold lower affinity than pNPY. At the zYb and zYc receptors, this compound bound with 70- and 9000-fold lower affinity than NPY. The p[Glu^{4}, Pro^{34}]NPY analogue bound with 2-, 3-, and 6-fold lower affinity, respectively, than pNPY. None of the three non-peptidic antagonists BIBP3226, SR120819A, and BIIE0246 bound to any of the three zebrafish receptors with detectable affinities. As a control, BIIE0246 was tested at our cloned guinea pig NPY receptors and found to compete with ^{125}I-pPYY binding at the guinea pig Y2 receptor (K_i = 2.4 ± 0.5 nM). No binding was detected at the guinea pig Y1, Y4, and Y5 receptors. The importance of the radioligand at the zYa receptor was explored in a competition experiment using ^{125}I-hPP as radioligand where the rank order of potencies was found to be indistinguishable from that given by the competition using ^{125}I-pPYY as radioligand (pNPY ≈ pNPY3–36 ≈ pNPY13–36 ≈ p[Ala^{34}]NPY > [Ahx$^{5–24}$]NPY > bPP >>> BIBP3226). The IC_{50} values were 58 ± 3 pM, 59 ± 6 pM, 70 ± 20 pM, 70 ± 5 pM, 120 ± 20 pM, and 200 ± 20 pM for pNPY, pNPY3–36, pNPY13–36, p[Ala^{34}]NPY, [Ahx$^{5–24}$]NPY, and bPP, respectively (n = 3 for all ligands). BIBP3226 at concentrations up to 3 μM did not compete with ^{125}I-hPP binding to the zYa receptor. The affinity of ^{125}I-hPP for the zYb, and zYc receptors was too low to enable the use of this radioligand at these receptors.

Sequence Analyses

Alignment of the whole Y1 receptor subfamily (zYa, zYb, zYc, cod Yb, Y1, Y4, and y6 receptors, Fig. 2) revealed that only 90 amino acids were completely conserved. Of the receptors that display a Y1-like binding profile (i.e. Y1, Yb, and Yc), 144 amino acids were conserved. All positions previously identified as important for BIBP3226 binding to the human Y1 receptor (Trp^{163}, Phe^{173}, Tyr^{211}, Gln^{219}, Asn^{283}, Phe^{286}, and Asp^{287}) [31] were conserved among the Y1, Yb, and Yc receptors. Only three differences were found in these positions when all the sequences of the Y1 receptor subfamily were compared (Fig. 2). The amino acid corresponding to Phe^{173} is a leucine in y6 receptors, Tyr^{211} is a phenylalanine in mouse y6, and Phe^{286} is an alanine in zYa and a glutamic acid in Y4 receptors.

DISCUSSION

Porcine NPY and PYY, Pro^{34}-substituted analogues of NPY and PYY, and zebrafish NPY bind to the zebrafish receptors Ya and Yc with similar affinities, whereas these ligands have about 10-fold higher affinity for the zYb receptor. The different properties of the zYb receptors are surprising as this receptor shares 75% identity with the zYc receptor,

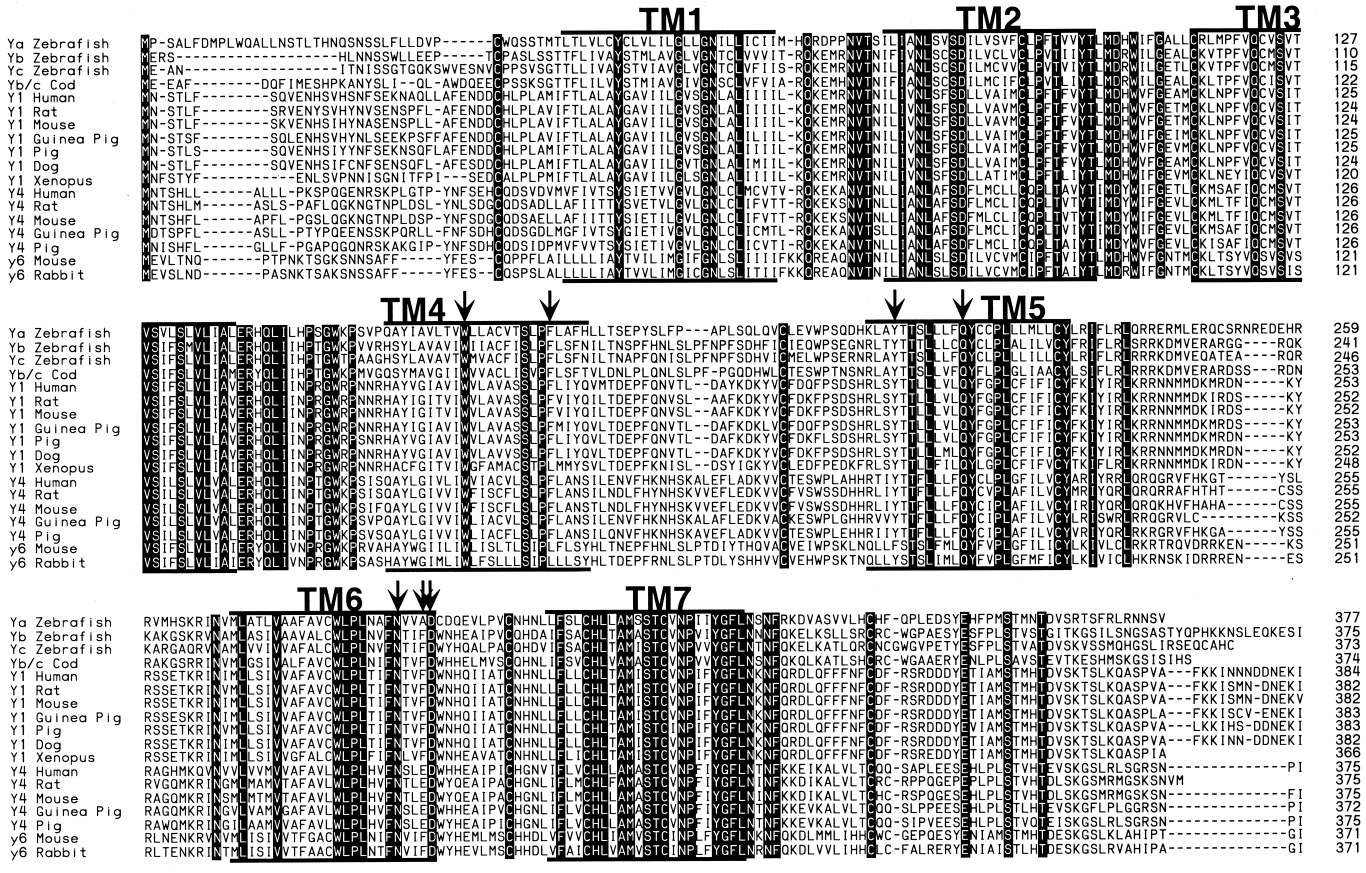

FIG. 2. Alignment of all published full-length sequences of the Y1 subfamily of receptors: Y1 from human, rat, mouse, guinea pig, pig, dog, and the frog (*Xenopus laevis*); Y4 from human, rat, mouse, guinea pig, and pig; y6 from mouse and rabbit; Ya, Yb, and Yc from zebrafish; and Yb from cod. Thick lines label TM regions. Conserved amino acids are white on black background and amino acids previously reported to be involved in BIBP3226 and NPY recognition [31] at the Y1 receptor are labeled with black arrows.

whereas the zYa receptor is only 50% identical to the other two. However, the zYa receptor does display some unique properties relative to zYb and zYc [12, 14] (see below). Zebrafish PYY bound with 2- to 3-fold lower affinity than zNPY to all three receptors [14].

According to earlier alanine scanning and structure–activity relationship (SAR) data of Y1 and Y2 receptors, the last four amino acids of the ligand are extremely important for the receptor–ligand interaction and subtype selectivity [19]. When each of the positions Arg^{33}, Gln^{34}, Arg^{35}, and Tyr^{36} of the NPY molecule was separately replaced by an alanine, the affinity decreased dramatically both for the human Y1 (300- to 65,000-fold) and Y2 (75- to 38,000-fold) receptors endogenously expressed in the neuroblastoma cell lines SK-N-MC and SMS-KAN, respectively [19]. At both receptors, the Arg^{35} to alanine substitution had the greatest impact. In fact, binding to the hY1 receptor was affected by Ala substitution at each of the 36-amino-acid positions of the NPY molecule. Only replacements at positions 4, 6, 7, and 26 resulted in a loss in affinity that was less than 10-fold [19]. At the hY2 receptor, however, binding was only affected more than 5-fold by replacement at Pro^5 and Pro^8 of the first 18 amino acids of the NPY molecule. The effects of the two Pro substitutions are probably secondary due to disturbance of the proline helix causing the carboxy-terminal part of the NPY molecule to adapt a conformation that is unfavorable for binding to Y2 receptors.

In the present study, Chinese hamster ovary cells stably expressing the zebrafish receptors were used. The expression levels differed 17-fold between the zYa receptor- and zYc receptor-expressing cell lines. However, all the expression levels were rather low (40–700 fmol/mg prot) and were not likely to have caused uncoupled receptors (G-protein depletion) or toxic effects that could have interfered with the binding experiments and changed the pharmacological profile of the receptors. As expected, all three zebrafish receptors were influenced by Ala substitutions of the NPY molecule at positions 33, 35, and 36. Similarly to the Y1 and Y2 receptors,the most dramatic effect regarding the zYb and zYc receptors was observed when Arg^{35} was substituted with an alanine. This modification caused the affinity to drop 250- and 6000-fold, respectively. At the zYa receptor, however, the p[Ala^{33}]NPY displayed the lowest affinity, K_i = 3 nM (i. e. 50-fold lower than pNPY), while p[Ala^{35}]NPY bound to the zYa receptor with a K_i of 1.5 nM (25-fold lower than pNPY). Interestingly, the p[Ala^{34}]NPY analogue bound to all three receptors with affinities similar to pNPY.

The loop-deleted peptides [Ahx^{8-20}]NPY [20], [Ahx^{8-20},

Pro[34]]NPY, and [Ahx[5–24]]NPY [21] all displayed a similar affinity for the zYa receptor as pNPY. Previous studies have also shown that the affinity of the smallest amino-terminally truncated peptide tested, pNPY13–36, was as high as for the native peptide to the zYa receptor [12, 14]. This indicates that only the 12 most carboxy-terminal amino acids, i.e. amino acids 25–36, are involved in the actual binding to the zYa receptor. This can be compared with the hY2 receptor where [Ahx[5–24]]NPY bound with about 10-fold lower affinity than NPY [21, 32] and where the more rigid cyclopeptide [Glu[28]-Lys[32], Ac[25–36]]NPY acts as a full agonist [33]. Molecular dynamics modeling of this compound has suggested that the carboxy-terminus and the amino-terminus have a truly NPY-like conformation [34].

NPY analogues bind to the zYb and zYc receptors with a profile reminiscent of that reported for mammalian Y1 receptors and form together with the cod Yb receptor [15, 16] a subgroup of receptors with a Y1-like binding profile characterized by a decreased affinity with progressive amino-terminal truncation of the NPY or PYY molecules and with Pro[34]- substituted analogues remaining as full agonists at these receptors. However, a few differences between the zYb and zYc receptors as compared to the mammalian Y1 receptors have been identified [14]. Human Y1 and Y2 receptors lost affinity by 300- and 75-fold, respectively, when Glu[34] was substituted with alanine [19], whereas the zYb receptor was unaffected and the zYc receptor only lost affinity by about 3-fold. On the other hand, zYb and zYc receptors are more sensitive to amino-terminal deletions than the Y1 receptor. When Tyr[1] of the NPY molecule was deleted, generating NPY2–36, the zYb and zYc receptors lost affinity about 1000- and 100-fold, respectively [13, 14], whereas mammalian Y1 receptors only lost affinity by about one order of magnitude [24, 35, 36]. Deletion of the second amino acid, Pro[2], caused the affinity to drop yet another order of magnitude at both the zYb and zYc receptors [13, 14].

The pharmacological studies clearly distinguish zYa as a receptor with a unique binding profile. NPY and PYY, as well as most modified NPY analogues, bind to the zYa receptor, identifying it as a rather promiscuous receptor. Even bovine PP binds [14] with high affinity to the zYa receptor (K_i = 0.36 nM), although fish lack this peptide. The affinity of bPP to the zYb and zYc receptors was 2.8 and 30 nM, respectively [14]. In order to explore the PP binding of the zYa receptor, an NPY analogue with two positions exchanged for the corresponding amino acids in PP, p[Glu[4], Pro[34]]NPY, was tested. This peptide bound with affinities 2-, 3-, and 6-fold lower than pNPY to the zYa, zYb, and zYc receptors, respectively.

Gregor *et al.* [37] have reported that the use of different radioligands can result in altered binding. We therefore tested ^{125}I-hPP as radioligand at the zYa receptor. The rank order of potencies was the same as when ^{125}I-pPYY was used: pNPY ≈ pNPY3–36 ≈ pNPY13–36 ≈ p[Ala[34]]NPY > [Ahx[5–24]]NPY > bPP >>> BIBP3226. ^{125}I-hPP at concentrations up to 500 pM did not bind significantly to the zYb and zYc receptors.

The Ya receptor also binds amino-terminally truncated peptides and loop-deleted peptides with high affinity, as does the Y2 receptor (although zYa is only 30% identical to Y2). However, the Y2 receptor does not bind NPY with a proline at position 34, whereas the zYa receptor does. To further distinguish the binding profile of the zYa receptor from the Y2 receptor, a recently published Y2-selective antagonist, BIIE0246 [30], was tested and found not to bind to the zYa receptor. This antagonist is highly selective, as it has been found to bind to the human Y2 receptor with an IC_{50} value of 3.3 nM [30] and to the guinea pig Y2 receptor with a K_i value of 2.4 nM (the present investigation). However, it does not bind to Y1, Y4, and Y5 receptors from human [30] and guinea pig. Y5 receptors also bind moderately amino- terminally truncated analogues, but since the Y5-selective agonist p[D-Trp[32]]NPY [38] does not bind to the zYa receptor, the binding profile of this receptor is clearly unique.

The Y1-selective non-peptidic antagonist BIBP3226 was designed by replacing amino acid 1–34 of the NPY molecule with diphenylacetylation after screening with various substituents [28]. Moreover, the other Y1-selective antagonist used in this study, SR120819A, displays similarities to the carboxy-terminal part of the NPY molecule. However, despite their Y1-like binding profiles for various peptides, neither of the two antagonists BIBP3226 and SR120819A bound to any of the zYa, zYb, zYc, or cod Yb receptors [16]. In a mutagenesis study of the human Y1 receptor where amino acids in the TM regions were mutated to alanine, six amino acids (Trp[163], Phe[173], Gln[219], Asn[283], Phe[286], and Asp[287]) in TM4–6 were identified as being important for the interaction with both NPY and BIBP3226 [31] (Fig. 2). Interestingly, all these positions are conserved in all three fish receptors that display a Y1-like binding profile (zYb, zYc, and cod Yb). In fact, out of all mammalian Y1, Y4, and y6 receptors together with the fish Ya–Yc receptors, only three replacements are found at these positions: Phe[173] at the top of TM4 is a leucine in y6 receptors and Phe[286] at the top of TM6 is an alanine in zYa and a glutamic acid in Y4 receptors. Tyr[211] in TM5 was the only amino acid that influenced antagonist binding without affecting the binding of NPY. Tyr[211] is conserved in all Y1 subfamily receptors except the mouse y6 receptor (but is present in rabbit y6 receptor). The binding of BIBP3226 to the y6 receptor, however, remains to be explored. The fact that BIBP3226 does not bind to Yb and Yc receptors might imply an influence from additional amino acid residues in the interaction between BIBP3226 and the Y1 receptor. It may also be that the fish receptors display slightly different conformations of the amino acid side chains as compared to Y1 receptors or that other amino acids prevent BIBP3226 from entering the binding cavity between TMs 4–6. TM4 and TM5 differ extensively between Y1 and the fish Yb and Yc receptors (50% of the positions are different, Fig. 2) and several candidates for such "blocking" amino acids are

likely to be located there. Also, the replacement of Thr^{280}, located one turn below Asn^{283} in TM6 of Y1 receptors, with an asparagine in all the fish receptors, could block the binding of BIBP3226. In fact, there are no fewer than 14 positions in TM4–6 that are conserved among all the functionally expressed Y1 receptors that differ in all cloned Y4 and fish receptors. Of these 14 amino acids, nine (Val^{167}, Ser^{169}, Ile^{175}, Tyr^{176}, and Gln^{177} in TM4, Ser^{210}, Leu^{214}, and Leu^{218} in TM5, as well as Thr^{280} in TM6 of the hY1 receptor) are located in the upper half of the TM region and thus represent strong candidates for providing additional interaction points for BIBP3226 in the Y1 receptor. To our knowledge, only Ser^{210}, and Thr^{280} have previously been targeted by site-directed mutagenesis [31].

In summary, we have identified the zYa receptor as a very promiscuous receptor, since all peptides of the NPY family as well as Pro^{34}-substituted, amino-terminally truncated, carboxy-terminally modified, and loop-deleted peptide analogues bind to the zYa receptor with high affinity. The zYb and zYc receptors have overall binding profiles more similar to the Y1 receptor. In the present study, the zYb receptor displays a binding profile intermediate to the zYa and zYc receptors. The zYc receptor was the most sensitive to all alanine substitutions and loop deletions of NPY tested here. Neither of the Y1 receptor-specific antagonists BIBP3226 and SR120819A binds to any of the three zebrafish receptors. This fact, together with the moderate (50%) sequence identity between the zebrafish receptors and Y1 receptors, will be highly useful for further modeling of mammalian Y1 receptors as well as for all other Y1-like receptors.

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