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Multiplicity of Neuropeptide Y Receptors: Cloning of a Third Distinct Subtype in the Zebrafish[1]

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Five different receptor subtypes for neuropeptide Y (NPY) have recently been cloned in mammals. We have discovered three distinct subtypes by PCR in the zebrafish, *Danio rerio*, and describe here one of these called zYc. The protein sequence identity is 46–51% to mammalian subtypes Y1, Y4 and Y6 and to zebrafish Ya, i.e., the same degree of identity as these subtypes display to one another. The identity to zYb is higher, 75%, indicating that zYb and zYc share a more recent ancestor. The zYc receptor binds NPY and PYY (peptide YY) from mammals as well as zebrafish with high affinities and has a Kd of 16 pM for ^{125}I-pPYY. The pharmacological profile is similar to, but distinct from, mammalian Y1. zYc inhibits cAMP synthesis. This work suggests that NPY has more receptor subtypes than any other peptide that binds to G protein-coupled receptors. Work is in progress to see if the zebrafish receptors are present in mammals.

Neuropeptide Y (NPY) and peptide YY (PYY) are 36-amino-acid peptides found in all vertebrates. They form a family of structurally related neuroendocrine peptides together with pancreatic polypeptide (PP) in tetrapods and peptide Y (PY) in certain fishes (1). NPY has widespread and abundant expression in the nervous system of all vertebrates investigated and gives rise to many physiological effects including stimulation of food intake and vasoconstriction (2). PYY has primarily endocrine functions in mammals, but is also expressed in brainstem neurons throughout vertebrate phylogeny (3-5) and is transiently expressed in embryonic rat dorsal root ganglia (6). PP is strictly endocrine, found only in tetrapod pancreatic islets (7), whereas fish PY, like PYY, seems to have both endocrine and neuronal expression (8).

The effects of the NPY-family peptides are mediated by receptors which belong to the rhodopsin superfamily of G protein-coupled receptors (9, 10). Several NPY receptor subtypes have been demonstrated in mammals by physiological studies of tissue preparations as well as binding studies with radiolabelled ligands (11). Most of these predicted subtypes have now been cloned in mammals, namely subtypes Y1, Y2, Y4 (for reviews see (9, 10, 12) and the Y5 "feeding" receptor (13, 14). In addition, an unexpected subtype was cloned in mouse, initially called Y5 (15) or PP2 (16), subsequently renamed Y6. This receptor is a truncated pseudogene in humans and other primates (16-18) and seems to be absent from rat genome, but is expressed in rabbit (17).

Nevertheless, some pharmacologically described receptor subtypes have yet to be cloned, namely the NPY-preferring Y3 receptor (previously reported clones have turned out not to bind NPY, (19, 20) and a PYY-preferring receptor (21). Furthermore, the cloned Y2 receptor is expressed only in brain (22, 23), why a presumed peripheral Y2 receptor seems to remain to be cloned. Also, a PP-binding receptor in addition to Y4 seems to be present in the rat brain (24).

To study the evolutionary events that have generated the various NPY receptor subtypes, and as an additional way to clone the remaining receptor subtypes, we have turned to non-mammalian vertebrate species. One of these is the zebrafish, *Danio rerio*, which in addition is an excellent model system for studies of development (25). We have previously isolated the genes for NPY and PYY from this species (5), why receptor clones would allow studies of co-distribution of ligands and receptors during ontogeny as well as in

[1] The sequence reported in this paper has been deposited with the GenBank Data Library.

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Abbreviations used: NPY, neuropeptide Y; PYY, peptide YY; PP, pancreatic polypeptide; PY, peptide Y; zNPY, zebrafish NPY; zPYY, zebrafish PYY; pNPY, porcine NPY; pPYY, porcine PYY; bPP, bovine PP; PCR, polymerase chain reaction; TM, transmembrane.

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adult animals. Surprisingly, all three receptors that we have discovered in the zebrafish display sequences and pharmacological properties that make them distinct from all five of the mammalian receptor cloned to date. We report here the sequence and pharmacological profile of one of these novel NPY receptor subtypes, the zYc receptor.

MATERIALS AND METHODS

Isolation and sequencing of receptor clones. Multiple degenerate primer pairs based on zebrafish receptor sequences zYa (26) and zYb (27) were used in different pairwise combinations for PCR on zebrafish genomic DNA. The following conditions were used: 1 minute at 94°C, 1.5 minute at 49°C and 2 min at 72°C for 25 cycles using Taq polymerase. One primer combination gave a product of the expected size. The 5′ primer was a 20-mer called YG3aF and had the sequence ATYGCWGTIGARMGICAYCA (mixed bases designated according to International Union of Biochemistry) corresponding to positions 362-382 413-432 (TM3) in the zebrafish zYc sequence. The 3′ primer was an 18-mer called YG7aR with the sequence CATIGCIGTIARRTGRCA complementary to positions 935-952 (TM7). The PCR product of approximately 600 bp was isolated, reamplified, and cloned into the plasmid vector pMOS-T Blue (Company). Several clones were sequenced and one clone was found to be highly homologous but dissimilar to the zYb receptor and was called zYc for "zebrafish Y-receptor c". A genomic library (kindly provided by A. Fjose, University of Bergen, Norway) in the vector EMBL3 was screened with a zYc fragment of 600 bp generated with the same degenerate primers as above. Hybridizations were done at high stringency at 65°C in 25% formamide, 6X SSC, 10% Dextran sulfate, 5X Denhardt's solution and 0.1% SDS. Filters were washed twice at room temperature in 2X SSC/0.1% SDS and twice for 30 min at 65°C in 0.5X SSC/0.1% SDS. Four phage clones were obtained and one was subjected to DNA sequencing with the AmpliCycle sequencing kit (Perkin Elmer) using [α-^{33}P]dATP and specific oligonucleotide primers.

Cloning into expression vector. A fragment containing the entire coding region was generated from the phage clone with PCR using Taq DNA polymerase. The 5′ primer is located in the 5′-untranslated region at positions 1-22 and contained an *Eco*RI cloning site and had the sequence CGGAATTCCGATTTCCTTCCACCCTTTA. The 3′ primer is located in the 3′-untranslated region at positions 1190-1209 and contained a *Bam* HI cloning site and the sequence CGGGATCCTATGATGACTTCTGCTCCTG. The reaction was phenol extracted, cut with *Eco*RI - *Bam*HI and the 1.2 kb fragment was purified on an agarose gel using the QIAquick gel extraction kit (Qiagen) and ligated into the expression vector pTEJ-8 (28). The resulting clone zYc-pTEJ was completely sequenced and found to be identical to the genomic sequence.

Transfection protocol. For stable transfections CHO (Chinese hamster ovary) cells were grown in DMEM containing 10% fetal calf serum. Cells were transfected with 100 μl Lipofectin (Gibco BRL) and 2 μg of the construct zYc-pTEJ, that had been linearized with PvuI, in 10 ml OptiMEM (Gibco BRL) with no fetal calf serum according to the manufacturer's recommendations. After 6 hours, 10 ml of DMEM containing 20% fetal calf serum was added. Positive clones were selected for with 500 μg/ml of Geneticin (Sigma) in the medium during a period of approximately 3 weeks. 12 cell clones resistant to Geneticin were selected. Each cell clone was grown in 6-well plates, harvested and pelleted by centrifugation. The pellets were resuspended in PBS and homogenized using an Ultra-Turrax homogenizer and frozen in aliquots at −80°C. Membranes were tested for pPYY binding. 9 clones were found to bind peptide.

Peptides. The zebrafish (z) NPY and PYY peptides were synthesized at Eli Lilly and Company as deduced from the sequences of the cloned zebrafish genes (5). The porcine (p) and bovine (b) peptides were purchased from Bachem, King of Prussia, PA.

Binding assays. The thawed aliquots were resuspended in 25 mM HEPES buffer (pH 7.4) containing 2.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 2 g/l Bacitracin and rehomogenized. Saturation experiments were performed in a final volume of 200 μl with 0.75 μg protein and ^{125}I-pPYY (Amersham) for 2 hours at room temperature. This radioligand is iodinated at tyrosines 21 and 27 and has a specific activity of 4000 Ci/mmol. Saturation experiments were carried out with serial dilutions of radioligand. Nonspecific binding was defined as the amount of radioactivity remaining bound to the cell homogenate after incubation in the presence of 100 nM unlabelled zNPY. Competition experiments were performed in a final volume of 100 μl. Various concentrations of the peptides zNPY, zPYY, pNPY, pPYY, p[Leu^{31}-Pro^{34}]NPY, pNPY 2-36, pNPY 3-36, pNPY 13-36, bPP and NPY[D-Trp^{32}] were included in the incubation mixture along with ^{125}I-pPYY. Incubations were terminated by filtration through GF/C filters, which had been presoaked in 0.3% polyethyleneimine, using a TOMTEC (Orange, CT) cell harvester. The filters were washed with 2.5 ml of 50 mM Tris (pH 7.4) at 4°C and dried at 60°C. The dried filters were treated with MeltiLex A (Wallac) melt-on scintillator sheets and the radioactivity retained on the filters counted using the Wallac 1450 Betaplate counter. The results were analyzed using the Prism 2.0 software package (Graphpad, San Diego, CA). Protein concentrations were measured using Bio-Rad Protein Assay (Bio-Rad) with BSA for standards.

Cyclic AMP assay. Cyclic AMP was assayed on transfected CHO cells. The cells were detached using EDTA (no Trypsin) in EBSS (Gibco), diluted to 1000 cells/μl and treated for 20 minutes at 37°C with 500 μM isobutylmethylxanthine. Cells were incubated with 50 μM forskolin and various concentrations of zNPY, zPYY or bPP for 20 minutes at 37°C in a total volume of 250 μl. Reactions were terminated by adding 25 ul perchloric acid ($HClO_4$, 4.4 M) and the suspension was neutralized by adding 25 μl potassium hydroxide (KOH, 5M). Membranes were pelleted by centrifugation and 50 μl of the supernatant was used to quantify cAMP using radioassay. After 2 hours the incubation was terminated by rapid filtration through a GF/B filter using a Brandel harvester. The radioactivity remaining on the filters were counted in a Liquid Scintillation Analyzer (Packard). cAMP-binding protein was extacted from bovine adrenal cortex (29).

RESULTS

Isolation of Zebrafish NPY Receptor Clones

Zebrafish genomic DNA was used as template for PCR with degenerate primers based upon the zebrafish receptor sequences zYa and zYb (26, 27). One primer combination from TM2 and TM7 generated a PCR product of the expected size. Approximately twenty clones were sequenced. One clone encoded a receptor with high identity to zYb. This novel zebrafish receptor was named zYc. The zYc PCR fragment was used to screen a genomic zebrafish library. Four positive phages were isolated, one of which was subcloned and sequenced.

The deduced protein sequence of the zYc receptor encompasses 377 amino acids (Fig. 1) and has 75% overall amino acid sequence identity to zYb. The identity to the structurally related subtypes Y1, Y4, Y6, and zYa is slightly lower at 46-51%. In the TM regions the identity of zYc to Yb is 84% and to Y1, Y4, Y6 and

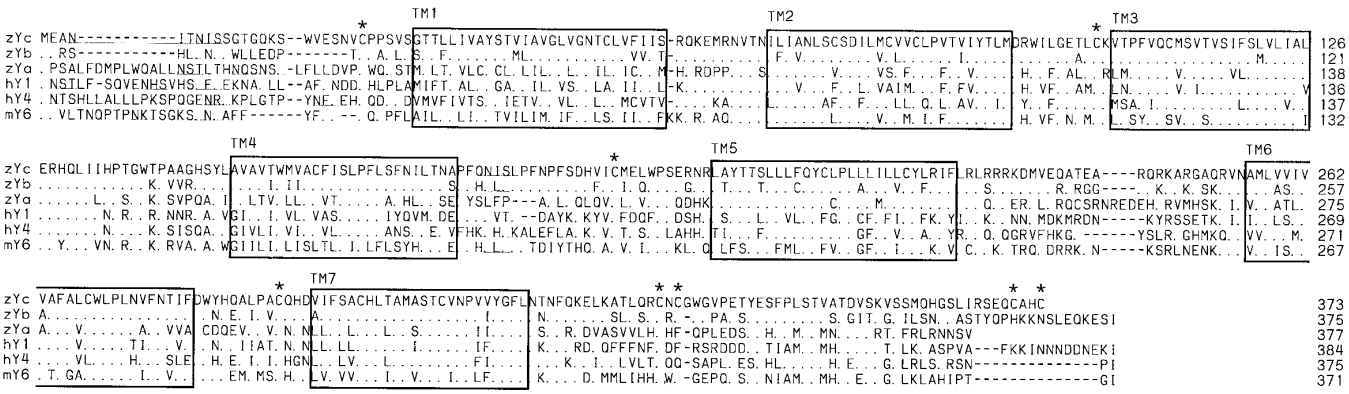

FIG. 1. Amino acid sequence alignment. The zebrafish zYc receptor serves as master sequence in alignment with zebrafish Yb and Ya, human Y1 and Y4, and mouse Y6. In the latter sequences only positions that differ from the zYa sequence are shown while *dots* mean identities. *Dashes* represent gaps introduced to optimize alignment. The hydrophobic segments assumed to be embedded in the cell membrane are *within boxes*. Tripeptides that are *underlined* in the extracellular parts conform to the consensus sequence for *N*-linked glycosylation, i.e., N-X-S/T. *Stars* show four extracellular cysteines and four cysteines in the cytoplasmic tail of zYc.

zYb in the range 55-66%. The sequence relationships are apparent also from the distance tree calculated with the neighbour joining method (Fig. 2). The identity of zYc to the Y2 and Y5 receptors is considerably lower at approximately 25% (for the entire sequence). The Y1-like receptor reported in *Drosophila melanogaster* (30) has even lower identity at 23%. The NPY receptor genes including zYc lack introns in the coding region, with the single exception of the Y1 gene which has one intron.

The predicted zYc amino acid sequence has many of the structural features of the Y1-like receptors; there are two consensus sites for N-linked glycosylation in the aminoterminal region and one site in the second extracellular loop. Four extracellular cysteine residues are conserved in all Y1-like receptors and presumably form two disulfide loops. Four cysteines are present in the cytoplasmic tail of zYc and probably at least one of these serves to anchor the tail to the membrane via palmitate. zYb has two cytoplasmic cysteines and the other Y1-like receptor all have a single cytoplasmic cysteine.

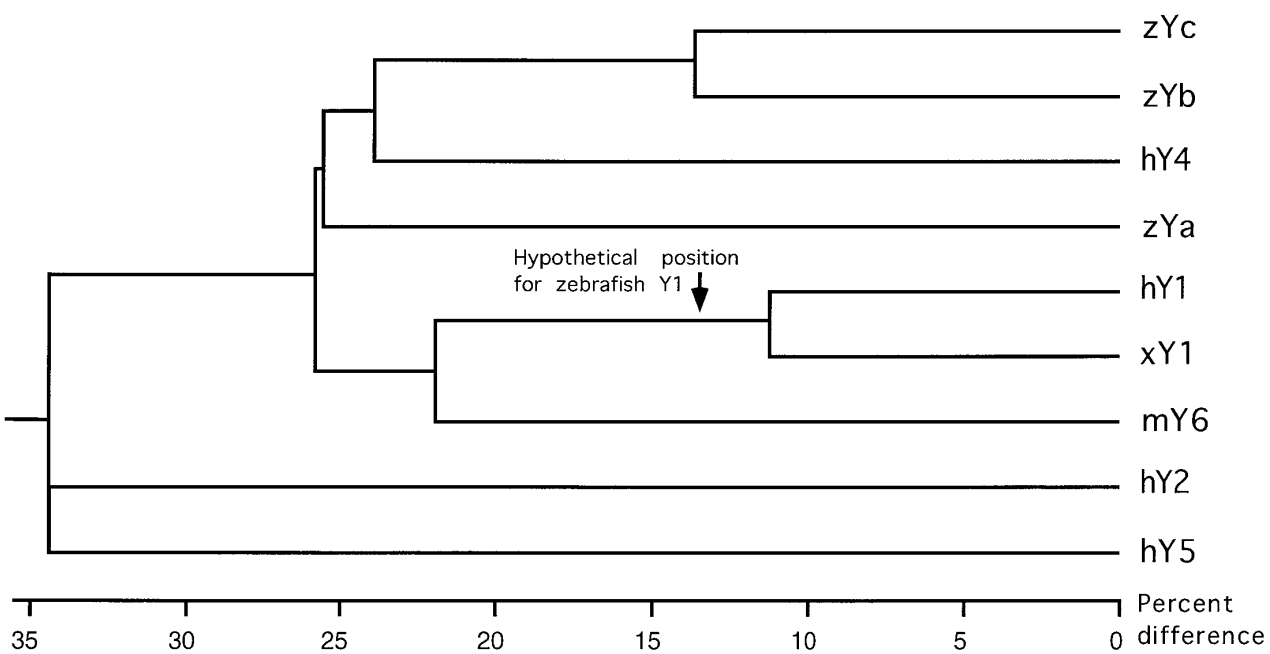

FIG. 2. Distance tree for the NPY family of receptors. The amino termini were excluded from comparison as they evolve very rapidly. Branch lengths correspond to sequence divergence as calculated with the neighbour joining method using the Lasergene DNASTAR Megalign software. The human neurokinin 3 receptor was used as outgroup.

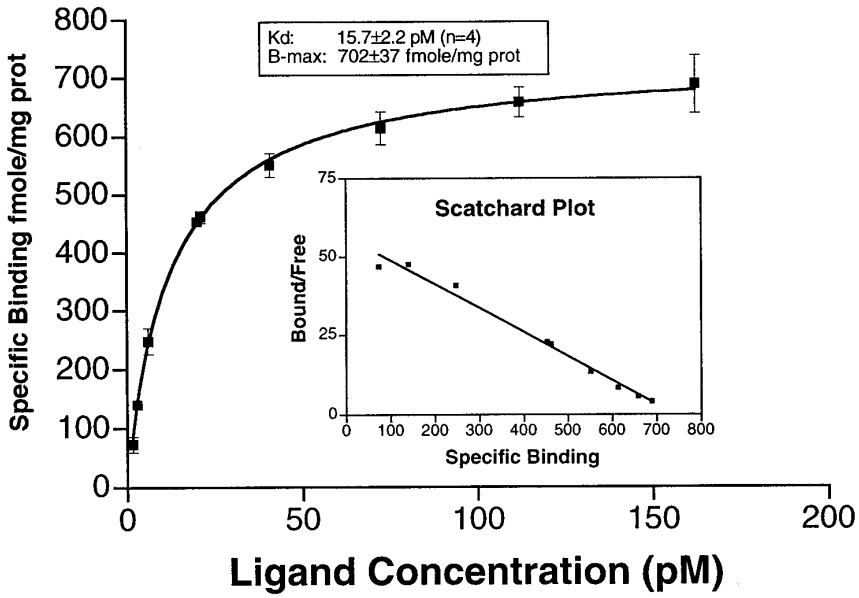

FIG. 3. Saturation and Scatchard (inset) analyses of ^{125}I-pPYY binding to membranes prepared from CHO cells stably transfected with the expression plasmid zYc-pTEJ. Result shown is one typical experiment performed in duplicate. Non-specific binding was defined by 100 nM zNPY.

Binding Properties

The expression construct zYc-pTEJ was stably transfected into CHO cells. Seven out of twelve clones resistant to G418 bound ^{125}I-pPYY. The clone with the highest receptor expression was called zYc:11 and was selected for further characterization. Membranes prepared from zYc:11 cells exhibited concentration-dependent binding of ^{125}I-pPYY (Fig. 3) while non-transfected cells exhibited no specific binding (data not shown). ^{125}I-pPYY identified a single class of high-affinity binding sites with a dissociation constant (Kd) of 15.7 ± 2.2 pM (n=4) and a Bmax of 702 ± 37 fmol/mg protein. Competition experiments were performed using both zebrafish and porcine NPY and PYY and various porcine peptide analogues (Table 1 and Fig. 4). NPY and PYY from both species showed strong and equipotent inhibition of binding of ^{125}I-pPYY in the low picomolar range. zNPY and zPYY had inhibition constants (Ki) of 25 pM and 83 pM, respectively. The porcine peptides as well as p[Leu^{31}-Pro^{34}]NPY (LP-pNPY) and h[Leu^{31}-Pro^{34}]PYY (LP-hPYY) had inhibition constants similar to zNPY. When the N-terminal tyrosine was left out (pNPY 2-36) the affinity dropped by two orders of magnitude. The affinities for pNPY 3-36 and pNPY 13-36 were more than three orders of magnitude lower than for intact pNPY. The Y5-selective analogue NPY[D-Trp^{32}] had relatively low affinity with a Ki value of approximately 200 nM.

Cyclic AMP assay

CHO cells stably expressing the zYc receptor were assayed for inhibition of forskolin-stimulated cAMP production. The peptides zNPY, zPYY, and bPP inhibited cAMP production in a dose-dependent fashion (not shown) with the same rank order of potency as in the competition experiments with ^{125}I-pPYY (Table 1). However, the EC50 values were much higher than the Ki values for zNPY and zPYY and slightly higher for bPP.

DISCUSSION

The zebrafish has become one of the most widely used species for studies of vertebrate development. To explore the possible roles of NPY and PYY in development, we have previously cloned these genes in the zebrafish (5). In order to correlate ligand and receptor expression, we also wished to isolate clones for the receptors of NPY and PYY. We have recently identified two such receptors called zYa and zYb (26, 27). PCR with degenerate primers based upon these sequences led us to discover a third zebrafish NPY/PYY receptor which we have named zYc and whose characteristics are reported here.

The three zebrafish receptors are equally distantly related to the previously cloned mammalian subtypes in the Y1-like subfamily (Y1, Y4 and Y6) with overall amino acid sequence identities in the range 46-51% (see Figs. 1 and 2). This degree of identity is lower than would be expected for zebrafish orthologues of these three mammalian NPY receptors. The expected position in the dendrogram of a zebrafish Y1 sequence is indicated in Fig. 2 as extrapolated from the frog sequence. Other G protein-coupled receptors that have

TABLE 1

Inhibition of ^{125}I-pPYY Binding

	zYa	zYb	zYc
Kd ^{125}I-pPYY	0.028 ± 0.001[1]	0.0037 ± 0.0004[2]	0.016 ± 0.002
zNPY	0.030 ± 0.008[1]	0.0034 ± 0.0012[2]	0.025 ± 0.007
zPYY	0.086 ± 0.004[1]	0.042 ± 0.008[2]	0.083 ± 0.010
pNPY	0.044 ± 0.013[1]	0.0028 ± 0.0012[2]	0.024 ± 0.006
pPYY	0.020 ± 0.006[1]	0.0026 ± 0.0015[2]	0.023 ± 0.008
LP-pNPY	0.039 ± 0.007[1]	0.012 ± 0.011	0.022 ± 0.005
LP-hPYY	0.036 ± 0.001	0.0064 ± 00001[2]	0.032 ± 0.005
pNPY 2-36	0.038 ± 0.009[1]	3.4 ± 0.8[2]	2.1 ± 0.7
pNPY 3-36	0.061 ± 0.011[1]	35 ± 12[2]	35 ± 17
pNPY 13-36	0.036 ± [1]	90 ± 4	63 ± 36
bPP	0.36 ± 0.05	2.8 ± 0.6[2]	29 ± 12
NPY [D-Trp^{32}]	114 ± 21[1]	6.8 ± 1.3	214 ± 94

Data represent inhibition constants (Ki) ± SEM in nM for three to five experiments performed in duplicate.
1) From ref (26).
2) From ref (27).

been cloned in fishes display much higher sequence identity to their mammalian orthologues, for instance the opioid receptors (31, 32). Furthermore, all three zebrafish receptors lack the intron present in the Y1 gene in mammals as well as in frog (33) and chicken (S. Mikko and D. Larhammar, unpublished). The zYc receptor is more closely related to zYb (75%) than to any of the other receptors, and thus appears to be the result of a more recent gene duplication. Studies of these genes in other fish species will be required to determine the time point for the gene duplication event. Taken together, these comparisons strongly suggest that the three receptors that we have discovered in the zebrafish represent distinct NPY receptor subtypes, and that mammalian orthologues should be expected for zYa, zYb and zYc (or at least one orthologue for zYa and one for zYb/c).

The zYc receptor displays most of the structural features of Y1-like receptors, but has four cysteines in the carboxyterminal tail, whereas most other receptors have just one (Fig. 1). Pharmacologically, the binding profile of zYc is reminiscent of zYb (Fig. 5) and mammalian Y1. Both Yc and Yb differ from Y1 in that they bind the radioligand ^{125}I-pPYY with a higher affinity, 15.7 pM for zYc. Secondly, zYc and zYb are more sensitive to aminoterminal deletions of the porcine NPY molecule than are Y1 receptors (Table 1). The affinities for pNPY2-36 to zYc is 2.1 nM and to zYb 3.4 nM. This represents a 100-fold and a 1000-fold loss of affinity, respectively, compared to native pNPY, whereas most

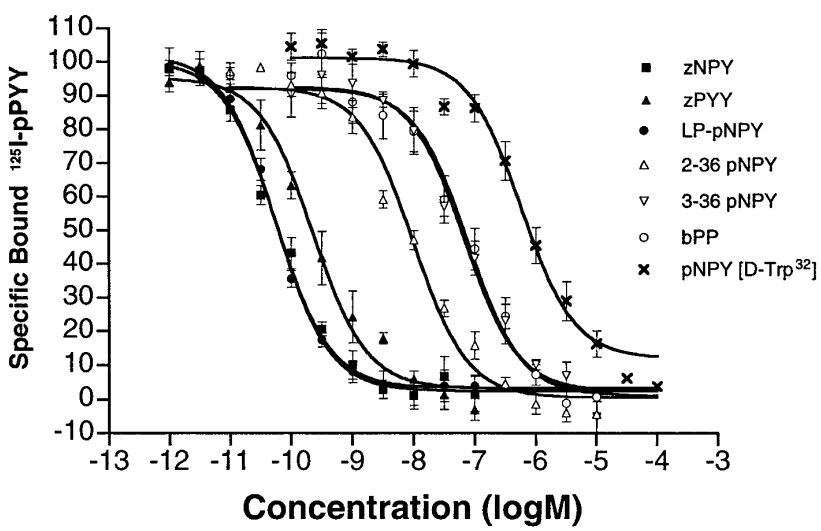

FIG. 4. Inhibition of ^{125}I-pPYY binding to membranes prepared from CHO cells stably transfected with the expression plasmid zYc-pTEJ. Competition data are expressed as percentages of the binding observed in the absence of competitor peptide. Results represent the mean ± SEM for three to four experiments performed in duplicate. Non-specific binding was defined by 100 nM zNPY.

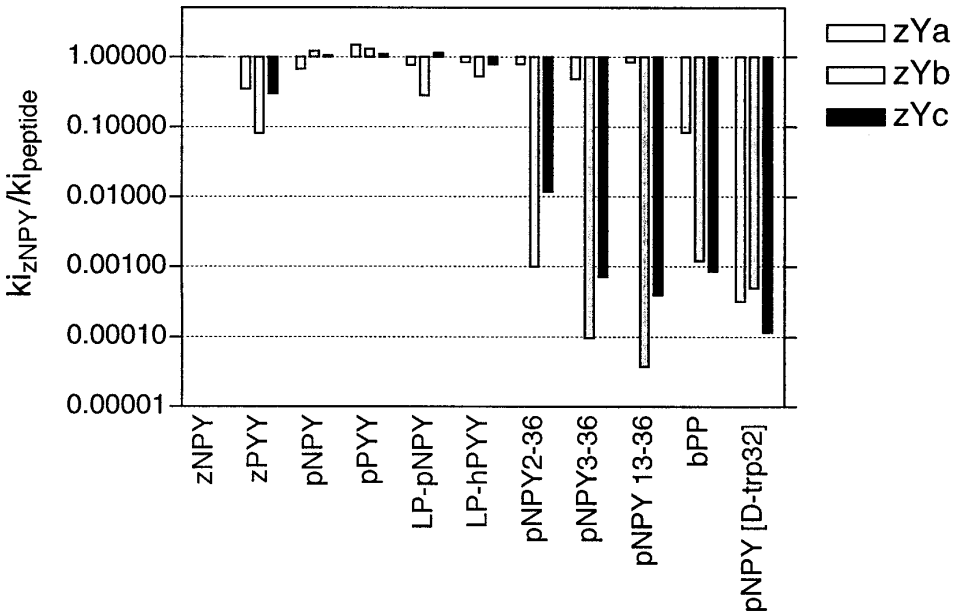

FIG. 5. The three zebrafish receptors Ya, Yb and Yc compared to one another. The Ki of each peptide has been divided with the Ki for zNPY to that receptor. A value below one indicates a loss of binding as compared to zNPY.

reports for Y1 describe losses in the range three-fold (34) to ten-fold (35) or 80-fold (36). Both zYc and zYb lose another order of magnitude when the second amino acid (proline) is deleted (see Table 1 and Fig. 5) as does Y1 (34).

An interesting feature displayed by all three zebrafish receptors is their lower affinity for zPYY (3-11 fold) than for zNPY, pNPY and pPYY (Fig. 5). This is surprising because zPYY differs at only six positions from zNPY whereas pPYY is in fact more different from the other three peptides. Structural modelling of receptor and ligand interactions along with site-directed mutagenesis of receptors is underway to find the basis for the lower affinity for zPYY.

Receptor Ya has a completely different binding profile from all other NPY receptors of the Y1 subfamily in that it is insensitive to aminoterminal truncation of the peptide ligands (Fig. 5), thereby resembling the mammalian Y2 receptor (which has only about 30% amino acid sequence identity to Ya), but also binds the Pro-34-substituted ligands LP-pNPY and LP-hPYY, once thought to be Y1-selective. Finally, Ya binds bPP with subnanomolar affinity and has some affinity (Ki about 100 nM, see Table 1) for the Y5-selective analogue NPY[D-Trp^{32}] (13). Overall, the pharmacological profile of zYa resembles the mammalian Y5 receptor, although these two receptors share only 27% amino acid identity (Fig. 2).

The Yc receptor has a much lower affinity for bPP (29 nM) than either Ya or Yb (Fig. 5), but nevertheless responds to this peptide by inhibiting forskolin-stimulated cAMP production in CHO cells stably expressing zYc (Table 2). The rank order of potency in the functional cAMP assay was the same as in the binding assays (zNPY>zPYY>bPP) but the difference between zNPY and bPP was smaller in the cAMP assay; the difference in affinity (Ki) to the zYc receptor between zNPY and bPP is 1800-fold, but difference in IC50 is only 40-fold (Tables 1 and 2). Similarly, zPYY was 12 times less potent in cAMP inhibition compared to zNPY while the difference in binding was only three-fold. These discrepancies between binding and functional assays might indicate that the G proteins of CHO cells do not interact as well with the zebrafish NPY receptors as they do with mammalian NPY receptors.

In summary, we report here a third zebrafish NPY receptor called zYc and provide structural and pharmacological data that this receptor as well as the two previously cloned zebrafish receptors represent subtypes distinct from the cloned mammalian receptor subtypes. zYc is more closely related to zYb than to the other NPY receptors. The sequence divergence indicates that at least two of the zebrafish receptors arose prior to

TABLE 2

Inhibition of Forskolin-Stimulated cAMP Production in CHO Cells Transfected with the zYc Receptor

Compound	EC50 (nM)	SEM (nM)	n
zNPY	2.27	0.91	4
zPYY	27.8	1.5	2
bPP	98.2	28.2	3

the divergence of ray-finned fishes and the mammalian lineage (tetrapods), which suggests that mammals should be expected to have orthologues of the zebrafish Ya and Yb/c receptors.

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