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A Y2 Receptor Mimetic Aptamer Directed against Neuropeptide Y*

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Neuropeptide Y (NPY) is a 36-amino acid neuropeptide that exerts its activity by at least five different receptor subtypes that belong to the family of G-protein-coupled receptors. We isolated an aptamer directed against NPY from a nuclease-resistant RNA library. Mapping experiments with N-terminally, C-terminally, and centrally truncated analogues of NPY revealed that the aptamer recognizes the C terminus of NPY. Individual replacement of the four arginine residues at positions 19, 25, 33, and 35 by L-alanine showed that arginine 33 is essential for binding. The aptamer does not recognize pancreatic polypeptide, a highly homologous Y4 receptor-specific peptide of the gut. Furthermore, the affinity of the aptamer to the Y5 receptor-selective agonist [Ala^{31},Aib^{32}]NPY and the Y1/Y5 receptor-binding peptide [Leu^{31},Pro^{34}]NPY was considerably reduced, whereas Y2 receptor-specific NPY mutants were bound well by the aptamer. Accordingly, the NPY epitope was recognized by the Y2 receptor, and the aptamer was highly similar. This Y2 receptor mimicking effect was further confirmed by competition binding studies. Whereas the aptamer competed with the Y2 receptor for binding of [^{3}H]NPY with high affinity, a low affinity displacement of [^{3}H]NPY was observed at the Y1 and the Y5 receptors. Consequently, competition at the Y2 receptor occurred with a considerably lower K_i value compared with the Y1 and Y5 receptors. These results indicate that the aptamer mimics the binding of NPY to the Y2 receptor more closely than to the Y1 and Y5 receptors.

Neuropeptide Y (NPY)[1] is a 36-amino acid neuropeptide and is one of the most conserved peptides during evolution (1, 2). It shows high homology to the other members of the pancreatic polypeptide hormone family, namely pancreatic polypeptide and peptide YY (3, 4). NPY is widely distributed in the central and peripheral nervous system (5). It modulates a variety of physiological processes such as the central regulation of food intake (6, 7), vasoconstriction, memory retention (8, 9), and regulation of circadian rhythm (10–12). NPY transmits its activity by at least three receptor subtypes (Y_1, Y_2, and Y_5), which all belong to the large family of G-protein-coupled receptors (GPCR). They are coupled to G_i proteins and accordingly inhibit adenylate cyclase (13, 14). The different receptor subtypes are distributed heterogeneously in various tissues in the central nervous system and the periphery and so far it is not understood how NPY selectively activates a particular receptor pathway.

In general, structural insight how GPCRs are activated is scarce. Only recently the first crystal structure of a G-protein-coupled receptor, rhodopsin, has been solved and provided insight into the molecular mechanism of GPCR activation (15). However, these structural analyses are highly advanced and cannot be considered routine. Furthermore, because the ligand is covalently bound in rhodopsin, the process of ligand approaching is still unknown. Accordingly, biochemical methods that facilitate our understanding of GPCRs are urgently required. All current information on structure/activity or structure/affinity relationships of NPY and its G-protein-coupled receptor subtypes have been obtained by indirect methods such as site-directed mutagenesis in transmembrane regions and extracellular loops of the receptors (16) and replacement of amino acid residues in NPY (17–19). Modified analogues and recombinant receptors are excellent tools to study ligand-receptor interaction *in vitro* or in cell lines. However, they cannot be applied to characterize interactions *in vivo* because the *in vivo* expression of each modification would require an individual transgenic animal. To circumvent these problems, selective low molecular weight antagonists have been developed (20, 21). These small organic molecules, however, do not necessarily bind to the receptor in a way similar to the endogenous ligand. For example, only partial overlapping of the binding site has been shown for BIBP3226, and in the substance P and angiotensin systems completely different binding sites for agonist and antagonist were identified (22). Mapping of the Y_1 receptor with anti-receptor antibodies has provided some insight into the hypothetical binding topology of NPY (23); however, the use of polyclonal antibodies directed against the 16–20-mer receptor segments only provided limited structural information.

We therefore chose an alternative approach to characterize the interaction of NPY with its receptors. Here we describe the selection of aptamers that specifically recognize NPY with good affinities. The binding of the aptamer to NPY in relation to a series of NPY agonists or antagonists was characterized. Finally, we investigated the quality of the aptamer as a func-

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[1] The abbreviations used are: NPY, neuropeptide Y; GPCR, G-protein-coupled receptor; FCS, fetal calf serum; PBS, phosphate-buffered saline; DMF, dimethylformamide; Me_2SO, DMSO, dimethyl sulfoxide; Fmoc, fluorenylmethoxycarbonyl; hPP, human pancreatic polypeptide; Ahx, aminohexoyl; Aib, amino isobutyric acid.

tional and selective NPY competitor by displacing NPY from its receptors Y1, Y2, and Y5.

EXPERIMENTAL PROCEDURES

Materials—The N^{α}-Fmoc-protected amino acids were obtained from Alexis (Switzerland) and Novabiochem. The side chain protecting groups are as follows: *tert*-butyl for Asp, Glu, Ser, Thr, and Tyr; *t*-butoxycarbonyl for Lys; trityl for Asn, Gln, and His; and 2,2,5,7,8-pentamethylchroman-6-sulfonyl for Arg. The 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy (Rink Amide) resin was obtained from Novabiochem.

Peptide Synthesis and Analysis—Peptides were synthesized by automated multiple solid phase peptide synthesis on a peptide synthesizer (Syro, MultiSynTech, Bochum, Germany) using Rink Amide resin (30 mg, resin loading 0.6 mmol/g). N^{α}-Fmoc amino acids were attached by a double coupling procedure with 10-fold excess of amino acid, 1-hydroxybenzotriazole (HOBt), 1,3-diisopropylcarbodiimide (DIC) in DMF (2×40 min). The Fmoc deprotection step was accomplished with 40% piperidine in DMF for 3 min, 20% piperidine for 7 min, and finally 40% piperidine for 5 min. Amino acid sequences of the peptides are listed in Table II. The sequences of the two random NPYs were determined by random combination of the 36 amino acids of NPY. Prior to cleavage of the truncated NPY variants from the resin, they were N-terminally biotinylated. The Ahx spacer was introduced as final step during automated synthesis. N-terminal biotinylation was achieved by using 5 eq of biotin; HOBt and DIC were dissolved in DMF (0.5 M). One hour after addition of this solution to the pre-swollen resin, 5 eq of N-ethyldiisopropanolamine (DIPEA) were added. The coupling reaction was stopped after 15 h by washing the resin with DMF, methanol, methylene chloride, DMF, and diethyl ether (4 times at 1 ml each). The peptides were cleaved from the resins with a mixture of trifluoroacetic acid/thioanisole/*p*-thiocresol (90:5:5 v/v), precipitated from ice-cold diethyl ether, collected by centrifugation, washed with diethyl ether 4 times, and lyophilized from water/*tert*-butanol (3:1 w/w). Characterization of the peptides was achieved by mass spectrometry and analytical high pressure liquid chromatography.

In Vitro Selection of Anti-NPY Aptamers—The RNA library was synthesized by *in vitro* T7 transcription from a PCR-amplified double-stranded DNA pool (MicMod 40N) as follows: 5′-TCTAATACGACTCACTATAGGGAGAAAGGG**AAGCTT**GAG-40N-AGAAGAAGGAACGAGCGTACGGATCCGATC-3′, 5′-PCR primer Mic-(1–39), 5′-TCTAATACGACTCACTATAGGGAGAAAGGG**AAGCTT**GAG-3′ (underlines indicate the T7 promoter and boldface indicates the *Hin*dIII restriction site), 3′-PCR primer Mic-(1–25), 3′-CTTCCTGCTCGCATG**CCTAGG**CTAG-5′ (boldface indicates the *Bam*HI restriction site) as described (24). The T7 *in vitro* transcription was done in a standard fashion by incubating the DNA template with T7 polymerase (250 units) and ATP, GTP, 2′NH_2-CTP, and 2′NH_2-UTP (all at 1 mM) for 10–12 h at 37 °C in 40 mM Tris/HCl buffer, pH 8, containing 12 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM spermidine, 0.002% Triton X-100, and 4% polyethylene glycol. The biotinylated NPY was immobilized on streptavidin-agarose (Pierce) in 0.25× PBS, pH 7.4, at 4 °C overnight. The coupling efficiency quantified spectroscopically was about 95%. In the first cycle 3.8 nmol (2.3 library equivalents) of the ^{32}P-labeled RNA were dissolved in PBS binding buffer (Invitrogen, 155 mM NaCl, 1.1 mM KH_2PO_4, 3 mM $Na_2HPO_4 \cdot 7H_2O$, pH 7.4), heated for 2 min at 90 °C and then renatured for 10 min on ice in the presence of 1 mM $MgCl_2$ to allow proper secondary structure formation. To remove RNAs with affinity for streptavidin-agarose material, a counter selection using streptavidin-agarose was performed in cycles 1–9. The initial two fractions of the streptavidin-agarose flow-through were incubated with the immobilized NPY for 1 h at 37 °C by mixing gently. The matrix was washed with 100 column volumes of binding buffer. NPY-bound RNA was eluted with 7 M urea containing 3 mM EDTA and quantified by scintillation counting. Further treatment and amplification was performed as described previously. In cycles following round 9, the immobilized NPY concentration was decreased gradually from 26 to 2.5 μM, and selections were done in the presence of 1% heparin and 10% fetal calf serum. After cycle 12 the enriched pool was PCR-amplified with primers Mic-(1–39) and Mic-(1–25), cloned, and sequenced as described (25).

Equilibrium Dissociation Constants—Binding studies were done with 1.5 nM 5′-^{32}P-labeled RNA. Representatives of each sequence group were incubated with peptide dilution series ranging from 5 nM to 7 μM in 50 μl of binding buffer (0.9 mM KH_2PO_4, 5 mM Na_2HPO_4, 70 mM NaCl, 1.3 mM KCl, 1% heparin, 20 μg of streptavidin, pH 7.6) for 1 h at 37 °C. Streptavidin was added to improve the retention of the peptide on the nitrocellulose filter, and filter-binding studies were performed as described. The percentage of input RNA retained on each filter was quantified by PhosphorImaging. For RNA ligands exhibiting monophasic binding equilibrium dissociation constants (K_d values) were determined using Equation 1 (26),

$$(f/2R_t) = (P_t + R_t + K_d) - ((P_t + R_t + K_d)^2 - 4P_tR_t)^{1/2} \quad \text{(Eq. 1)}$$

Binding to Peptide Analogues—The various NPY derivatives (Table II) were immobilized on streptavidin-agarose. The internally ^{32}P-labeled RNA (50 pmol) was incubated with the coupled peptides (7 μM derivatized agarose material). After 1 h of incubation at 37 °C the mixture was placed into a Bio-Rad column, and the nonbinding aptamers were removed by washing with 100 column volumes of binding buffer, and the amount of the complexed RNA was determined by scintillation counting.

Binding to Cells—For competition of NPY-receptor interaction on cells, SK-N-MC cells (neuroblastoma cell line) expressing endogenously the hY1 receptor, SMS-KAN cells (neuroblastoma cell line) expressing endogenously hY2, and baby hamster kidney cells transfected with the rY5 receptor were cultured as described (27, 28). Cells were harvested at 100% confluence in phosphate-buffered saline containing 0.02% EDTA (SK-N-MC cells and SMS-KAN cells) or in the presence of trypsin (baby hamster kidney cells), resuspended in incubation buffer (minimal essential medium, 1% bovine serum albumin, 1% Pefabloc, and 1% Bacitracin), and counted. After centrifugation (800 rpm for 5 min) the pellet was resuspended in the incubation buffer and diluted to 2×10^6 cells/ml. 200 μl of cell suspension (5×10^5 cells) were incubated simultaneously with 25 μl of oligonucleotide solution in increasing concentrations and a constant amount of [^{3}H]propionyl-NPY to give a total volume of 250 μl. After 1.5 h at room temperature, incubation was terminated by centrifugation for 5 min at 3200 rpm at 4 °C. The pellets were washed with cold PBS, resuspended in PBS, and mixed with scintillation mixture, and the ^{3}H radioactivity was measured. To test for and to quantify the unspecific binding of ^{3}H-labeled NPY to the cells containing the NPY receptors, a 1000-fold increased concentration of unlabeled NPY was added. Experiments were repeated independently three to four times in triplicate each. Values of IC_{50} were calculated with the program GraphPadPrism 3.02 using the "sigmoidal dose response (variable slope)" Equation 2,

$$Y = (\text{resp}_{\text{Bottom}} + (\text{resp}_{\text{Top}} - \text{resp}_{\text{Bottom}})) \times (1 + 10^{(\log \text{IC}_{50} - x) \cdot \text{Hillslope}})^{-1} \quad \text{(Eq. 2)}$$

where Y indicates response, whereas Y starts at bottom ($\text{resp}_{\text{Bottom}}$) and goes to top ($\text{resp}_{\text{Top}}$) with sigmoidal shape; x indicates logarithm of concentration of aptamer. Equation 2 is also designated as the "four parameter logistic equation."

Values of K_i were calculated according to the method of Cheng and Prusoff (29) using the Equation 3,

$$K_i = \text{IC}_{50}(1 + L \times K_d^{-1})^{-1} \quad \text{(Eq. 3)}$$

where L is the concentration of competed [^{3}H]NPY which was 1 nM for Y1 and Y5 and 2 nM for Y2 receptors.

RESULTS

In vitro selection of random oligonucleotide libraries has been used to isolate RNA, single-stranded DNA, or modified RNA ligands with high affinity for diverse targets (30–33). To be applicable in cell culture studies, we generated nuclease-resistant aptamers by screening oligonucleotide libraries in which all pyrimidine residues were substituted by 2′-amino-2′-deoxy-modified monomers (34–38). This modification is known to protect against the majority of serum nucleases so that such aptamers can be applied in the presence of biological materials in which normal RNA would rapidly degrade (39, 40).

Aptamers Bind to NPY with High Affinity and Specificity—As the C-terminal pentapeptide of NPY has recently been shown (19) to be essential for its interaction with all receptor subtypes, we immobilized NPY via N-terminal biotinylation on streptavidin-agarose to ensure maximal accessibility of the C terminus (Fig. 1*A*). From an RNA pool with a complexity of 9×10^{14} different sequences in which all pyrimidine residues were substituted by 2′-amino-2′-deoxy derivatives, anti-NPY-aptamers were selected in 12 cycles of iterative selection and

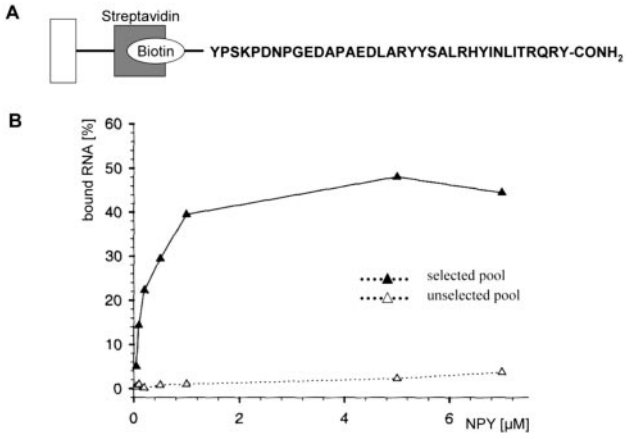

FIG. 1. **The selection target NPY and its binding to the enriched library from cycle 12.** *A*, sequence of the N-terminally biotinylated and C-terminally amidated NPY used in the selection. *B*, binding study of the selected modified RNA library after cycle 12 (*filled triangles*) compared with the unselected pool (*open triangles*). The relative proportion of complexed RNA (%) is shown as a function of the NPY concentration used in the filter binding assay.

Sequence-family I

DP11 (11)	CAAGGCCGGGUUAGGUGGGACCGUGAUCUGAUGUGGCGUG
DP1	CAAGGCCGGGUUAGGCGGGACCGUGAUCUGAUGUGGCGUG
DP27	CAAGGCCGGGUGAGGUGGGACCGUGAUCUGAUGUGGCGUG
DP12 (2)	CAAGGCCGGGUUAGGUGGGACCGUGAUCUGAUGUGACGUG
DP28 (2)	CAAGGCCGGGUUAGGGGGACCGUGAUCUGAUGUGGCGUG
DP2	CAAGGCCGGGUUAGGUGGGACGUGAUACUGAUGUGGGGAG
DP5 (2)	CAAGGCCGGGUUAGG-GGGACCGUGAUCUGAUGUGGCGUG
DP20	CAAGGCCGGGUUAGG-GGGACCGUGAUCUGUAGUGGCGUG
DP26 (2)	CAAGGCCGGGUUAGGUGGGAC-GUGAUCUGAUGUGGCGUG
DP22	CAAGGCCGGGUUAGGUGGGACUGUGAUUUCGAUGUGGCUG
DP4	CAAGG---GGUUAGG-GGGACCGUGAUCUGAUGUGGCGUG

Sequence-family II

DP3	CAGCAGGAGGGCCGGCGUUAGGGUUAGCGAGCCGAUUGAA
DP8	CAGCAGGAGGGCCGGCGUUAGGGUUAGCGAGCCGAU-GAA

orphans

DP14	CUGCCAAAGGUUGGCCAUGUGUGGGGGAAGCUCCAACGU
DP19	GCCACCAAUGCGCACCCACCCAGACACGUCAAGUCAAGU
DP25	GGAUACUUAGCGUAAGUNAGUGGCUGGGGGGUGACGAAG

FIG. 2. **Sequences of 2′-aminopyrimidine RNA ligands selected from the cycle 12 pool.** For each clone only the variable 40-nucleotide region is shown. *Parentheses* show the number of identical clones. The 2′-amino-2′-deoxycytidine and 2′-amino-2′deoxyuridine residues are shown simply as *C* or *U*. *N* in clone 25 denotes an ambiguous position on the sequencing gel. Nucleotides highlighted in *gray* reflect point mutations; − indicates deletions or insertions in relation to the most abundant clone DP11.

amplification (Fig. 1). Fig. 1*B* shows the concentration-dependent binding of NPY to the unselected pool and the enriched pool from cycle 12, determined by nitrocellulose filtration. Whereas the cycle 12 library reaches saturation at an NPY concentration of 5 μM, no significant retention of the unselected pool was detected. Therefore, the affinity of the cycle 12 pool for NPY had improved at least 3 orders of magnitude compared with the starting pool. Cloning and sequencing of 30 clones from cycle 12 revealed one major (>80%) and a minor (7%) family of sequences and three orphans (Fig. 2) indicating that the pool diversity was decreased considerably after cycle 12.

We next determined K_d values of representative clones from each family for binding to full-length NPY by titration experiments using nitrocellulose filtration. As shown in Table I, DP3 from sequence class II exhibited a K_d of 370 nM, representing the tightest NPY binding aptamer. DP11, a member of the most abundant class I aptamers bound with 470 nM, orphans showed only weak (DP25) or no affinity (DP14 and DP19; Table I, and data not shown). As negative controls, we also tested the unselected pool RNA for binding to NPY and the binding behavior of DP3 to a version of NPY with randomly scrambled sequence (scNPY, Table I). We found no affinity in either case showing that NPY recognition occurs with high sequence specificity both for the RNA and the peptide. Further mapping and specificity experiments were performed with clone DP3 (see below).

Binding of Aptamers to NPY Variants—We next sought to identify the epitope on NPY recognized by the aptamer DP3 by comparing its binding behavior to a variety of truncated analogues of NPY with that of the full-length peptide. First, we mapped the discrete binding region on NPY minimally required for complex formation with the aptamer. The results are summarized in Tables I and II. NPY fragments comprising central domains of the N and C terminus, respectively, do not show any detectable interaction with DP3 (entries 1–7, Table II). Similarly, the C-terminal construct 18–34 (entry 7, Table II), which lacks the two C-terminal amino acid residues, Arg^{35} and Tyr^{36}, shows no detectable interaction with DP3. Interestingly, construct 18–36 (Table I and II, entry 10) containing Arg^{35} and Tyr^{36}, which is otherwise identical to 18–34, is recognized by DP3 with the same order of magnitude as full-length NPY. The same is true for construct 13–36 (Table I and II, entry 9). In this sense, DP3 binding to NPY correlates with the requirements of NPY complexation by all of its receptor subtypes which also do not bind NPY constructs lacking the C-terminal amino acid (19).

Peptide 25–36 did not show any detectable affinity to DP3 (Table II, entry 4). Likewise, this peptide competes only weakly NPY bound to any receptor subtype (data not shown). In contrast, the well characterized centrally truncated Y2 receptor-selective agonist, Ahx-(5–24) (Table II, entry 8), in which amino acid residues 5–24 are substituted by an aminohexyl spacer shows restoration of the binding to DP3 in the same order of magnitude as full-length NPY.

We next investigated a set of analogues in which arginine residues that are thought to play an important role for receptor-ligand interaction were replaced, in order to determine the role of individual amino acid residues for NPY/DP3 complexation. All four arginines of NPY were individually substituted by alanines (Table II, entries 11–14). These residues were chosen because we initially assumed that positively charged arginine side chains are likely to contribute strongly to the binding affinity between DP3 and the NPY C terminus. The R19A substitution did not affect aptamer binding (Table II, entry 11), whereas a substantial reduction of binding was observed for the R25A and the R35A substitutions (Table II, entries 12 and 14). In contrast, when arginine 33 was substituted for alanine, the peptide completely lost its affinity to the aptamer (Table II, entry 13)

To test for specificity of the aptamer DP3 within the pancreatic polypeptide family, its binding to the human pancreatic polypeptide (hPP) was investigated. Although hPP exhibits 50% sequence identity to NPY, it does not interact with the neuropeptide Y receptors except of the Y4 subtype for which it is actually the endogenous ligand (41, 42). Whereas recognizing Y4 with picomolar K_d, hPP binds to Y5 receptors with considerably lower affinity, whereas the Y1 and Y2 receptors are not recognized at all (41, 42). Interestingly, no affinity of hPP to the aptamer DP3 was detected (Table I) indicating that DP3 can easily discriminate between different peptide hormones within related families.

To explore further the relationship between receptor and aptamer specificity, we tested DP3 binding to other analogues that have been shown earlier to interact selectively with some Y receptor subtypes. For example, the Y5 receptor selective analogue [Ala^{31},Aib^{32}]NPY (18) was considerably less efficiently

TABLE I

K_d values of anti-NPY aptamers and the unselected pool to full-length NPY, scrambled NPY, truncated versions of NPY, and human pancreatic peptide (hPP)

K_d values were determined in the presence of streptavidin and heparin by filter binding assays and calculated as described.

Aptamer clone	K_d					
	NPY[a]	ScNPY[b]	18–36[c]	13–36[d]	Ahx5–24[e]	hPP[f]
			μM			
DP3	0.37	NA	1.16	0.76	0.71	NA
DP4	0.41					
DP11	0.47	NA				
Pool	NA	NA	NA	NA	NA	NA

[a] Biotinylated full-length NPY.
[b] Sequence of the scrambled NPY, SKPQRDANREPTRYAIYDYSNPDIELHYLRPAYALG-NH_2.
[c] Amino acids 1–17 were deleted.
[d] Amino acids 1–12 were deleted.
[e] Amino acids 5–24 were substituted by an aminohexanoic acid spacer.
[f] Sequence of hPP, APLEPVYPGDNATPEQMAQYAADLRRYINMLTRPRY-NH_2.
[g] NA, no affinity.

TABLE II

Binding of anti-NPY aptamer (DP3) was probed with a panel of immobilized peptide variants of porcine NPY (pNPY)

In constructs 1–7, 9, and 10 the remaining amino acid residues of several N- and C-terminally truncated porcine NPY variants are indicated on the left. Construct 8, centrally truncated amino acid residues 5–24 were substituted by an aminohexanoic acid spacer. Several point mutations compared with the wild-type NPY are denoted in brackets. Binding activities are denoted in relation to the interaction with the original NPY target; −, 0–10% of the NPY control; +, 11–80% of the NPY control; ++, 81–100% compared with the NPY control (top sequence). The two amino acid residues RQ, which are absolutely essential for the RNA recognition are underlined and in bold (top sequence).

Entry	Name	Peptide sequence	Binding
	NPY	Biotin-YPSKPDNPGEDAPAEDLARYYSALRHYINLIT**RQ**RY-NH_2	++
1.	1–10	Biotin-YPSKPDNPGE-$CONH_2$	−
2.	5–20	Biotin- PDNPGEDAPAEDLARY-$CONH_2$	−
3.	25–34	Biotin- RHYINLITRQ-NH_2	−
4.	25–36	Biotin- RHYINLITRQRY-NH_2	−
5.	18–28	Biotin- ARYYSALRHYI-NH_2	−
6.	18–32	Biotin- ARYYSALRHYINLIT-NH_2	−
7.	18–34	Biotin- ARYYSALRHYINLITRQ-NH_2	−
8.	$Ahx^{5–24}$	Biotin-YPSK--------Ahx---------RHYINLITRQRY-NH_2	++
9.	13–36	Biotin- PAEDLARYYSALRHYINLITRQRY-NH_2	++
10.	18–36	Biotin- ARYYSALRHYINLITRQRY-NH_2	++
11.	[Ala^{19}]18–36	Biotin- A**A**YYSALRHYINLITRQRY-NH_2	++
12.	[Ala^{25}]18–36	Biotin- ARYYSAL**A**HYINLITRQRY-NH_2	+
13.	[Ala^{33}]18–36	Biotin- ARYYSALRHYINLIT**A**QRY-NH_2	−
14.	[Ala^{35}]18–36	Biotin- ARYYSALRHYINLITRQ**A**Y-NH_2	+
15.	[Leu^{31},Pro^{34}]	Biotin-YPSKPDNPGEDAPAEDLARYYSALRHYINL**L**TR**P**RY-NH_2	−
16.	[Ala^{31},Aib^{32}]	Biotin-YPSKPDNPGEDAPAEDLARYYSALRHYINL**AAib**RQRY-NH_2	+

bound by DP3 (Table II, entry 16), whereas the Y1/Y5 receptor selective agonist [Leu^{31},Pro^{34}]NPY was not recognized by the aptamer at all (Table II, entry 15). Interestingly, the replacement of Gln^{34} by Pro also leads to loss of affinity at the Y2 receptor. Taken together, the binding behavior of the aptamer to all tested NPY constructs reflects that of the Y2 receptor.

Inhibition of NPY/NPY Receptor Binding by DP3 Shows Selectivity for the Y2 Receptor—DP3 efficiently binds to NPY *in vitro* and shows preference for NPY analogues that are also bound preferentially by the Y2 receptor. We therefore investigated whether and how the *in vitro* binding behavior of DP3 is reflected in a competitive situation in which the aptamer competes with various receptor subtypes for NPY binding. We used cell lines that individually produce the different receptor subtypes as follows: the neuroblastoma cell lines SK-N-MC and SMS-KAN endogenously expressing the hY1 and the hY2 receptors, respectively. For competition at the Y5 receptor, baby hamster kidney cells stably transfected with the rY5 receptor were used. Competition experiments were performed by using ^{3}H-labeled NPY, and quantification of receptor-bound NPY was performed by scintillation counting in the presence of increasing concentrations of DP3. As a negative control, the same experiment was performed in the presence of increasing concentrations of the unselected RNA library, which did not bind NPY. The results are summarized in Fig. 3. In general, DP3 shows concentration-dependent inhibition of specific binding of NPY to all receptor subtypes investigated. However, the binding kinetics of the Y1 and Y5 receptors differed significantly from those of the Y2 receptor. The competition between DP3 and Y2 receptors for NPY clearly reflects a monophasic competition curve indicating that DP3 directly competes with the Y2 receptor for NPY binding (Fig. 3*B*). At a concentration of 73 nM, half-maximal inhibition of the competition is observed (Table III). In contrast, the competition curves at the Y1 and Y5 receptors exhibit a completely different shape (Fig. 3, *A* and *C*). Complete loss of receptor affinity is only obtained at 1000 nM. From the fitted curves we calculated IC_{50} values of NPY competition by the aptamer for the various receptor subtypes and subsequently K_i values (Table III). Thus, the aptamer-dependent inhibition of NPY/Y2 receptor interaction occurs with an K_i of 0.65 nM which is at a 70–400-fold lower concentration than for the Y1 and Y5 receptors, respectively, although Y2 shows the highest affinity to NPY (19).

DISCUSSION

We describe here two main classes of novel 2′-amino-2′-deoxypyrimidine-modified RNA aptamers that bind to neuropeptide Y with affinities between 370 and 470 nM. These binding affinities compare well with other *in vitro* selections performed with peptide targets. For example, the anti-HIV-1

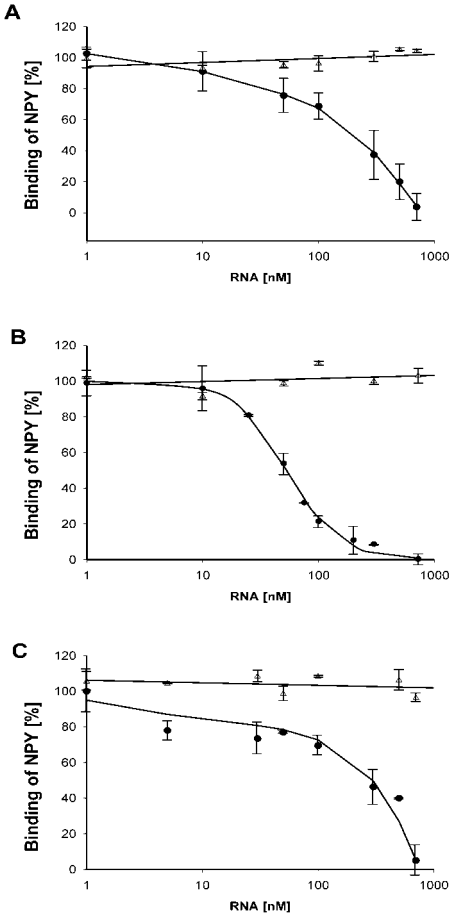

FIG. 3. **Aptamer DP3 competes with Y2 receptor for NPY binding.** Influence of high affinity ligand DP3 (*filled circles*) and random pool RNA (*open triangles*) on specific binding of ^{3}H-labeled NPY to G-protein-coupled transmembrane receptor subtypes Y1 (*A*), Y2 (*B*), and Y5 (*C*) expressed on cell surfaces as a function of RNA concentration. Individual data points are averaged from three independent measurements each measured as triplicates.

TABLE III
IC_{50} and K_i values resulting from the competition studies in cell culture

Receptor	IC_{50}[a]	K_d[b]	K_i[c]
	nM	*nM*	*nM*
Y1	328 ± 141	0.18	50 ± 21
Y2	73 ± 25	0.018	0.65 ± 0.2
Y5	416 ± 63	1.7	262 ± 40

[a] Values of IC_{50} were calculated from the data points in Fig. 3 with the program GraphPadPrism 3.02 using the "sigmoidal dose response (variable slope)" variant.

[b] Values of K_d for NPY binding to individual receptor subtypes are obtained from the following references: Y1 receptor (48), Y2 receptor (49), Y5 receptor (50).

[c] Values of K_i were calculated according to the method of Cheng and Prussoff (29).

Rev-peptide aptamer binds its target with a K_d of 19 nM, the substance P-aptamer with 190 nM, the CD18cyt-aptamer with 500 nM, and the vasopressin aptamer with 900 nM (43–46).

Correlation of Aptamer and Receptor Recognition—We have applied a series of peptides previously shown to act as selective NPY receptor agonists, and we investigated their binding to the DP3 aptamer. DP3 binds with high affinity to peptide NPY-(18–36). This peptide is bound by the Y2 receptor subtype with an IC_{50} of 0.25 nM, whereas the Y1 receptor exhibits only weak binding affinity to 18–36 (IC_{50} of 2.7 μM) (19). The next candidate in this series was [Ahx$^{5-24}$]NPY, in which four N-terminal amino acids are linked to the C-terminal NPY fragment NPY-(25–36) via an amino hexanoic acid linker. [Ahx$^{5-24}$]NPY constitutes a biologically active conformation of NPY that binds with an IC_{50} of 2 nM to Y2 receptors, whereas Y1 and Y5 receptors are bound with considerably weaker affinity with an $IC_{50} > 4$ μM and 795 nM, respectively. According to structure-activity studies of linear and constraint analogues, and circular dichroism experiments, the N-terminal segment does not exhibit a direct effect but rather stabilizes the C-terminal conformation (23, 47). As shown in Tables I and II, this centrally truncated construct is also well bound by the aptamer (Table I and Table II, entry 8), which, like Y2, fails to bind just the C-terminal fragment NPY-(25–36) alone (Table II, entry 4). This is also a good indication that the aptamer recognizes a defined conformation of NPY. It has been suggested that the biologically active conformation of NPY that is bound selectively by Y2 forms a hairpin-like structure in which the N and C termini are located in close proximity to each other. Therefore, the C terminus represents a functional region whereas the N terminus constitutes a structural component required for stabilization of the orientation of the C terminus (19).

Even more striking is that [Leu^{31},Pro^{34}]NPY is not recognized by the Y2 receptor and by the aptamer (Table II, entry 15). This analogue is widely used to distinguish between Y2 and "not-Y2" receptor-mediated functions of NPY. The Y5 receptor-selective ligand [Ala^{31},Aib^{32}]NPY (Table II, entry 16) and the Y4 preferring peptide hPP (Table I) both bind only weakly or not at all to both the aptamer and the Y2 receptor. These data indicate that DP3 shows only weak affinity to analogues that are bound well by the NPY receptors Y1, Y5, or Y4. Accordingly, DP3 shows very similar binding behavior as found for the Y2 receptor subtype.

More important for a comparison of DP3/NPY recognition with the possible binding mode of NPY to its receptors, however, is the finding that the C-terminal arginine residues are essential for receptor recognition. Arg^{33} and Arg^{35} are essential for NPY binding by all receptors. Substitution of Arg^{19} and Arg^{25} by alanine residues reduces the affinity for Y1 receptor binding greater than 1000- and 50-fold, respectively, whereas the affinity of the Y5 receptor for the R25A mutant is reduced 100-fold (27). As shown in entry 11 of Table II the aptamer tolerated the substitution R19A completely, whereas mutants R25A and R35A located closer to the C terminus showed substantially reduced binding (entries 12 and 14, Table II). Interestingly, the most critical position required for aptamer/NPY binding appeared to be Arg^{33} (entry 13, Table II), one of the seven positions found to be absolutely conserved within the whole pancreatic polypeptide families among all species known to express these regulatory peptides.

In summary, the binding studies that applied NPY variants that are specific for individual NPY receptor subtypes suggest that NPY binding by the aptamer DP3 is strikingly similar to that of the Y2 receptor. This in turn justifies the assumption that NPY may exist in different biologically active conformations *in vivo* including the possibility that the Y2 receptor-specific NPY conformation differs from the conformation that is required for the activation of the Y1 or Y5 receptors.

The aptamers were selected by using N-terminally biotinylated analogues of NPY. We chose the N-terminal position because biologically relevant residues mainly have been identified at the C-terminal segment. However, at the Y1 and Y5 receptors, N-terminal truncation of one or two residues, respectively, also considerably reduces receptor affinity, whereas at the Y2 receptor C-terminal segments still are active up to NPY-(18–36). Accordingly, the Y2 receptor preferring conformation of NPY might

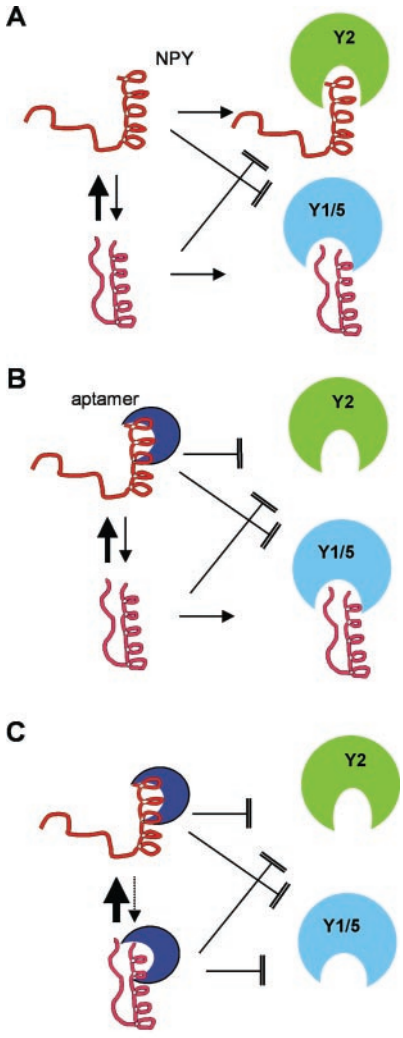

FIG. 4. **Models for the interaction of NPY with the Y receptors and the aptamer.** According to this model, NPY exists in at least two different conformations (*A*). Model of NPY receptor interaction in the absence of aptamers. *B*, at low concentration of aptamers the Y2 receptor binding conformation is blocked. *C*, at high concentrations of aptamer either the equilibrium is shifted toward the aptamer binding conformation or the aptamer binds to further NPY conformations.

have been induced by the N-terminal immobilization of NPY for aptamer selection, and the subsequent inhibition of the N- to C-terminal arrangement that is required for high receptor affinity at the Y1 and Y5 receptor subtypes.

Competition of NPY Binding by the DP3 Aptamer and NPY Receptor Subtypes—The competition experiments in which NPY binding to cells expressing the Y1, Y2, or Y5 receptor subtypes was quantified at different aptamer concentrations clearly showed a differential modulation for the Y2 *versus* Y1 and Y5 receptors, respectively (Fig. 3 and Table III). DP3 showed the lowest K_i at the Y2 receptor, which was 70- and 400-fold lower than the K_i values obtained at the Y1 and Y5 subtypes, respectively. This competition was DP3-specific, as the unselected pool did not lead to any detectable inhibition at either receptor subtype.

How can these data be explained? As shown by NMR spectroscopy, NPY exists in different conformations in solution. In the presence of up to 10 nM DP3, no effect is found at any receptor. Increasing concentrations of DP3 clearly show that the binding of NPY to the Y2 receptor is inhibited, whereas only little displacement is recognized at Y1 and Y5 receptors. Obviously the aptamer stabilizes a similar conformation of NPY as the Y2 receptor (Fig. 4*A*). At a DP3 concentration of about 200 nM [^{3}H]NPY does not bind to the Y2 receptors at all, whereas more than 50% of binding is still maintained at Y1 and Y5 receptors (Fig. 4*B*). Further increase (>1 μM DP3) also abolished the affinity of NPY to Y1 and Y5 receptors. This might either be due to a change of the equilibrium of the ligand conformation in solution toward the DP3-bound Y2 receptor-mimicking structure or to the reduced selectivity of DP3 at higher concentrations. In the latter case, DP3 would bind to further NPY conformations at higher RNA concentrations. In any case, however, the data clearly show that the aptamer can distinguish between different conformations of the peptide ligand. Whereas this ability is crucial for enzymes and receptors and has been found for antibodies as well, it has so far not been described for aptamers. Accordingly, for the first time, we identified a so-called anti-idiotypic aptamer, that mimics the Y2 receptor. NPY binds at the Y2 receptor with the highest affinity and 10–100-fold better than at Y1 and Y5 receptors as indicated by the different K_d values (Table III). Despite this difference in affinity, competition was 5–6-fold more efficient at Y2 receptors, which lead to the surprisingly high selectivity of 70–400-fold with respect to K_i values. However, even without considering the preference of NPY for Y2 receptors, the 5–6-fold more efficient blocking of Y2 receptor-binding sites could be shown.

Although we can speculate on the consequences of the peptide-aptamer complex at the receptor, little is known about the formation of this complex. It is possible that the aptamer recognizes NPY by an adaptive binding mechanism, as observed for many aptamer-ligand complexes. Adaptive binding of DP3 could account for the different modes of inhibition of NPY binding to receptors Y2 and Y1/Y5, respectively. Consequently, different NPY conformations recognized by DP3 may also induce different conformations at the aptamer. Detailed structural characterizations of the NPY-aptamer complex are required to support these hypotheses.

These results, together with the specificity determinations of Y2-specific NPY analogues (Table II), strongly suggest that the aptamer DP3 mimics the binding behavior of the Y2 receptor more closely than that of the Y1 and Y5 receptors. Whether the different conformations of NPY are induced by the Y2 receptor and are then preferentially recognized by the aptamer, or whether they are induced or stabilized by the aptamer itself cannot be answered at the moment. Our data strongly suggest that neuropeptide Y exhibits different biologically active conformations *in vivo* whereby the conformation specific for Y2 receptor subtype recognition differs from the one that is specifically bound by the Y1 or Y5 subtypes.

Further studies will reveal the suitability of the aptamers in *in vivo* experiments as well as in brain slices. In the hypothalamus, Y1, Y2 and Y5 receptors are expressed. By selectively blocking the interaction at the Y2 receptor of the released NPY, the aptamers might contribute to the overall understanding of the Y2 receptor in brain areas that express multiple Y receptor subtypes.

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